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VAVILOV JOURNAL OF GENETICS AND BREEDING

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Уважаемые коллеги, дорогие читатели! Очередной номер «Вавиловского журнала генетики и селекции» посвящен актуальным направлениям исследований в различных областях генетики растений, животных и микроорганизмов.

В рубрике «Молекулярная генетика» привлекает внимание оригинальное исследование, в котором описана разработка препаратов для профилактики и лечения такого опасного заболевания, как клещевой энцефалит. Результаты проверки эффективности действия химерного антитела ch14D5 по отношению к гликопротеину Е вируса клещевого энцефалита дальневосточного, сибирского и европейского субтипов позволяют прогнозировать протективную активность антитела к различным штаммам вируса.

Основные пути биосинтеза L-аскорбиновой кислоты (витамина С) и пути ее рециркуляции в тканях различных растений рассмотрены в обзорной статье раздела «Генетика растений». Описаны ключевые гены, участвующие в биосинтезе и накоплении аскорбиновой кислоты в плодах. Показаны различия, которые наблюдаются в путях биосинтеза в зависимости от вида растения и стадии его развития. Одна из экспериментальных статей этого же раздела знакомит с молекулярно-филогенетическими исследованиями сибирских и дальневосточных видов пырейника (*Elymus* ssp.) с использованием ITS-последовательностей генов 35S рРНК, на основе которых сделано заключение о необходимости пересмотра используемых на данный момент признаков для классификации этого вида.

Традиционную для журнала рубрику «Селекция растений на иммунитет и продуктивность» открывает оригинальное исследование, в котором представлены результаты фитопатологического и молекулярно-генетического тестирования сортов и селекционных линий мягкой пшеницы на устойчивость к патогену стеблевой ржавчины. Анализируя полевую и лабораторную устойчивость образцов, авторы установили влияние различных генов и их комбинаций на уровень восприимчивости растений к патогену. Интересны также две работы, выполненные на картофеле. Одна из них посвящена диагностике мозаичных вирусов картофеля у дикорастущих видов из коллекции генбанка ВИР, а в другой с помощью биоинформатического подхода выявлены гены-кандидаты, определяющие устойчивость к биотическим и абиотическим стрессовым факторам.

К настоящему времени возросла актуальность исследований по поиску и изучению штаммов микроорганизмов, используемых для биоремедиации вод и грунтов. В экспериментальной статье раздела «Генетика и селекция микроорганизмов» представлены данные по отбору и тестированию активности штамма рода *Trichoderma* для ускоренной утилизации полимеров растительных остатков и целлюлозосодержащих материалов с целью оздоровления почвы и биоконтроля почвообитающих фитопатогенных видов. В следующей статье описан методический подход для количественного анализа различных образцов, содержащих вирусы животных, растений и микроорганизмов. Для диагностики вирусов предлагается использовать электронную микроскопию, которая имеет ряд преимуществ в сравнении с традиционными методами.

В обзорной статье раздела «Генетика животных» анализируются данные, раскрывающие природу меланиновой окраски оперения у птиц на примере кур, и молекулярно-генетические механизмы появления разных типов окраски. Известно, что межвидовая гибридизация – один из способов расширения генетического разнообразия животных. Протекание мейотического процесса и нарушения, которые возникают при отдаленных скрещиваниях, рассмотрены в оригинальном исследовании на примере домашней овцы *Ovis aries*.

Заключительная рубрика «Экологическая генетика» включает две экспериментальные статьи. В первой обсуждаются вопросы генетической дифференциации популяций евразийской свиязи (*Mareca penelope*) – одного из многочисленных перелетных видов уток. Проведено сравнение генетической дифференциации вида, полученное с помощью данных кольцевания птиц и результатов секвенирования митохондриальной ДНК. Во второй статье дан анализ нуклеотидных последовательностей митоходриального генома у природных популяций *Drosophila melanogaster*. Полученные авторами результаты рассмотрены с точки зрения адаптивности различных митогаплотипов дрозофил.

Академик В.К. Шумный

Вавиловский журнал генетики и селекции. 2019;23(3):256-261
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Влияние различий в третьем домене гликопротеина Е вируса клещевого энцефалита дальневосточного, сибирского и европейского субтипов на связывание рекомбинантных белков D3 с химерным антителом

И.К. Байков[1] ✉, А.Л. Матвеев[1, 2], Л.А. Емельянова[1, 2], Г.Б. Каверина[1], С.Е. Ткачёв[1], Н.В. Тикунова[1, 2] ✉

[1] Институт химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
✉ e-mail: tikunova@niboch.nsc.ru; ivan_baykov@mail.ru

Целью настоящей работы было исследование влияния различий аминокислотной последовательности рекомбинантных доменов D3 гликопротеина Е вируса клещевого энцефалита дальневосточного, Сибирского и европейского субтипов на связывание протективного антитела ch14D5 с этими белками. Методами вестерн-блот анализа и поверхностного плазмонного резонанса было показано, что наибольшее сродство (K_D = 1.7 ± 0.5 нМ) антитело ch14D5 проявляет к домену D3 вируса клещевого энцефалита штамма «Софьин-Ru», принадлежащего к дальневосточному субтипу вируса. В то же время сродство к аналогичным белкам D3, полученным на основе штаммов «Заусаев», «1528-99» и «Абсеттаров» сибирского и европейского субтипов вируса клещевого энцефалита, оказалось заметно ниже (K_D = 25 ± 4, 300 ± 50 и 250 ± 50 нМ соответственно). Кроме того, информация о пространственном расположении аминокислотных остатков, которыми отличаются полученные рекомбинантные белки, указывает на то, что узнаваемый антителом ch14D5 эпитоп находится в области бокового ребра домена D3 гликопротеина Е.
Ключевые слова: вирус клещевого энцефалита; гликопротеин Е; домен D3; антитело; рекомбинантный белок; поверхностный плазмонный резонанс; картирование эпитопа.

Для цитирования: Байков И.К., Матвеев А.Л., Емельянова Л.А., Каверина Г.Б., Ткачёв С.Е., Тикунова Н.В. Влияние различий в третьем домене гликопротеина Е вируса клещевого энцефалита дальневосточного, сибирского и европейского субтипов на связывание рекомбинантных белков D3 с химерным антителом. Вавиловский журнал генетики и селекции. 2019;23(3):256-261. DOI 10.18699/VJ19.490

The effect of differences in the third domain of the glycoprotein E of tick-borne encephalitis virus of the Far Eastern, Siberian and European subtypes on the binding of recombinant D3 proteins with a chimeric antibody

I.K. Baykov[1] ✉, A.L. Matveev[1, 2], L.A. Emelianova[1, 2], G.B. Kaverina[1], S.E. Tkachev[1], N.V. Tikunova[1, 2] ✉

[1] Institute of Chemical Biology and Fundamental Medicine, SB RAS, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
✉ e-mail: tikunova@niboch.nsc.ru; ivan_baykov@mail.ru

Currently, a therapeutic drug based on recombinant antibodies for the prevention and treatment of tick-borne encephalitis virus (TBEV) is developed in ICBFM SB RAS, and the chimeric antibody ch14D5 is considered as one of the key components of this drug. It was previously shown that this antibody is directed to the domain D3 of the glycoprotein E of TBEV. It was previously shown that this antibody is able to protect mice from the European subtype of TBEV, strain "Absettarov", and the presence of virus-neutralizing activity against the Far Eastern subtype of TBEV, strain 205 was also shown for this antibody. However, it remains unclear whether this antibody exhibits selectivity for different subtypes of TBEV. The aim of this study was to investigate the effect of amino acid sequence differences of recombinant D3 domains derived from the glycoprotein E of TBEV of the Far Eastern, Siberian and European subtypes on the binding of the protective antibody ch14D5 to these proteins. Using Western blot analysis and surface plasmon resonance, it was shown that ch14D5 antibody has the highest affinity (K_D = 1.7 ± 0.5 nM) for the D3 domain of the TBEV of the "Sofjin-Ru" strain belonging to the Far Eastern subtype of the virus. At the same time, the affinity of ch14D5 antibody for similar D3 proteins derived from "Zausaev", "1528-99" and "Absettarov" strains of the Siberian and European subtypes of TBEV was noticeably lower (K_D = 25 ± 4, 300 ± 50, 250 ± 50 nM, respectively). In addition, information about the spatial arrangement of amino acid residues that are different for the studied

recombinant proteins indicates that the epitope recognized by the ch14D5 antibody is in close proximity to the lateral ridge of D3 domain of E glycoprotein.
Key words: tick-borne encephalitis virus; glycoprotein E; domain D3; antibody; recombinant protein; surface plasmon resonance; epitope mapping.

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Введение

Вирус клещевого энцефалита (ВКЭ) относится к роду *Flavivirus*, переносится иксодовыми клещами и вызывает у людей тяжелые нейроинфекции. Ежегодно в мире регистрируют около 10 тыс. случаев заболевания клещевым энцефалитом (Heinz, Stiasny, 2012). В настоящее время единственным специфическим препаратом для профилактики и лечения клещевого энцефалита является «Иммуноглобулин человека против клещевого энцефалита» (далее ВКЭ-Ig), представляющий собой антитела IgG, получаемые из сыворотки крови иммунизированных доноров, а также людей, проживающих на эндемичных по клещевому энцефалиту территориях. Этот препарат, производимый в Российской Федерации, используется только на территории России, а также в Казахстане, в то время как в большинстве стран Европы специфические средства профилактики и лечения клещевого энцефалита отсутствуют. Поскольку препараты сывороточных антител имеют ряд ограничений, связанных с нестабильностью характеристик и повышенным уровнем биологического риска при использовании, в мировой практике наблюдается тенденция замены таких препаратов более перспективными и безопасными лекарственными средствами на основе рекомбинантных антител. Разработка препарата для профилактики и лечения клещевого энцефалита – одно из приоритетных направлений Института химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук (ИХБФМ СО РАН).

Ранее был получен набор мышиных моноклональных антител против вируса клещевого энцефалита (Tsekhanovskaya et al., 1993) и продемонстрированы высокие противовирусные свойства некоторых из них как *in vitro*, так и *in vivo* (Levanov et al., 2010; Baykov et al., 2014). В случае антитела ch14D5, которое наряду с другими антителами может быть использовано для создания современного иммунопрепарата против ВКЭ, протективная активность была исследована только в отношении европейского субтипа вируса клещевого энцефалита, штамм «Абсеттаров» (Baykov et al., 2014), в то время как штаммы других субтипов ВКЭ не исследованы. Кроме того, было установлено, что узнаваемый антителом ch14D5 эпитоп расположен в домене D3 гликопротеина Е (Байков и др., 2018). Хотя последовательность аминокислотных остатков этого домена высоко консервативна для ВКЭ, при сравнении различных штаммов этого вируса иногда выявляются единичные аминокислотные замены в этой части гликопротеина Е. В случае, если эти различия попадают в область связывания антитела, они могут существенно влиять на величину сродства антитела ch14D5 к белку Е, и, возможно, на противовирусные свойства этого антитела. Таким образом, в настоящее время остается открытым вопрос о том, проявляет ли ch14D5 избирательность по отношению к различным субтипам вируса клещевого энцефалита.

Цель исследования – выяснить, влияют ли различия в последовательности аминокислотных остатков рекомбинантных доменов D3, полученных на основе гликопротеина Е вируса клещевого энцефалита дальневосточного, сибирского и европейского субтипов, на связывание протективного антитела ch14D5 с этими белками.

Материалы и методы

Материалы. Химерное антитело ch14D5a было получено и очищено согласно методике, опубликованной ранее (Baykov et al., 2014). Использованная в работе кДНК различных штаммов ВКЭ была получена в реакции обратной транскрипции, совмещенной с ПЦР, на основе суммарной РНК, выделенной из индивидуальных клещей, собранных на территории Сибири и Дальнего Востока.

Получение генетических конструкций, кодирующих белки D3_Eu, D3_ZauM и D3_Bal. На основе кДНК, полученной для образцов TBEV-2781 (штамм «Заусаев» сибирского субтипа ВКЭ), 126-17 (штамм «Абсеттаров» европейского субтипа ВКЭ), 1528-99 (балтийская линия сибирского субтипа ВКЭ) из коллекции ИХБФМ СО РАН с помощью олигонуклеотидов D3_NcoI_dir: 5′-GCGCCATGGCCGGCGGTGGCTCGGGTCTTACATACACAATGTGCG-3′; и D3_his_NotI_rev: 5′- TTAGCGGCCGCTTAGTGATGGTGATGATGATGACTCCCTTTTTGGAACCATTG-3′ были получены ПЦР-фрагменты размером около 330 п. н. Фрагменты ДНК, кодирующие белки D3_Eu, D3_ZauM и D3_Bal, были встроены в плазмидную ДНК pHEN2 по сайтам узнавания эндонуклеаз рестрикции *Nco*I и *Not*I. Правильность конструкций pHEN2-D3_ZauM, pHEN2-D3_Eu и pHEN2-D3_Bal подтверждали секвенированием.

Получение рекомбинантных белков. Бактерии *E. coli* HB2151, трансформированные соответствующей плазмидной ДНК, растили в среде LB с добавлением ампициллина до концентрации 100 мкг/мл и 0.1 % глюкозы при скорости перемешивания 180 об./мин при 37 °С. При достижении оптической плотности OD_{600} = 0.7–0.9 индуцировали синтез белка добавлением изопропил-бета-тиогалактозида до конечной концентрации 0.5 мМ; культивирование продолжали при скорости перемешивания 180 об./мин и температуре 30 °С. Через 4 ч биомассу отделяли от культуральной жидкости центрифугированием в течение 10 мин при 6000 g, осадок ресуспендировали в буфере, содержащем 20 % сахарозы, 1 мМ этилендиаминтетраацетата натрия (ЭДТА) и 10 мМ Трис-HCl pH 7.5, взятом в количестве 1/10 исходного объема жидкой культуры. После инкубации 5 мин при комнатной температуре и 5 мин при 0 °С клетки осаждали 2 мин при 10000 g и температуре 6 °С. После удаления супернанта

клеточный осадок ресуспендировали в 5 мМ растворе $MgSO_4$, взятом в количестве 1/10 исходного объема жидкой культуры, и инкубировали 5 мин при 0 °С. Осадок сферопластов отделяли центрифугированием 2 мин при 10000 g и температуре 6 °С, а супернатант, содержащий периплазматические белки, фильтровали через полиэфирсульфоновый фильтр с размером пор 0.22 мкм и анализировали электрофорезом в 15 % полиакриламидном геле.

Вестерн-блот анализ. Растворы периплазматических белков, содержащие целевые белки D3_Sof, D3_Bal, D3_ZauM и D3_Eu, фракционировали электрофорезом в 15 % денатурирующем полиакриламидном геле, после чего белки переносили на нитроцеллюлозную мембрану (Bio-Rad) методом электропереноса. Далее анализ проводили аналогично тому, как это описано в (Байков и др., 2018). Рекомбинантные белки выявляли раствором антитела ch14D5 в концентрации 1 мкг/мл. Иммунные комплексы выявляли вторичным антителом, конъюгированным со щелочной фосфатазой, Anti-Human IgG (whole molecule)−Alkaline Phosphatase antibody produced in goat, A1543 (Sigma). Окрашенную мембрану промывали дистиллятом и сканировали.

Исследование взаимодействия антитела с рекомбинантными белками D3 методом поверхностного плазмонного резонанса. Взаимодействие антитела ch14D5 с рекомбинантными белками D3 исследовали на оптическом биосенсоре ProteOn XPR36, в качестве системного буфера использовали фосфатно-солевой буферный раствор с добавлением 0.005 % твин-20 и 0.1 мМ ЭДТА. Поверхность HTG-чипа активировали пропусканием 1 мМ водного раствора $Ni(NO_3)_2$ в течение 120 с. Образцы периплазматических белков, содержащие какой-либо из целевых белков, использовали для иммобилизации на поверхность HTG-чипа до достижения уровня сигнала 50−70 единиц отклика. Неспецифически связавшиеся белки отмывали пропусканием 25 мМ раствора имидазола. Последовательные трехкратные разведения антитела ch14D5a анализировали на связывание с рекомбинантными белками. После первоначального скрининга для белков D3_Eu и D3_Bal был выбран диапазон концентраций 405, 135, 45, 15 и 5 нМ, для белков D3_ZauM и D3_Sof – 81, 27, 9, 3 и 1 нМ. Диапазон концентраций выбирали так, чтобы он охватывал концентрацию, равную по значению K_D для исследуемого взаимодействия. В качестве референсного сигнала использовали сигнал, зарегистрированный для буфера, не содержащего антитела, а также сигнал, полученный при пропускании разведений антитела в той части чипа, где не было иммобилизовано белков. Скорректированный таким образом сигнал использовали для вычисления кинетических и равновесных констант методом глобального выравнивания с использованием простой модели односайтового связывания с помощью программного обеспечения PreoteOn Manager 3.1.0.

Анализ последовательностей гена Е вируса клещевого энцефалита и визуализация различий на пространственных моделях гликопротеина Е и вириона ВКЭ. Последовательности аминокислотных остатков белков D3_Sof, D3_Bal, D3_ZauM и D3_Eu выравнивали с помощью программы MEGA 5 методом Clustal. Для визуализации расположения различий на пространственной модели гликопротеина Е использовали программу PyMol 1.8 и файл координат pdb_id: 1svb.

Результаты

На первом этапе на основе вирусной РНК штаммов «Заусаев» и «1528-99», относящихся к сибирскому субтипу вируса клещевого энцефалита, а также штамма «Абсеттаров», относящегося к европейскому субтипу ВКЭ, были получены фрагменты ДНК, кодирующие домен D3 гликопротеина Е этих вирусов. Фрагменты были встроены в плазмидную ДНК pHEN2-rED3_301, использованную нами ранее для получения фрагмента D3 штамма «Софьин-Ru» (Байков и др., 2018). После индукции синтеза белка из клеток были выделены фракции периплазматических белков, содержащих целевые белки D3_Sof, D3_Eu, D3_Bal и D3_ZauM (рис. 1), соответствующие штаммам «Софьин-Ru», «Абсеттаров», «1528-99» балтийской линии и «Заусаев».

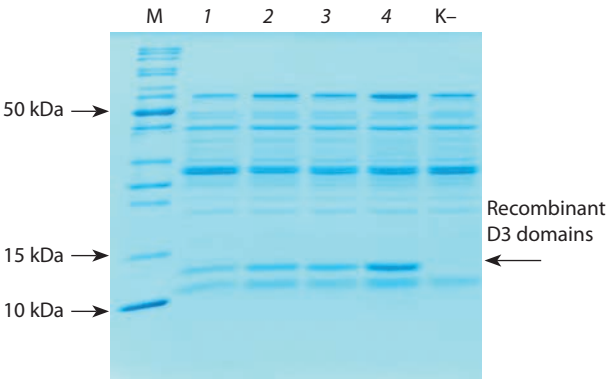


Fig. 1. 15 % PAGE image of periplasmic fractions of bacterial cells containing plasmids. Lanes: *1*, pHEN2-D3_Eu; *2*, pHEN2-D3_Bal; *3*, pHEN2-D3_ZauM; *4*, pHEN2-rED3_301.

K is the periplasmic fraction of cells containing no plasmid DNA; M, protein molecular weight ladder (Thermo scientific #26614).

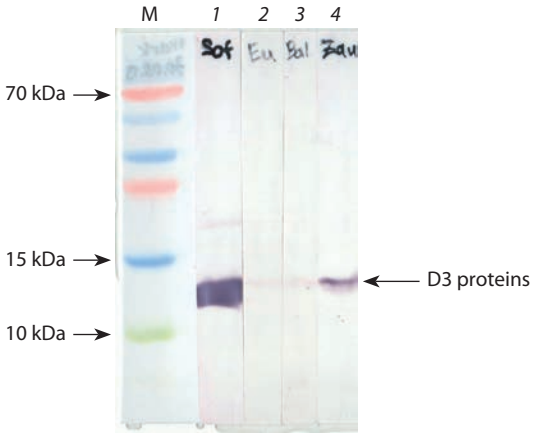


Fig. 2. Western blot analysis of recombinant D3 proteins with ch14D5 antibody.

Periplasmic fraction protein samples probed with ch14D5 antibody. Lanes: *1*, D3_Sof protein; *2*, D3_Eu protein; *3*, D3_Bal protein; *4*, D3_ZauM protein. M, molecular weight ladder (Thermo scientific #26619). Protein complexes were visualized using Sigma #A1543 secondary antibody conjugate.

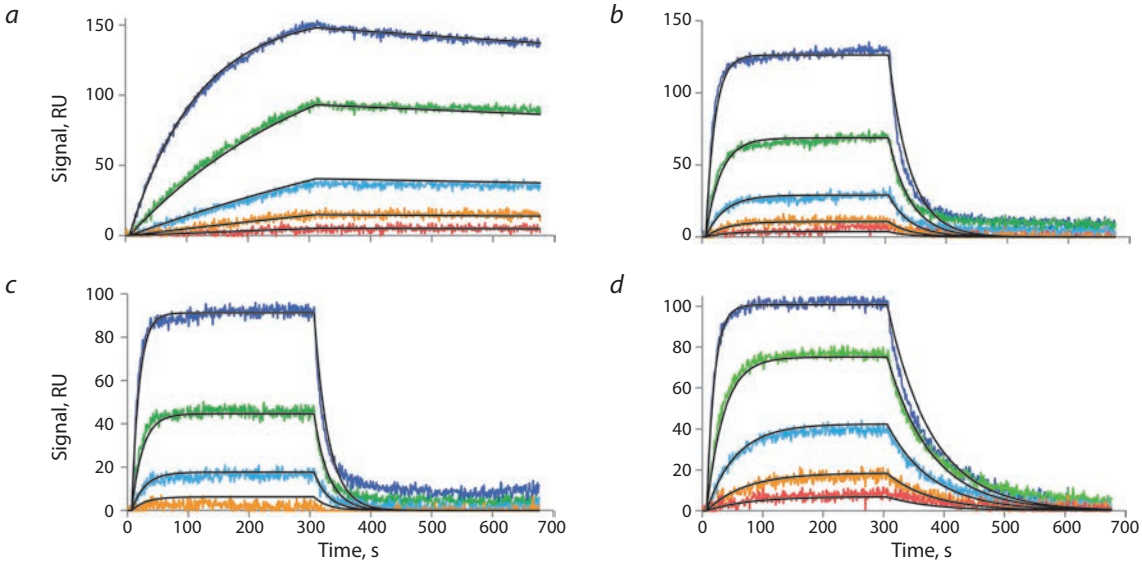


Fig. 3. Surface plasmon resonance (SPR) analysis of ch14D5 antibody binding to recombinant proteins. (*a*) D3_Sof, (*b*) D3_Eu, (*c*) D3_Bal and (*d*) D3_ZauM.

Experimental traces are shown in colors, approximation lines are shown in black.

Далее методом вестерн-блот анализа было исследовано взаимодействие химерного антитела ch14D5 с полученными рекомбинантными белками. Количество материала для переноса белков было выбрано так, чтобы на нитроцеллюлозной мембране оказалось равное количество целевых белков. В результате было показано, что химерное антитело проявляет наибольшее сродство к варианту D3_Sof. Вариант D3_ZauM окрашивался со значительно меньшей интенсивностью, а варианты D3_Eu и D3_Bal окрасились наиболее бледно (рис. 2).

Кроме того, сродство антитела ch14D5 к полученным рекомбинантным белкам было определено методом поверхностного плазмонного резонанса (рис. 3). Значения равновесных констант диссоциации составили 1.7±0.5 нМ для белка D3_Sof, 250±50 нМ для белка D3, _Eu, 300±50 нМ для белка D3_Bal и 25±4 нМ для белка D3_ZauM, что хорошо согласуется с качественными данными, полученными методом вестерн-блот анализа.

Обсуждение

Один из современных подходов при профилактике и лечении вирусных инфекций – использование препаратов на основе специфических вируснейтрализующих либо протективных антител (Lambour et al., 2016; Salazar et al., 2017). Для флавивирусных инфекций этот подход также применим, и в настоящее время разрабатывают терапевтические антитела против вируса клещевого энцефалита, вируса Западного Нила, вируса лихорадки Денге, вируса Зика, вируса желтой лихорадки и других флавивирусов (Oliphant et al., 2005; Lai et al., 2010; Sautto et al., 2013; Baykov et al., 2014; Julander et al., 2014; Fuzik et al., 2018). В зависимости от того, какой эпитоп на поверхности вирусного белка узнает то или иное антитело, антитела могут либо быть протективными, либо, наоборот, усиливать развитие инфекции. Так, антитела, направленные к третьему домену флавивирусного гликопротеина Е, часто обладают выраженными противовирусными свойствами (Roehrig, 2003; Oliphant et al., 2005; Sánchez et al., 2005; Dai et al., 2016). Это вызвано тем, что именно третий домен гликопротеина Е флавивирусов участвует в связывании с клеточными рецепторами. Антитела, направленные к доменам D1 и D2, часто усиливают инфекцию, что делает их не только бесполезными, но даже опасными (Dowd, Pierson, 2011; Halstead, 2014; Haslwanter et al., 2017; Katzelnick et al., 2017).

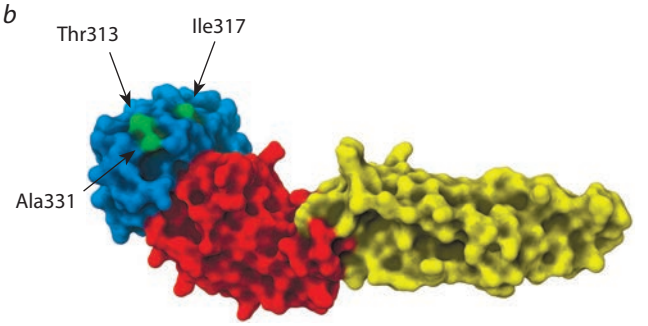
В настоящей работе мы изучили связывание антитела ch14D5 с рекомбинатными белками, представляющими собой фрагменты гликопротеина Е ВКЭ европейского, сибирского и дальневосточного субтипов. Поскольку ранее было установлено, что антитело ch14D5 связывается с доменом D3 гликопротеина Е (Байков и др., 2018), то в исследовании были использованы рекомбинантные домены D3, продуцируемые бактериями *E. coli* в растворимом мономерном виде. Методами вестерн-блот анализа и поверхностного плазмонного резонанса было обнаружено, что сродство антитела ch14D5 к различным вариантам домена D3 различается более чем на два порядка. При постановке экспериментов мы постарались исключить возможное влияние на результаты экспериментов факторов, связанных с продукцией белка: белки D3_Sof, D3_Eu, D3_Bal и D3_ZauM нарабатывали и выделяли одновременно в идентичных условиях. Дизайн эксперимента на биосенсоре ProteOn XPR36 был выбран таким образом, что рекомбинантные белки D3 были иммобилизованы на поверхность, а анализируемое антитело находилось в растворе. Соответственно, в случае потенциально гетерогенного образца, в котором часть молекул целевого белка имеет далекую от нативной конформацию, изменились бы количество образующихся комплексов и уровень детектируемого сигнала, но не детектируемые кинетические константы k_{on}, k_{off} и равновесная константа диссоциации K_D, характеризующая степень сродства антитела

a

```
        ↓301                                          ↓340
D3_Sof  GLTYTMCDKT KFTWKRIPTD SGHDTVVMEV AFSGTKPCRI PVRAVAHGSP
D3_ZauM .......... ..A...T... .......... M......... ..........
D3_Bal  .......... ......T... .......... T......... ..........
D3_Eu   .......... ......A... .......... T......... ..........

        |350                                                 |397
D3_Sof  DVNVAMLITP NPTIENNGGG FIEMQLPPGD NIIYVGELSH QWFQKGS
D3_ZauM ..........  ..........  ..........  ..........  .......
D3_Bal  ..........  ..........  ..........  ..........  .......
D3_Eu   ..........  ..........  ..........  ..........  .......
```

b



c

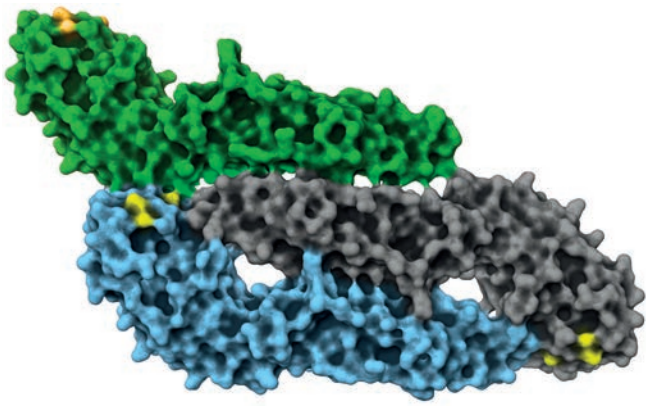


Fig. 4. Amino acid sequence differences between D3_Sof, D3_Bal, D3_ZauM and D3_Eu proteins located on the surface of TBEV E glycoprotein.

(*a*) Amino acid sequence alignment; (*b*) TBEV E glycoprotein spatial structure. Differences between D3_Sof, D3_Bal, D3_ZauM and D3_Eu proteins are shown in green. D1, D2, and D3 domains are shown in red, yellow, and blue, respectively; (*c*) TBEV virion frag-ment that illustrates the spatial accessibility of Thr313, Ile317, and Ala331 amino acid residues (shown in yellow). Three molecules of E glycoprotein are shown in green, light blue and gray.

к рекомбинантным белкам. Кроме того, эксперименты по наработке рекомбинантных белков и анализу на биосенсоре проводили в нескольких повторах с получением сходных результатов.

Сродство антитела к рекомбинантным фрагментам вирусного гликопротеина Е различных штаммов ВКЭ, вероятно, коррелирует со способностью антитела нейтрализовать инфекционность вируса или обеспечивать протекцию животных. Естественно, механизмы противовирусного действия каждого конкретного антитела достаточно гибкие и могут включать множество путей, поэтому корреляция, скорее всего, далека от 100 %. Вместе с тем в случае нескольких антител против ВКЭ было показано, что разница в сродстве антитела к антигену хорошо согласуется с разницей в нейтрализующей активности *in vitro* этих антител (Tsekhanovskaya et al., 1993; Levanov et al., 2010; Baykov et al., 2014). В проведенных нами экспериментах наиболее слабое сродство, около 250 нМ, было зарегистрировано по отношению к белкам D3_Eu и D3_Bal, полученным на основе гена Е штамма «Абсеттаров» европейского субтипа ВКЭ и штамма «1528-99» балтийской линии сибирского субтипа ВКЭ соответственно. Ранее было установлено, что антитело ch14D5 обладает высокой протективной активностью в отношении штамма «Абсеттаров» и способно блокировать развитие инфекции на мышиной модели клещевого энцефалита при однократном введении в дозировке 80 мкг/мышь (Baykov et al., 2014). Поскольку сродство к белкам D3_ZauM и D3_Sof, полученным на основе штаммов «Заусаев» и «Софьин_Ru» сибирского и дальневосточного субтипов (K_D = 25±4 и 1.7±0.5 нМ соответственно), оказалось выше сродства к белку D3_Eu, полученному на основе штамма «Абсеттаров» (K_D = 250± ±50 нМ), то мы полагаем, что протективная активность антитела ch14D5 по отношению к большинству штаммов сибирского и дальневосточного субтипов ВКЭ либо окажется на том же уровне, что и протективная активность этого антитела по отношению к штамму «Абсеттаров» (Baykov et al., 2014), либо будет выше.

Следует отметить, что поскольку штаммы как сибирского, так и дальневосточного субтипов обладают некоторой вариабельностью последовательности аминокислотных остатков гликопротеина Е, то в случае некоторых отдельных штаммов ВКЭ с нетипичными аминокислотными остатками в области эпитопа антитело ch14D5 может проявлять сниженную активность. В то же время, как только эпитоп, узнаваемый антителом ch14D5, будет определен с точностью до отдельных аминокислотных остатков, подобные случаи можно будет прогнозировать на основе данных о нуклеотидной последовательности гена Е каждого конкретного штамма ВКЭ.

Зарегистрированные в настоящем исследовании различия в прочности связывания антитела ch14D5 с белками D3 разных субтипов ВКЭ могли быть вызваны либо тем, что отличающиеся аминокислотные остатки находились в области эпитопа, узнаваемого антителом, либо тем, что белки D3 обладали разной стабильностью и, соответственно, пространственная структура была более подвижна в случае менее стабильных белков, что могло приводить к ослаблению сродства антитела к белку. Анализ различий в последовательностях аминокислотных остатков исследованных белков D3 (рис. 4, *а*) показал, что соответствующие аминокислотные остатки пространственно сгруппированы и расположены на поверхности домена D3 (см. рис. 4, *б*). Более того, аминокислотные остатки Thr313 и Ala331 находятся в области бокового ребра домена D3, известного тем, что антитела к этой области домена D3 обладают наиболее выраженными противовирусными свойствами (Roehrig, 2003; Oliphant et al., 2005; Sánchez et al., 2005).

Поскольку аминокислотные остатки Thr313, Ile317 и Ala331 расположены на поверхности и не затрагивают внутреннюю структуру домена D3, то крайне малове-

роятно, что они влияют на стабильность этого домена, который сам по себе стабилен (Zidane et al., 2013). По-видимому, эти аминокислотные остатки находятся в области эпитопа, узнаваемого антителом ch14D5, тем более что этот участок домена D3 расположен на поверхности вириона и пространственно доступен для связывания антител (см. рис. 4, *в*).

Заключение

В результате проведенного исследования показано, что сродство химерного антитела ch14D5 к домену D3 гликопротеина Е различных субтипов вируса клещевого энцефалита существенно различается. Наименьшее сродство антитело проявляет к белку, полученному на основе штамма «Абсеттаров», и в то же время известно, что антитело ch14D5 обладает высокими протективными свойствами по отношению к штамму «Абсеттаров». Поэтому есть все основания полагать, что протективная активность этого антитела по отношению к штаммам сибирского и дальневосточного субтипов будет также высокой. Кроме того, установлено, что эпитоп, узнаваемый антителом ch14D5 на поверхности гликопротеина Е, находится в области бокового ребра домена D3, что, по-видимому, обусловливает высокие противовирусные свойства этого антитела.

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Association of haplotypes for SNPs in the LTR regions of bovine leukemia virus with hematological indices of cattle

N.V. Blazhko[1], S.Kh. Vyshegurov[1], A.S. Donchenko[1, 2], K.S. Shatokhin[1] ✉, T.I. Krytsyna[1], V.A. Ryabinina[1]

[1] Novosibirsk State Agrarian University, Novosibirsk, Russia
[2] Siberian Federal Research Centre for AgroBiotechnology, RAS, Krasnoobsk, Novosibirsk region, Russia
✉ e-mail: true_genetic@mail.ru

Molecular typing of *BLV* samples isolated from Holsteinized Russian Black Pied cattle was carried out, and various cytofluorometric and morphological blood indices were examined. We performed the total count of white blood cells (WBC), lymphocyte (lymf), granulocyte (gran), monocyte (mon), red blood cell (RBC), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet crit count (PCT). The LTR-region of *BLV* was haplotyped. Only viruses of haplotypes I (0.33 ± 0.03) and III (0.67 ± 0.03) of the eight possible were detected. The ratio of hematologically sick, healthy, and suspected carriers of *BLV* of haplotypes I and II was comparable with the results of other researchers. The numbers of leukocytes, erythrocytes and platelets in the blood of carriers of haplotype III exceeded the corresponding parameters of cattle affected by the virus of haplotype I. It is interesting to note that the difference in the hemolytic status of animals was manifested not only by the concentration of leukocytes as direct immune agents but also by the count of erythrocytes and platelets, which are not directly involved in the immune response. The number of particles of haplotype III of the *BLV* circulating in the blood of infected individuals exceeded that of the carriers of haplotype I. In this connection, an assumption was made about the evolutionary advantage of the more virulent haplotype III. However, the results of our own research in conjunction with the data of other scientists indicate that the high virulence of individual virus strains is a consequence of the tendency to implement the maximum possible intensity of the synthesis of virus particles but not of the high damaging effect alone. It is shown that high lethality is evolutionarily disadvantageous for viruses, since the extinction of the carrier as a biological species is fraught with the disappearance of the virus itself.
Key words: *BLV*; LTR-region; haplotypes; hematological indices; leukocytes; cattle.

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Связь гаплотипов SNP LTR-области *BLV* с гематологическими показателями крови крупного рогатого скота

Н.В. Блажко[1], С.Х. Вышегуров[1], А.С. Донченко[1, 2], К.С. Шатохин[1] ✉, Т.И. Крыцына[1], В.А. Рябинина[1]

[1] Новосибирский государственный аграрный университет, Новосибирск, Россия
[2] Сибирский федеральный научный центр агробиотехнологий Российской академии наук, р. п. Краснообск, Новосибирская область, Россия
✉ e-mail: true_genetic@mail.ru

Проведено молекулярно-генетическое типирование образцов вируса лейкоза крупного рогатого скота (*BLV*), выделенного из образцов крови черно-пестрых голштинизированных коров, у которых были исследованы различные цитофлюорометрические и морфологические показатели крови. Оценивали общее содержание лейкоцитов (WBC), содержание лимфоцитов (lymf), гранулоцитов (gran), моноцитов (mon), эритроцитов (RBC), гемоглобина (HGB), гематокрит (HTC), средний объем эритроцитов (MCV), среднее содержание гемоглобина в одном эритроците (MCH), концентрацию гемоглобина в эритроцитарной массе (MCHC), индекс распределения эритроцитов (RDW), количество тромбоцитов (PLT), средний объем тромбоцитов (MPV), индекс распределения тромбоцитов (PDW) и тромбокрит (PCT). Определены гаплотипы SNP LTR-области *BLV*. Из восьми возможных были обнаружены только вирусы гаплотипов I (0.33 ± 0.03) и III (0.67 ± 0.03). Соотношение гематологически больных, здоровых и подозрительных носителей вируса лейкоза крупного рогатого скота I и III гаплотипов было сопоставимо с результатами других исследователей. Количество лейкоцитов, эритроцитов и тромбоцитов в крови носителей III гаплотипа превышало аналогичные параметры крупного рогатого скота, пораженного вирусом I гаплотипа. Интересно отметить, что разница

гематологического статуса животных проявилась не только в концентрации лейкоцитов, непосредственных иммунных агентов, но и в содержании эритроцитов и тромбоцитов, не имеющих к иммунному ответу непосредственного отношения. Количество частиц III гаплотипа *BLV*, циркулирующих в крови зараженных особей, превышала таковое значение носителей I гаплотипа. В связи с этим выдвинуто предположение об эволюционном преимуществе III гаплотипа как более вирулентного. Впрочем, результаты настоящего исследования в совокупности с данными других ученых показывают, что высокая вирулентность отдельных штаммов вируса есть следствие стремления к реализации максимально возможной интенсивности синтеза вирусных частиц, а не достижения высокого поражающего эффекта как такового. Показано, что высокая смертоносность эволюционно невыгодна вирусам, так как вымирание носителя как биологического вида чревато исчезновением и самого вируса.
Ключевые слова: *BLV*; LTR-область; гаплотипы; гематологические показатели крови; лейкоциты; крупный рогатый скот.

Introduction

The bovine leukosis epizootic situation inspires intensive development of strategies aimed at preventing the spread of the disease. It comes down to the isolation of sick animals from healthy ones or to the slaughter of infected individuals (Knapen et al., 1993; Nuotio et al., 2003; Acaite et al., 2007). The latter method proved to be very effective in the countries of Western Europe, New Zealand and Australia, where the purification of herds from the pathogen (*BLV*) is complete or almost complete (Polat et al., 2017).

Despite these measures, bovine leukosis is by far the most common epizootic disease in Russia and some other countries (Juliarena et al., 2017). It was identified in 28 regions of the Russian Federation (175 adverse sites) in 2017. The largest number of adverse sites for the disease, 45, was found in Kaluga region; in the Republic of Crimea, 32; Novosibirsk oblast, 27; and Moscow oblast, 20 (Novikova et al., 2018). According to some data (Kozyreva, Gulyukin, 2017), leukosis constituted about 65–66 % of the cases of infectious diseases in 2015.

One of the likely reasons for the low efficiency of bovine leukosis control is the high percentage (70 to 90 %) of animals with the asymptomatic stage (Ernst et al., 1997; Smirnov Yu.P. et al., 2015; Gyles, 2016; Juliarena et al., 2017), as characterized, among other things, by the normal nonpathological number of leukocytes, in particular, lymphocytes. The clinical stage is typically observed in 4 to 5-year-old animals, where, in the overwhelming majority of cases, the economic use of dairy cows is nearing its completion (Smirnov P.N. et al., 2015). Sometimes the latent period can be delayed to 8 years of age (Kettmann et al., 1994). In some cases, slaughter of animals infected with *BLV* but culled for other reasons not related to the clinical manifestations of leukosis was recorded (Mishchenko et al., 2018).

PCR diagnostics (Smirnov P.N. et al., 2015) and enzyme-linked immunosorbent assay (ELISA) (Syurin et al., 2001) are effective methods for identifying *BLV* carriers, but their high cost significantly hinders widespread use.

Another complicating factor is the high mutational variability associated with viruses (Lewin, 2008). In particular, there is a hypothesis about the accumulation of *BLV* mutations that allow the virus to avoid the host's immune response (Blood et al., 1979; Syurin et al., 2001; Buehring et al., 2003; Smirnov, 2007; Smirnov et al., 2011; Batenyova, 2015). For the virus itself, such mutations are undoubtedly beneficial and therefore must be supported by natural selection.

The LTR region contains the so-called housekeeping genes, among which there are regulators of mRNA transcription and translation. The collinear nucleotide sequence of the virus studied shows that at least some of the evolutionary phenotypic "acquisitions" stem from mutations not in protein-coding genes, but in the household genes, due to which mutagenesis is accelerated (Barrick et al., 2009). Indeed, mutations in the nucleotide sequence of the LTR region can activate mutagenesis in *BLV* (Merezak et al., 2001). A study of the LTR regions of other viruses gave similar results (Moelling, 2016). It is logical to assume that it was mutations in the LTR-region of *BLV* that could contribute to the evolutionary flexibility of the virus and provoke its ability to avoid the host's immune response. It is likely that more virulent strains that efficiently translate mRNA have the maximum advantage, thereby causing the greatest damage to the carrier. The aim of our research is to evaluate the hypothesis of the evolutionary advantage of mutant *BLV* strains due to higher virulence.

Materials and methods

Experiments were done with total DNA samples isolated from 780 cows of the Holsteinized Russian Black Pied breed. Blood samples were taken from the tail vein with sterile catheters using EDTA as an anticoagulant in 2015–2016. DNA was isolated with the DNA-Sorb-B kit (Central Research Institute of Epidemiology, Russia).

Cytofluorimetric and morphological blood indices were determined with an automatic veterinary haematology analyzer PCE-90 Vet.

Oligonucleotide primers were designed with regard to the mutational status of the isolate sequences. The annealing temperature of the primers was calculated from the percentages of nucleotides in the oligonucleotide sequences (Table 1).

Primers were synthesized according to the sequence in an automatic oligonucleotide synthesizer. Primer purity was monitored by High Sensitivity Gas Chromatography and found to be no less than 95 %. Primers were stored at –20 °C for no more than six months.

Table 1. Characteristics of oligonucleotide primers flanking the LTR region of *BLV*, 443 bp

Criterion	Forward primer	Reverse primer
Sequence (5′->3′)	CCCCATRCGACCGGTTACAC	AGAGRRCTCRGGACCGAGAG
Flanking start site	8021	8444
Flanking end site	8040	8463
Annealing t°	60.18	60.11
GC, %	60.00	60.00

Table 2. The amplification mode for LTR 443 bp

Number of cycles	Temperature, °C	Time, min
1	95	3.0
35	95	0.5
	61	0.5
	72	0.5
1	72	3.0

Table 3. Composition of the PCR mixture (per one reaction)

Components	Required volume, μL
PCR buffer	2.5
$MgCl_2$	1.0
dNTP	1.0
Pr 1, 50 ng	1.0
Pr 2, 50 ng	1.0
Taq pol	1.5
Water	15.0
DNA, 50 ng	2

Before starting the polymerase chain reaction, the amplification mode was programmed. Based on the calculated annealing temperature of the primers, a special program was used to program the amplifier (Table 2).

The required number of components of the reaction was calculated for the required number of samples in order to prepare the PCR mixture. We determined the total number of reactions as $n + 3 + 1$, where n is the number of DNA samples that need to be diagnosed; 3 is the number of controls used in the reaction (IC is the internal control of the PCR setting; NC is the negative control reactions; PC is the positive control of the reaction); 1 is for the PCR mixture for an additional calculated sample. The calculation of the volume of each component of the mixture was made in accordance with Table 3.

The following reagents were added to the control tubes: positive control (PC), internal control (IC), and negative control (NC). Standard BLV FLK was used as a PC; a pair of specific primers for bovine DNA was used as an IC; DNA buffer was used as a NC.

Twenty microliters of mineral oil were layered on top of the mixture (in case of using amplifiers with a nonheated lid). The amplification products were resolved by electrophoresis in an agarose gel slab and visualized on a transilluminator.

To purify the amplification products from nonspecific fragments, the luminous strips were cut out of the gel on a transilluminator and the amplificates were isolated by the spin column method.

Maps of hypothetical LTR-region restriction sites were compiled. The restriction sites were: *BstMA* I (237 bp), *Bse*1 (378, 370 bp), and *BspAC* I (262 bp). To analyze possible combinations of substitutions on the selected sequence of the *BLV* genome region, a typing method was developed. Substitutions at the genome sites of 8034 and

Table 4. The formation of haplotype layout

Haplotype	The products of restriction fragments		
	BstMA I – GTCTCN↑CAGAG$(N)_5$↓	*Bse*1 – ACTGGN↑TGAC↓CN	*BspAC* I – C↑CGC GGC↓G
I	237 (A)	378 (GC)	262 (CG)
II	237 (A)	378 (GC)	262 (AG)
III	237 (A)	370 (CN)	262 (CG)
IV	237 (A)	370 (CN)	262 (AG)
V	– (N)	378 (GC)	262 (CG)
VI	– (N)	378 (GC)	262 (AG)
VII	– (N)	370 (CN)	262 (CG)
VIII	– (N)	370 (CN)	262 (AG)

8139 base pairs were analyzed by restriction fragment length polymorphism. According to the results of PCR, two viral haplotypes were identified: I and III (Table 4).

Statistical processing of the data obtained was carried out by conventional methods (Lakin, 1973; Zhivotovsky, 1991) using the STATISTICA 10 software package.

Results

Infected animals of the studied population ($n = 780$) were represented by carriers of haplotypes I (0.33 ± 0.03) and III (0.67 ± 0.03) out of the eight possible haplotypes of *BLV*. When analyzing the blood indices of animals infected with leukemia of different haplotypes, it was found that the animals carrying haplotype III had a higher ($p < 0.001$) absolute content of all types of leukocytes. However, the percentage of monocytes did not show significant differences (Table 5). The following hematological indices were estimated: total leukocyte count (WBC), lymphocyte count (lymf), granulocyte (gran), monocyte (mon), erythrocyte (RBC), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), erythrocyte distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet crit count (PCT) (See Table 5).

It is noteworthy that the difference was noticed not only in the concentration of leukocytes but also in the number of erythrocytes and platelets, which do not have a direct relationship to the immunity of animals (See Table 5).

The facts indicate that the recognition of a particular strain of leukemia virus by the immune system of cattle begins even before the transition of the disease to the clinical stage. The level of leukocytes in the blood of animals affected by the virus of haplotype III was higher ($p < 0.001$) regardless of whether the animals had clinical symptoms of leukemia, had a latent stage, or belonged to the group with suspected leukemia (Table 6). In animals with a high content of leukocytes in the blood, which are classified as leukemia suspects, the levels of leukocytes in carriers of different haplotypes of *BLV* differed significantly. However, due to the clear limitation of the level of the indicator in this group the difference between the groups was more than 2×10^9/L.

On the average, differences in the leukocyte count in the diseased and the suspected animals exceed those between the suspected and the healthy animals (See Table 6). This is especially noticeable in visual comparison, when the cluster of infected individuals shows a varying degree of dependence on which *BLV* haplotype infected the studied cattle (Fig. 1). Differences in leukocyte count between animals of different hemolytic status were significant

Table 5. Cytometric and morphological blood indices of animals carrying different *BLV* haplotypes

Index	Haplotype I				Haplotype III				t_ϕ
	$\overline{X}$	$S_{\overline{X}}$	$D[\overline{X}]$	95 %	$\overline{X}$	$S_{\overline{X}}$	$D[\overline{X}]$	95 %	
WBC, 10^9/L	8.87	0.10	5.47	0.26	14.12	0.27	18.65	0.54	$p < 0.001$
lymf, 10^9/L	5.98	0.07	2.25	0.17	9.85	0.21	11.50	0.42	$p < 0.001$
mon, 10^9/L	0.73	0.01	0.06	0.03	1.15	0.03	0.17	0.05	$p < 0.001$
gran, 10^9/L	4.16	0.06	1.71	0.15	5.89	0.10	2.66	0.20	$p < 0.001$
lymf, %	54.33	0.42	93.28	1.09	67.72	0.76	147.13	1.50	$p < 0.001$
mon, %	8.43	0.08	3.55	0.21	8.55	0.19	9.11	0.37	nsd
gran, %	43.24	0.42	92.25	1.09	33.56	0.73	132.16	1.43	$p < 0.001$
RBC, 10^{12}/L	5.10	0.04	0.78	0.10	5.39	0.07	1.15	0.13	$p < 0.001$
HGB, g/L	90.23	0.75	293.64	1.93	93.20	1.21	365.85	2.38	$p < 0.05$
HTC, %	26.50	0.20	20.67	0.51	27.55	0.32	25.45	0.63	$p < 0.01$
MCV, fl	52.26	0.18	17.49	0.47	51.65	0.35	30.85	0.69	nsd
MCH, pg	17.78	0.13	9.50	0.35	17.57	0.23	13.75	0.46	nsd
MCHC, g/L	341.58	2.30	2772.97	5.94	337.98	3.92	3875.45	7.72	nsd
RDW, %	15.43	0.05	1.29	0.13	15.93	0.07	1.24	0.14	$p < 0.001$
PLT, 10^9/L	236.78	4.52	10725.82	11.69	214.60	5.22	6854.52	10.27	$p < 0.001$
MPV, fl	6.93	0.03	0.47	0.08	6.78	0.04	0.44	0.08	nsd
PDW	16.99	0.02	0.30	0.06	16.85	0.03	0.28	0.07	nsd
PCT, %	1.26	0.64	211.71	1.64	0.13	0.00	0.00	0.01	nsd

Notes: t_ϕ – the significance of differences determined by Student's test; nsd – no significant difference.

Table 6. Decomposition of hypotheses about the influence of the LTR haplotype on the course type of infection

LTR	HS	Leukocytes, 10^9/L				Lymphocytes,10^9/L				*n*
		$\overline{X}$	$S_{\overline{x}}$	–99.90 %	+99.90 %	$\overline{X}$	$S_{\overline{x}}$	–99.90 %	+99.90 %	
III	Suspected	12.762	0.128	12.333	13.190	5.788	0.107	5.431	6.145	240
III	Diseased	21.889	0.938	18.536	25.243	12.982	0.697	10.490	15.473	7
III	Healthy	10.900	0.125	10.711	11.240	3.700	0.105	3.500	4.000	281
I	Suspected	10.423	0.104	10.077	10.769	4.682	0.086	4.396	4.969	211
I	Diseased	17.314	0.040	17.074	17.555	10.014	0.571	6.612	13.416	38
I	Healthy	7.339	0.082	7.066	7.612	3.222	0.052	3.049	3.396	3

Notes: HS is the hematological status; $\lambda = 0.982$ at $p < 0.001$; F (4.1546) = 3.3451.

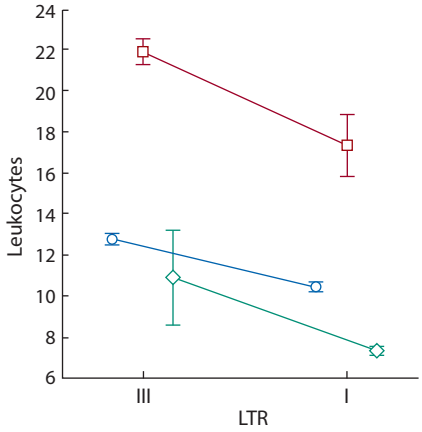

Fig. 1. The haplotype influence on the leukocyte count in healthy, suspected, and diseased animals ($\lambda = 0.982$; F (4.154) = 3.345; $p = 0.0098$).

The red line indicates the number of leukocytes in animals with clinical symptoms of leukemia, blue – in individuals with suspected leukemia, green – in healthy.

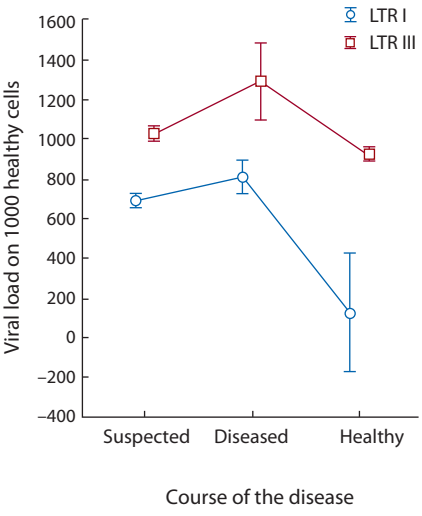

Fig. 2. Viral load in animals with different *BLV* haplotypes.

($p < 0.001$) except for the "healthy – suspected" difference for haplotype III. The proportion of infected animals is 15.08 % in cattle infected by the virus of haplotype I and 1.33 % in carriers of haplotype III.

A similar picture was observed in lymphocyte count in the blood of the animals. Haplotype certainly affects the change in the level of lymphocytes, although the groups had a low level of discrimination (See Table 6).

The level of viral load was in direct proportion to the status of the animals. The greatest number of viral particles was found in the blood of infected animals and the smallest, in animals without clinical signs of infection. It is interesting to note that in general, viral load was higher in cattle infected with haplotype III (Fig. 2).

For comprehensive evaluation of the hypothesis of the influence of categorical features on continuous variables, multidimensional criteria were used (Table 7). The values of the Wilks criteria (Wilks' Lambda, WL), Pillai's criteria (Pillai's Trace, PT), Hotelling's criteria (Hotelling's Trace, HT), and Roy's criteria (Roy's Largest Root, RLR) reveal a significant association of *BLV* LTR haplotypes with the process type of the infection, which is expressed, among other indices, in the count of leukocytes.

Discussion

A greater number of different types of leukocytes were found in animals infected with *BLV* haplotype III compared to the carriers of haplotype I (See Table 5). This observation points to a stronger immune response of the cattle organism to this particular type of the virus. It is possible that the virus of haplotype III is more virulent, as also indicated by the larger number of virus particles in comparison to haplotype I (See Fig. 2). The fact that the former haplotype is more common is indicative of an evolutionary advantage of more virulent strains over less virulent. Similar results were obtained in many experiments with RNA viruses of mice, rats, and rabbits (Furió et al., 2012; Elsworth et al., 2014; Korboukh et al., 2014; Fitzsimmons et al., 2018). Moreover, in some cases there was an increase in the evolutionary flexibility of viruses. For example, *H273R* – due to changes in the nucleotide sequences of mutant genes that accelerate mutagenesis (Korboukh et al., 2014).

The assumption of the evolutionary advantage of more virulent strains of the bovine leukemia virus looks convincing. The main objective of viruses, including *BLV*, is not the destruction of the cells of the host organism but the production of the maximum possible number of their own particles, which is perfectly reasonable (Agol, 2015). It has been proven that the evolutionary advantage of any mutations is determined primarily not by qualitative changes but by the survival rate of the greatest possible number of descendants of mutation carriers. This rule applies not only to viruses but to all representatives of all taxonomic kingdoms (Markov, Naimark, 2015). And one of the truly harmful qualities of viruses is not their virulence as such but the immune responses provoked by a

Table 7. Testing the null hypothesis about the absence of haplotype influence on the type of the infection

Index	Criterion	Important	F	dfEffect	dfError	*p*
Free term	WL	0.197743	1568.054	2	773	0.000000
	PT	0.802257	1568.054	2	773	0.000000
	HT	4.057061	1568.054	2	773	0.000000
	RLR	4.057061	1568.054	2	773	0.000000
LTR	WL	0.935624	26.593	2	773	0.000000
	PT	0.064376	26.593	2	773	0.000000
	HT	0.068805	26.593	2	773	0.000000
	RLR	0.068805	26.593	2	773	0.000000
Hematological status	WL	0.619193	104.675	4	1546	0.000000
	PT	0.380882	91.038	4	1548	0.000000
	HT	0.614883	118.672	4	1544	0.000000
	RLR	0.614686	237.884	2	774	0.000000
LTR+hematological status	WL	0.982912	3.345	4	1546	0.009764
	PT	0.017150	3.347	4	1548	0.009730
	HT	0.017322	3.343	4	1544	0.009799
	RLR	0.012124	4.692	2	774	0.009430

Notes: dfEffect – degree of freedom of the test; dfError – degree of freedom of the residual error.

large number of viral particles: degradation of RNA (both viral and cellular), suppression of protein synthesis (both viral and cellular), self-destruction (apoptosis and other types of programmed cell death), and, finally, inflammation (Debacq et al., 2004; Lezin et al., 2009; Agol, 2015). When we assume that *BLV* does not alter the host complex of the synthesized proteins without introducing anything of "its own" (Kettmann et al., 1980), such a theory looks quite plausible. Since *BLV* is not a carrier of a program encoding foreign proteins, its harmfulness can increase only through accelerated synthesis of its own copies. The result is the self-destructive response of the immune system of cattle.

The fact that the higher virulence of *BLV* haplotype III is a consequence of the acceleration of its replication and not vice versa is confirmed by the following data. The virus does not program carrier cells for the synthesis of extraneous particularly harmful proteins; in contrast, it carries genetic elements that activate the immunity of the cattle (Lagarias, Radke, 1989; Juliarena et al., 2017). At the first glance, it is a "suicidal" evolutionary acquisition that should have led to the extinction of *BLV* as such. But in reality, this provoked the creation of a certain complex of coexistence of cattle and *BLV*, where the virus is the carrier of the genetic system aimed at neutralizing the inevitable harmful presence of the virus in the carrier. As a result, 70–90 % of the cattle do not show any clinical signs of leukemia, which in general is shown in the present study (See Table 6).

Thus, the *BLV* genetic program provides not only the intensification of the synthesis of its own particles, but also mitigation of the negative consequences for the cattle. And the evolutionary strategy can be traced quite clearly: the virus does not benefit from the extinction of cattle as a biological species since this would lead to its own extinction.

Conclusions

The research results are consistent with the presented concept. Mutations in the genes of the LTR region of *BLV* initially provoked an acceleration of viral particle synthesis, which in turn caused a more intense immune response in cattle (See Table 5, Fig. 1). Thus, the evolutionary advantage of haplotype III of *BLV* over haplotype I is expressed in the accelerated reproduction of its copies, which is ultimately reflected in the higher prevalence of haplotype III (0.67 ± 0.03) compared to haplotype I (0.33 ± 0.03).

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Vitamin C in fleshy fruits: biosynthesis, recycling, genes, and enzymes

D.Y. Tyapkina[1], E.Z. Kochieva[1, 2], M.A. Slugina[1, 2] ✉

[1] Institute of Bioengineering, Research Center of Biotechnology, RAS, Moscow, Russia
[2] Lomonosov Moscow State University, Moscow, Russia
✉ e-mail: mashinmail@mail.ru

L-ascorbic acid (vitamin C) is a plant secondary metabolite that has a variety of functions both in plant tissues and in the human body. Plants are the main source of vitamin C in human nutrition, especially citrus, rose hip, tomato, strawberry, pepper, papaya, kiwi, and currant fruits. However, in spite of the biological significance of L-ascorbic acid, the pathways of its biosynthesis in plants were fully understood only in 2007 by the example of a model plant *Arabidopsis thaliana*. In the present review, the main biosynthetic pathways of vitamin C are described: the L-galactose pathway, L-gulose pathway, galacturonic and myo-inositol pathway. To date, the best studied is the L-galactose pathway (Smyrnoff–Wheeler pathway). Only for this pathway all the enzymes and the entire cascade of reactions have been described. For other pathways, only hypothetical metabolites are proposed and not all the catalyzing enzymes have been identified. The key genes participating in ascorbic acid biosynthesis and accumulation in fleshy fruits are highlighted. Among them are L-galactose pathway proteins (GDP-mannose phosphorylase (GMP, VTC1), GDP-D-mannose epimerase (GME), GDP-L-galactose phosphorylase (GGP, VTC2/VTC5), L-galactose-1-phosphate phosphatase (GPP/VTC4), L-galactose-1-dehydrogenase (GalDH), and L-galactono-1,4-lactone dehydrogenase (GalLDH)); D-galacturonic pathway enzymes (NADPH-dependent D-galacturonate reductase (GalUR)); and proteins, controlling the recycling of ascorbic acid (dehydroascorbate reductase (DHAR1) and monodehydroascorbate reductase (MDHAR)). Until now, there is no clear and unequivocal evidence for the existence of one predominant pathway of vitamin C biosynthesis in fleshy fruits. For example, the L-galactose pathway is predominant in peach and kiwi fruits, whereas the D-galacturonic pathway seems to be the most essential in grape and strawberry fruits. However, in some plants, such as citrus and tomato fruits, there is a switch between different pathways during ripening. It is noted that the final ascorbic acid content in fruits depends not only on biosynthesis but also on the rate of its oxidation and recirculation.

Key words: L-ascorbic acid; vitamin C; fruits; metabolism; the key genes of ascorbic acid biosynthesis.

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Накопление витамина С в сочных плодах: биосинтез и рециркуляция, гены и ферменты

Д.Ю. Тяпкина[1], Е.З. Кочиева[1, 2], М.А. Слугина[1, 2] ✉

[1] Институт биоинженерии Федерального исследовательского центра «Фундаментальные основы биотехнологии» Российской академии наук, Москва, Россия
[2] Московский государственный университет им. М.В. Ломоносова, Москва, Россия
✉ e-mail: mashinmail@mail.ru

L-аскорбиновая кислота (витамин С) – вторичный метаболит растений, выполняющий множество разнообразных функций как в растительных тканях, так и в организме человека. Основным источником витамина С в питании человека служат растения, и прежде всего плоды цитрусовых, шиповника, перца, смородины, томата, клубники, папайи, киви. Однако, несмотря на то что L-аскорбиновая кислота – важное биологически активное вещество, путь ее биосинтеза в растительной клетке был описан лишь в 2007 г. на примере модельного растения *Arabidopsis thaliana*. В настоящем обзоре рассмотрены известные на сегодняшний день пути биосинтеза L-аскорбиновой кислоты в тканях растений. Это L-галактозный, L-гулозный, галактуроновый и мио-инозитоловый пути. Наиболее изучен из них L-галактозный путь (путь Смирнова–Уилера), для которого определены все ферменты, катализирующие последовательную цепь реакций. Для других путей известна лишь предположительная последовательность метаболитов, при этом многие ферменты, катализирующие их превращение, еще не выявлены. Выделены ключевые гены, которые участвуют в биосинтезе и накоплении аскорбиновой кислоты в сочных плодах. Среди них ферменты L-галактозного пути (ГДФ-маннозофосфорилаза (GMP, VTC1), ГДФ-D-маннозо-3′5′-эпимераза (GME), ГДФ-L-галактозофосфорилаза (GGP, VTC2/VTC5), L-галактозо-1-фосфатфосфатаза (GPP/VTC4), L-галактозо-1-дегидрогеназа (GalDH) и L-галактоно-1,4-лактондегидрогеназа (GalLDH)); ферменты D-галактуронового пути (NADPH-зависимая D-галактуронатредуктаза (GalUR)) и ферменты рециркуляции АК (дегидроаскорбатредуктаза (DHAR1) и монодегидро-

аскорбатредуктаза (MDHAR)). До сих пор нет однозначного описания всех путей биосинтеза и накопления L-аскорбиновой кислоты в плодах. В настоящее время нельзя однозначно утверждать, что какой-то из четырех известных путей биосинтеза аскорбиновой кислоты является преобладающим в плодах растений. Так, в плодах персика и киви основным является L-галактозный путь, тогда как в плодах винограда и клубники – по всей видимости, D-галактуроновый. В то же время у ряда растений, например цитрусовых или томата, по мере созревания плодов может происходить смена различных путей биосинтеза. Отмечается, что уровни накопления аскорбиновой кислоты зависят не только от биосинтеза, но и от скорости ее окисления и рециркуляции.
Ключевые слова: L-аскорбиновая кислота; витамин С; плоды; метаболизм; гены биосинтеза аскорбиновой кислоты.

Introduction

L-Ascorbic acid (vitamin C) is a secondary plant metabolite involved in manifold functions in the cell (Davey et al., 2000; Iqbal et al., 2009; Smirnoff, 2018). L-Ascorbic acid (L-AsA) acts as an expression regulator for many genes, influences plant growth and development via phytohormones, and, which is no less important, is involved in the plant cell response to the impact of biotic and abiotic stress factors (Pastori et al., 2003; Gest et al., 2013; Li et al., 2013). Some plant species utilize ascorbates as a substrate for biosynthesis of other important metabolites, for example, oxalates and tartrates (Loewus F.A., Loewus M.W., 1987; Loewus F.A., 1999; Cruz-Rus et al., 2010).

Vitamin C is especially valuable in human diet since humans and other higher primates had lost the ability to produce L-AsA because of a mutation in one of the enzymes involved in its biosynthesis (Nishikimi, Yagi, 1996). Vitamin C plays an important role in the normal functioning of the body, being a coenzyme in several metabolic processes. It also possesses antioxidant properties and eliminates free radicals, which contribute to carcinogenesis and body aging (Figueroa-Méndez, Rivas-Arancibia, 2015). In addition, vitamin C improves human immunity by activating phagocytes, prevents the cardiovascular diseases associated with atherosclerosis, and enhances collagen formation and development of the cartilage tissue (Diplock et al., 1998). The main source of this vitamin in human diet is plants; fruits of citruses, sweet-brier, actinidia (kiwi), sand thorn, papaya, strawberry, mountain ash, sweet pepper, and tomato display the highest content of vitamin C (Iqbal et al., 2009; Streltsina et al., 2010).

Biosynthesis of ascorbic acid in plant cell

Strange it may seem but the biosynthesis of L-AsA in plant cell was finally described in a model plant, *Arabidopsis thaliana*, only as late as 2007 (Linster et al., 2007) despite its evident importance in the life of plants and human health. Unlike animals, which synthesize L-AsA from glucuronic acid, the plant cell has at least four alternative pathways of its biosynthesis, namely, L-galactose, L-gulose, galacturonic, and myo-inositol pathways (see the Figure) (Li et al., 2010; Yang et al., 2011).

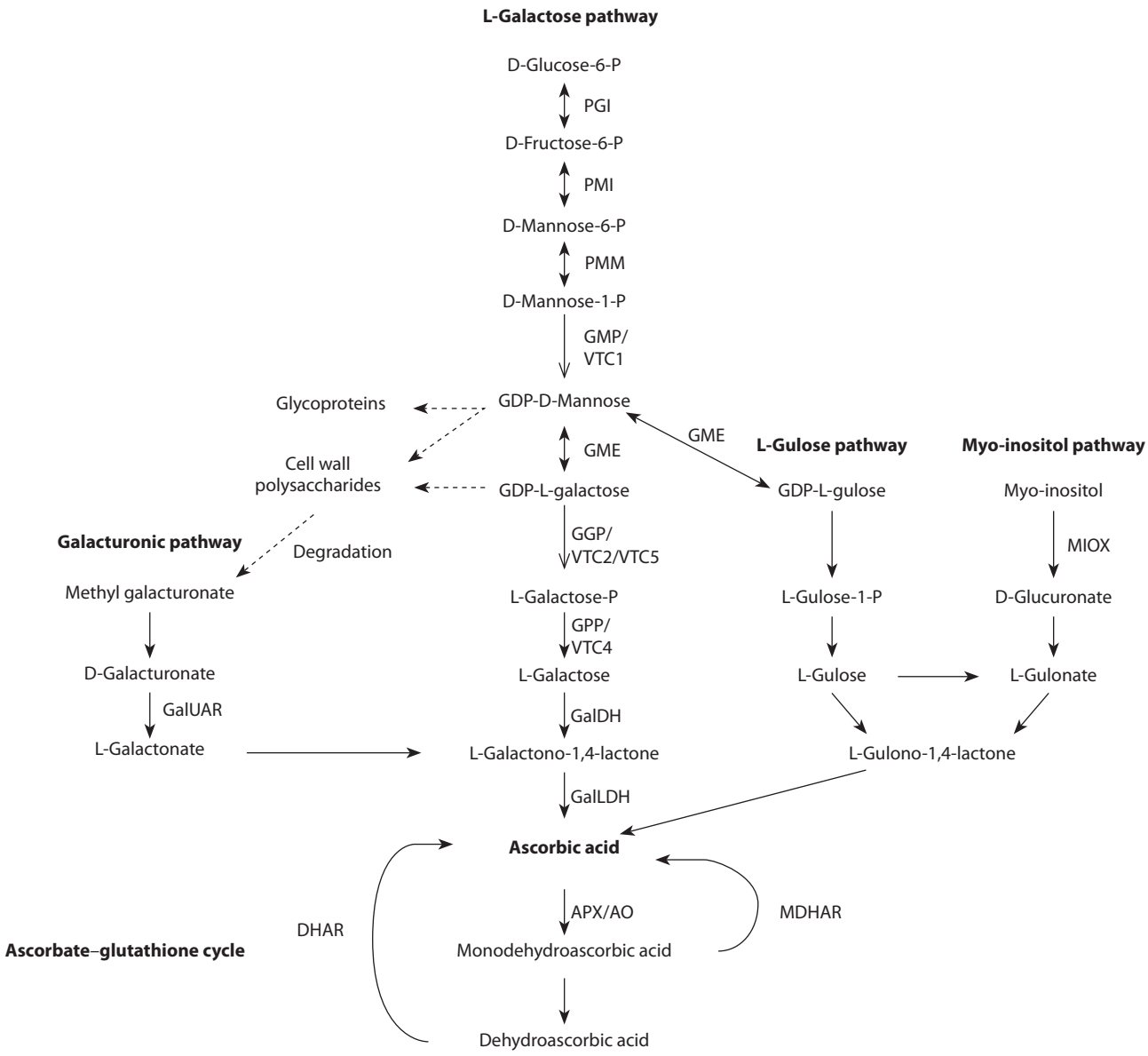
L-Galactose pathway

The L-galactose pathway, or Smirnoff–Wheeler pathway (Wheeler et al., 1998), is currently regarded as the main pathway of L-AsA biosynthesis in plants. The initial substrate for this pathway is a glucose molecule. The Smirnoff–Wheeler pathway comprises 10 successive stages. Interestingly, the first eight stages transform D-glucose into L-galactose, which differs from D-glucose only by the spatial positions of hydrogen and hydroxyl groups at the fourth carbon atom (Linster et al., 2007).

GDP-D-mannose and GDP-L-galactose are important metabolites of this pathway. The enzyme GDP-D-mannose-3′,5′-epimerase controls their mutual transformation. It is important here that the main part of the products in this reaction is spent for the primary metabolic reactions, namely, the biosynthesis of cell wall polysaccharides (Roberts, 1971; Baydoun, Fry, 1988), which is maximally active in growing organs and tissues. This suggests that the initial stages of this metabolic pathway are mainly involved in the growth of organs (leaves, fruits, and so on). In the formed (mature) organs, the secondary metabolic reactions are switched on and provide further transformation of GDP-L-galactose to AsA. Therefore, the limiting stage in this metabolic pathway of vitamin C synthesis is the reaction that directly generates L-AsA and is controlled by the enzyme GDP-L-galactose phosphorylase, VTC2 (see the Figure). It is believed that VTC2 is of a key importance for production of vitamin C and that its activity depends on the need of a cell in synthesizing cell wall polysaccharides (Bulley et al., 2012; Wang et al., 2014).

L-Gulose pathway

The case study of arabidopsis has shown that one of the enzymes of the above briefed pathway, GDP-D-mannose-3′,5′-epimerase (GME), putatively possesses 5′-isomerase activity and is able to catalyze GDP-D-mannose transformation to GDP-L-gulose (Wolucka, Van Montagu, 2003) along with the 3′,5′-isomerase activity, converting GDP-D-mannose to GDP-L-galactose. The next assumption was that GDP-L-gulose in the course of subsequent transformation into L-gulose-1-phosphate, L-gulose, and L-gulono-1,4-lactone could be also converted to L-AsA (see the Figure). However, the corresponding catalytic enzymes of the L-gulose pathway of vitamin C synthesis in plants have not been yet discovered except for L-gulono-1,4-lactone oxidase of arabidopsis (Maruta et al., 2010). Interestingly, the overexpression of rat L-gulono-1,4-lactone oxidase (*ALO*) elevated the L-ascorbic acid content in tobacco and lettuce (Jain, Nessler, 2000). An overexpression of rat *ALO* in the arabidopsis plants carrying a mutation in *VTC* gene (deficient in vitamin C) completely restored the L-AsA level (Radzio et al., 2003). This suggests that the L-gulose pathway may be regarded as one of the alternative pathways in L-AsA biosynthesis in plants.



Pathways of L-AsA biosynthesis in plant cells, according to (Li et al., 2010; Suekawa et al., 2017) with some modifications.

Myo-inositol pathway

Myo-inositol is a carbohydrate metabolite synthesized by most cells and necessary for a normal plant growth and development. In a form of inositol phosphates and phosphatidylinositol lipids, myo-inositol is involved in intracellular signal transduction in various cascades (Michell, 2007).

The myo-inositol pathway of L-AsA biosynthesis in plants comprises four stages (see the Figure). Myo-inositol is oxidized by myo-inositol oxygenase to D-glucuronic acid, which is further converted by glucuronate reductase to L-gulonic acid with subsequent transformation to L-gulono-1,4-lactone, which is catalyzed by aldonolactonase. The last reaction in this line is the transformation of L-gulono-1,4-lactone to L-AsA, catalyzed by L-gulonolactone dehydrogenase (Lorence et al., 2004).

Myo-inositol oxygenase (MIOX) is the key enzyme in this pathway: it is shown by the case study of arabidopsis that an overexpression of *MIOX* doubles the vitamin C content in flowers and leaves (Lorence et al., 2004). The remaining enzymes involved in the subsequent reactions in plants are still not determined.

D-Galacturonic pathway

As was shown in the early 1960s, galacturonic acid methyl ester is convertible to L-AsA. The existence of this metabolic pathway was for the first time demonstrated in the case study of a protist, *Euglena gracilis* (Shigeoka et al., 1979). In plants, exogenous application of D-galacturonic acid methyl ester elevates the ascorbic acid content in different tissues and arabidopsis cell culture (Loewus, Kelly, 1961; Davey et al., 1999), thereby demonstrating the presence of this pathway of ascorbic acid biosynthesis.

Note that the initial substrates for the D-galacturonic pathway are the degradation products of cell wall polysac-

charides. D-Galacturonic acid is a necessary player in two concurrent biochemical processes – synthesis of pectins, an important cell wall component, and AsA biosynthesis. According to the current view, this pathway comprises several key enzyme-catalyzed stages providing the reduction of D-galacturonic acid to L-galactonic acid or L-galactono-1,4-lactone by galacturonate reductase and subsequent formation of L-AsA (see the Figure). An important role of this metabolic pathway has been demonstrated for the fruits of several plants, such as the strawberry (Agius et al., 2003), grape (Cruz-Rus et al., 2010), orange (Xu et al., 2013), and apple (Mellidou et al., 2012). Since the D-galacturonic pathway is considerably shorter as compared with the L-galactose one, which is regarded the main pathway of AsA biosynthesis, it is assumed that the former may well supplement the prevalent biosynthesis pathway in fruits under stress conditions (Cruz-Rus et al., 2011).

AsA recycling (ascorbate–glutathione cycle)

The current data volume accumulated while studying the AsA metabolism in plant cells suggests that the AsA levels depend not only on its biosynthesis, but also on it oxidation and further recycling (Li et al., 2013).

The L-AsA formed in one of the above described biosynthesis cycles acts in the plant cell as an antioxidant, protecting from oxidative stress (Akram et al., 2017). In these processes, oxidized forms (monodehydroascorbic and dehydroascorbic acids) are formed. During the recycling, the oxidized forms are again reduced to AsA with the help of two reductases – monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). This is known as the ascorbate–glutathione cycle (see the Figure). The biological function of this cycle is determined by that the cell contains reactive oxygen species, on the one hand, and AsA has antioxidant properties, on the other.

The first stage in this pathway is detoxification of reactive oxygen species with the help of ascorbate peroxidase (APX) or ascorbate oxidase (AO). This gives monodehydroascorbic acid, which is either again reduced to AsA by MDHAR, or dehydroascorbic acid, which is reduced by DHAR.

Thus, the AsA final content in plant organs and tissues depends on both its biosynthesis and recycling.

Specific features of AsA biosynthesis and accumulation in fruits

L-AsA is involved in manifold functions in the life of a plant organism. The AsA biosynthesis is triggered in response to various endogenous and exogenous impacts and takes place in almost all plant tissues and organs. Therefore, the presence of at least four pathways for AsA synthesis is not surprising as well as switching between these pathways depending on the particular demands and conditions in the cell. However, our interest is naturally focused on how vitamin C is accumulated in edible parts of the plants; correspondingly, it is necessary to clearly understand which metabolic pathway of AsA biosynthesis is major in the fruits of different plant species. Although this issue is of considerable importance, the studies on the specific features of the vitamin C accumulation in plant fruits are few. Currently, the pathways of AsA biosynthesis and accumulation in fruits have been examined in sufficient detail in some cultivated plants, such as the strawberry, tomato, grape, kiwi, apple, pear, sweet cherry, and citruses (Agius et al., 2003; Hancock et al., 2007; Bulley et al., 2009; Cruz-Rus et al., 2010; Di Matteo et al., 2010; Li et al., 2010; Walker et al., 2010; Badejo et al., 2012; Alós et al., 2014).

As a rule, AsA is gradually accumulated with an increase in the mass of the developing fruit with the highest rate on days 75–100 after anthesis. This pattern of AsA accumulation has been observed, for example, for the tomato (Ioannidi et al., 2009). However, AsA more rapidly accumulates at early stages of fruit development in kiwi (Bulley et al., 2009) and black currant (Hancock et al., 2007) fruits, when the cell biosynthetic activity is maximal (Li et al., 2011).

A significant role of the major (L-galactose) pathway has been shown for several species. In particular, it has been shown that the content of vitamin C in the black currant (*Ribes nigrum*) fruits considerably varies depending on both climatic conditions and genotype; note that the correlation of expression with vitamin C accumulation is shown for only one gene, the gene coding for GDP-D-mannose-3′5′-epimerase (Walker et al., 2010).

The maximum content of vitamin C in kiwi (*Actinidia deliciosa* variety Qinmei) fruits is observable on day 30 after anthesis with a gradual decrease by day 60. Expression patterns are similar for most of the studied key genes except for the genes encoding L-galactono-1,4-lactone dehydrogenase (*GalLDH*) and L-galactose-1-phosphate phosphatase (*GPP*/*VTC4*). Note that only *GPP* expression correlates with AsA accumulation (Li et al., 2010).

An important role of the enzymes involved in the D-galacturonic pathway has been also demonstrated for the content of vitamin C in tomato (*Solanum lycopersicum*) fruits, typically reaching 35 mg per 100 g. Moreover, an increase in AsA with tomato (cultivar Micro-Tom) fruit ripening is inversely correlated with the expression of the genes involved in the major (L-galactose) pathway (Badejo et al., 2012). The L-galactose and D-galactouronate treatment of tomato plants increased the vitamin C content in the mature fruits; however, this result was unachievable by treating the plants with L-gulono-1,4-lactone, formed in the L-gulose and myo-inositol pathways (see the Figure) (Badejo et al., 2012). This suggests that the AsA synthesis during tomato fruit ripening may start with the Smirnoff–Wheeler pathway to further switch to the D-galacturonic pathway, the enzymes of which work at the late stage of fruit ripening. D-Galacturonic acid is produced via the cleavage of cell wall pectin; this suggests that the pathway in question is activated during the maceration of tomato fruits (Badejo et al., 2012). As has been earlier demonstrated, expression of the genes coding for pectinesterases and polygalacturonases (the enzymes involved in pectin degradation) is very high in an introgression line, IL 12-4 (*S. pennellii* and *S. lycopersicum*), differing from the parental line by a considerably higher vitamin C content. This is another evidence for that the D-galacturonic pathway is directly involved in the accumulation of vitamin C in tomato fruits (Di Matteo et al., 2010).

AsA is a most valuable component in the grapes (*Vitis vinifera*), acting as the substrate for synthesis of tartaric acid (Cholet et al., 2016). Similar to the strawberry and tomato fruits, the AsA content increases with grape ripening reaching

its maximum in fully mature fruits. Expression analysis of the genes controlling different AsA biosynthesis pathways in grape fruits demonstrates a strong correlation of the transcription of D-galactouronate reductase gene (*GalUR*) and the quantitative content of vitamin C (Cruz-Rus et al., 2010). Thus, the D-galacturonic pathway is also regarded as a major pathway for vitamin C biosynthesis during grape fruit development and ripening (Cruz-Rus et al., 2010).

It is known that the mature strawberries (*Fragaria* sp.) are rich in vitamin C, containing on the average 60 mg AsA per 100 g fresh tissue (Agius et al., 2003). As is shown, the D-galacturonic pathway is significant for the AsA accumulation in strawberry fruits. Expression of one of the enzymes of this pathway, NADPH-dependent D-galacturonate reductase (GalUR), in strawberry fruits increases proportionally to the accumulation of vitamin C there. Overexpression of the strawberry *GalUR* gene in arabidopsis leaves gives a twofold increase in vitamin C content there, suggesting an important role of this particular enzyme in AsA biosynthesis (Agius et al., 2003).

Note that the final vitamin C content in fruits depends not only on the rate of AsA biosynthesis, but also on its recycling (see the Figure). In particular, it has been shown that the expression of *MDHAR* gene (ascorbate–glutathione pathway) in strawberries positively correlates with the accumulation of vitamin C in developing fruits (Cruz-Rus et al., 2011).

The maximum AsA content in sweet cherry (*Prunus avium* cultivar Hongdeng) fruits is observed during their setting followed by a gradual decrease during fruit development and a moderate increase in the mature fruit (Liang et al., 2017). Nonetheless, AsA content continues to increase with the weight of fresh fruits. Full-sized cDNAs of 10 genes involved in the L-galactose pathway of AsA biosynthesis have been described as well as of 10 genes involved in AsA recycling. The expression levels of the genes encoding GDP-L-galactose phosphorylase (*GGP2*), L-galactono-1,4-lactone dehydrogenase (*GalLDH*), ascorbate peroxidase (*APX3*), ascorbate oxidase (*AO2*), glutathione reductase (*GR1*), and dehydroascorbate reductase (*DHAR1*) correlate with the quantitative content of vitamin C during fruit development, which suggests that the joint work of all these genes involved in AsA biosynthesis, degradation, and recycling together regulates the AsA accumulation in sweet cherry fruits (Liang et al., 2017).

The enzymatic activities involved in the AsA biosynthesis via the Smirnoff–Wheeler pathway and its recycling in different fruit tissues have been comprehensively studied in pear (*Pyrus pyrifolia* cultivar Aikansui) fruits (Huang et al., 2013). Biochemical analysis demonstrates that the AsA content increases with fruit development to reach the maximum 30 days after anthesis and then decreases and is maintained at the same level. The highest AsA concentration is observed in the pear peel, which is a result of a high L-galactose dehydrogenase and L-galactono-1,4- lactone dehydrogenase activities, on the one hand, and dehydroascorbate reductase and monodehydroascorbate reductase (involved in AsA recycling), on the other. An exogenous application of the precursors of AsA biosynthesis has shown that the peel displays a higher ability to synthesize the target substance via the Smirnoff–Wheeler and D-galacturonic pathways, whereas the pulp and core were less capable of synthesizing AsA (Huang et al., 2013).

According to a study of apple (*Malus domestica*) fruits, AsA can be synthesized via the L-galactose pathway (Li et al., 2008). Further studies of the dynamics of vitamin C accumulation during fruit (cultivar Gala) ripening have shown that the transcription levels of the genes coding for GDP-L-galactose phosphorylase, GDP-L-mannose pyrophosphorylase, D-galacturonate reductase, and L-galactono-1,4-lactone dehydrogenase, regulated on a posttranscriptional level, do not correlate with vitamin C accumulation. On the other hand, the expression patterns of L-galactose dehydrogenase, L-galactose-1-phosphate phosphatase, and GDP-D-mannose-3′5′-epimerase are in general similar to the AsA accumulation pattern. Interestingly, the expression and activity of the genes of monodehydroascorbate reductase and dehydroascorbate reductase, which degrade AsA in fruits, do not correlate with the AsA accumulation during fruit development (Li et al., 2011).

In addition, the search for the quantitative trait loci (QTL) responsible for the vitamin C accumulation in the *M. domestica* fruits (Mellidou et al., 2012) has succeeded in finding a linkage group that comprises the genes of GDP-L-galactose phosphorylase (*GGP*) and dehydroascorbate reductase (*DHAR*). Of special interest are three *GGP* paralogous genes, all residing within the AsA-QTL cluster. The association between some allelic variants of the *GGP* gene and increased vitamin C content has been observed. Comparison of the *GGP* expression patterns in the specimens displaying high and low vitamin C contents suggests a key role of *GGP* in the accumulation of vitamin C. Molecular markers (SNPs) have been found; these markers are helpful when breeding new cultivars with an increased vitamin C content in apple fruits (Mellidou et al., 2012). In addition, a correlation between DHAR and a QTL associated with the resistance to flesh browning has been shown (Mellidou et al., 2012).

Citruses are known as an important source of vitamin C. Expression patterns of 13 genes involved in vitamin C metabolism (including its synthesis, degradation, and recycling) in two citrus fruits – orange (*Citrus sinensis*) and tangerine (*C. unshiu*) – have been studied. The L-galactose pathway is shown to be major for the synthesis of vitamin C. Note that AsA accumulation is maximum in the peel and pulp, correlating with the expression profiles of the genes involved in the L-galactose pathway, whereas the myo-inositol pathway is prevalent for the AsA synthesis in immature fruit peel. *GGP* and *GPP* are regarded as the key genes controlling the AsA synthesis in the pulp; as for the peel, the function of *GMP* and *MIOX* is also important (Alós et al., 2014). In addition, a relative expression of the *MDHAR* and *DHAR* genes (involved in recycling) correlates with the AsA accumulation during fruit ripening and the cultivars with an elevated AsA content displayed an upregulation of these genes (Alós et al., 2014).

Thus, it is currently impossible to unambiguously state which of the four known pathways of AsA biosynthesis is prevalent in plant fruits. In particular, the L-galactose pathway is prevalent in peach (Imai et al., 2009) and kiwi (Bulley et al., 2009) versus grapes (Cruz-Rus et al., 2010) and strawberries (Agius et al., 2003), where the L-galacturonic pathway is

likely to be prevalent. On the other hand, the prevalence of AsA pathways changes during fruit ripening in several plants, for example, citruses, or tomatoes (Bajero et al., 2012; Alós et al., 2013).

Key enzymes determining L-AsA biosynthesis and accumulation in berries, vegetables, and fruits

Enzymes of L-galactose pathway

GDP-mannose phosphorylase (GMP or VTC1), EC 2.7.7.22, displays a mannose-1-phosphate guanylyltransferase activity. The gene coding for this enzyme was for the first time detected in and cloned from a mutant arabidopsis plant with a decreased AsA content (Conklin et al., 1999). As has been later shown, an inhibition of GMP in the tomato decreases the AsA content in fruits (Keller et al., 1999).

The NCBI database contains *VTC1* gene sequences of the arabidopsis (*A. thaliana*), tomato (*S. lycopersicum*), turnip (*Brassica rapa*), cabbage (*B. oleracea*), potato (*Solanum tuberosum*), papaya (*Carica papaya*), sweet cherry (*P. avium*), rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), jujube (*Ziziphus jujuba*), and pepper (*Capsicum annuum*). The gene with a total length of approximately 2500 bp is rather conserved in its structure, comprising four exons and four introns.

GDP-D-mannose-3′5′-epimerase (GME), EC 5.1.3.18, catalyzes the reversible epimerization of GDP-D-mannose, which is among the main reactions in the L-galactose pathway of AsA biosynthesis. In this reaction, a high-energy glycosyl-pyrophosphoryl linkage is hydrolyzed. GME is able to catalyze two different reactions with formation of either GDP-L-galactose or GDP-L-gulose from GDP-D-mannose. GDP-L-gulose is the initial substrate for the alternative gulose pathway of AsA biosynthesis. The other reaction product, GDP-L-galactose, can be further utilized not only for synthesizing vitamin C, but also in biosynthesis of the cell wall and glycoproteins, which is primarily necessary for the development of vegetative organs (Lukowitz et al., 2001; Gilbert et al., 2009; Mounet-Gilbert et al., 2016). As has been shown, this enzyme activity can also influence pollen development and seed formation (Mounet-Gilbert et al., 2016).

Currently, GME is regarded as one of the most important enzymes in the L-AsA biosynthesis in plants. The genes coding for this enzyme have been isolated and characterized in the arabidopsis (Wolucka et al., 2001; Wolucka, Van Montagu, 2003), rice (Watanabe et al., 2006), and tomato (Zhang C.J. et al., 2011; Zhang Y.Y. et al., 2011). Interestingly, the rice and tomato genomes carry two paralogous genes each (Watanabe et al., 2006; Zhang C.J. et al., 2011; Zhang Y.Y. et al., 2011). A positive correlation between expression of this gene and AsA content is observed in the apple (Li et al., 2010) and blueberry (Liu et al., 2015). However, this pattern is unobservable in the kiwi (Bulley et al., 2009), peach (Imai et al., 2009), and tomato (Ioannidi et al., 2009; Mellidou et al., 2012). Taking into account the need in maintenance of the metabolic balance between the syntheses of AsA and cell wall, competing for the common substrate (GDP-L-galactose), it is shown that an overexpression of the *GME* gene does not lead to an increase in the AsA content in arabidopsis (Yoshimura et al., 2014). On the other hand, a joint overexpression of *GME* and the gene coding for the enzyme catalyzing the limiting stage of the L-galactose pathway, *GPP*, results in a considerably increase AsA accumulation as compared with the overexpression of *GPP* alone (Bulley et al., 2009). Analogous data have been also obtained for kiwi fruits (Bulley et al., 2009). This demonstrates a highest importance of the *GME* and *GPP* genes and their joint control in the L-galactose pathway.

In plants, two paralogous genes are known, *GME1* and *GME2*. These genes have a length of approximately 1500 bp, display a high homology, and consist of six exons and five introns. Among the horticultural crops, these genes are known in the grape vine (*V. vinifera*), tomato (*S. lycopersicum*), pineapple (*Ananas comosus*), melon (*Cucumis melo*), and mulberry (*Morus notabilis*).

GDP-L-galactose phosphorylase (GGP or VTC2/VTC5), EC 2.7.7.69, catalyzes the GDP-L-galactose phosphorylation to L-galactose-1-phosphate. An important role of the *VTC2* gene was demonstrated in the arabidopsis (Dowdle et al., 2007). The recombinant *VTC2* alleles of the wild-type arabidopsis and two mutants were expressed in *Escherichia coli*. The product of one mutant allele did not lead to vitamin C synthesis and the product of the other mutant allele displayed only 2 % of the total wild-type *VTC2* activity. However, a comprehensive analysis of the plants has shown that the *vtc2* mutants of arabidopsis still accumulated AsA at the level of 10–20 % of its content in wild-type plants. This suggested existence of certain other pathways of AsA biosynthesis (Dowdle et al., 2007; Laing et al., 2007; Linster et al., 2007, 2008).

In addition, the changes in AsA level in response to the *VTC2* overexpression in arabidopsis, tomato, strawberry, potato, and rice have been experimentally studied (Bulley et al., 2012; Wang et al., 2014). The plants transformed with the constructs carrying *VTC2* gene displayed a considerable increase in the AsA content (Bulley et al., 2012; Wang et al., 2014).

Certain data demonstrate that not only the coding sequence of *VTC2* gene, but also a region in its promoter sequence influences the AsA accumulation in fruits. As has been shown, the AsA biosynthesis may be controlled via a posttranscriptional repression of GDP-L-galactose phosphorylase. This regulation involves an additional reading frame (uORF). Its "switch-off" leads to the synthesis of GDP-L-phosphorylase and, as a consequence, to an increase in the AsA concentration. This posttranslational AsA regulation is most likely a rather ancient control mechanism, since the uORF is retained in *GGP* genes from the mosses to angiosperms (Laing et al., 2015).

All this suggests that *GGP/VTC2* in some plants is the key regulator of AsA biosynthesis; many biotechnological studies aimed at an increase in the vitamin C content have been performed utilizing this particular gene (Zhou et al., 2012).

So far, the NCBI database contains the sequence of several *GGP* paralogs from the arabidopsis (*A. thaliana*), turnip (*B. rapa*), field mustard (*Brassica arvensis*), cabbage (*B. oleracea*), maize (*Zea mays*), sunflower (*Helianthus annuus*), potato (*S. tuberosum*), and tobacco (*N. attenuata*). The lengths of these DNAs vary around 2740 bp and they comprise seven exons and six introns.

L-galactose-1-phosphate phosphatase (GPP/VTC4), EC 3.1.3.B9, catalyzes dephosphorylation with formation of L-galactose. GPP is also regarded as an efficient enzyme for

regulation of AsA synthesis. This was first assumed for kiwi fruits (Laing et al., 2004; Bulley et al., 2009), apple (Mellidou et al., 2012), and tomato (Ioannidi et al., 2009). However, note that AsA is synthesized even in the case of *GPP* gene knockout although in a smaller quantity, which suggests that either there are other functional phosphatases involved in AsA biosynthesis or AsA is synthesized via other pathways (Conklin et al., 2006; Torabinejad et al., 2009).

It has been shown that GPP is a bifunctional enzyme able to catalyze the biosynthesis not only of AsA, but also of myo-inositol. Thus, GPP activity may represent the junction point of the L-galactose and myo-inositol pathways of AsA biosynthesis (Torabinejad et al., 2009).

Homologous *GPP* genes comprising 12 exons and 11 introns in the arabidopsis (length, 2413 bp) and nine exons and nine introns in turnip (length, 7196 bp) are known in the kingdom of plants.

L-galactose-1-dehydrogenase (GalDH), EC 1.1.1.316, and **L-galactono-1,4-lactone dehydrogenase (GalLDH)**, EC 1.3.2.3. GalDH oxidizes L-galactose to L-galactono-1,4-lactone using NAD^+ as electron acceptor. GalLDH is the final enzyme of the L-galactose pathway and directly leads to AsA synthesis. So far, the *GalLDH* gene has been isolated from the arabidopsis, pea, kiwi, and apple (Gatzek et al., 2002; Laing et al., 2004; Mieda et al., 2004). As has been shown for pear fruits, a high AsA concentration in the peel is in part determined by high activities of GalDH and the next enzyme in the biosynthesis pathway, GalLDH (Huang et al., 2013). GalLDH has been characterized for several plant species, including the sweet potato (Imai et al., 1998), cauliflower (Oesterhelt et al., 1997), spinach (Mutsuda et al., 1995), tobacco (Yabuta et al., 2000), strawberry (do Nascimento et al., 2005), melon (Pateraki et al., 2004), tomato (Zhang C.J. et al., 2011; Zhang Y.Y. et al., 2011), and arabidopsis (Leferink et al., 2008). Before the discovery of VTC2, it was believed that these particular enzymes, involved in the final stages of AsA biosynthesis, could play the key role in at least tomatoes (Alhagdow et al., 2007; Mellidou et al., 2012). However, GalDH has been recently assumed to influence AsA accumulation being involved in the AsA transport between different organs (Mellidou, Kanellis, 2017; Rodríguez-Ruiz et al., 2017).

Homologs of the *GalDH* gene have been found in the genomes of maize (*Z. mays*), sweet cherry (*P. avium*), barley (*Hordeum vulgare*), and arabidopsis (*A. thaliana*). This gene on the average is 4300 bp long and comprises five exons and four introns. As for the homologs of the *GalLDH* gene, they have been annotated in the genomes of sweet cherry (*P. avium*), sweet pepper (*C. annuum*), and apple (*M. domestica*); have lengths of 5139, 7667, and 6329 bp, respectively; and comprise six exons and five introns.

Enzymes of D-galacturonic pathway

NADPH-dependent D-galacturonate reductase (GalUR). The substrate for this enzyme is D-galacturonate, which is the product of degradation of cell wall pectins; the GalUR-catalyzed reaction gives L-galactonate (see the Figure). As is shown, the *GalUR* expression in grape (*V. vinifera*) fruits correlates with the vitamin C accumulation during fruit ripening (Cruz-Rus et al., 2010); analogous pattern is observed in the strawberry (Agius et al., 2003) as well as the key role of this enzyme in the AsA biosynthesis. Note that the reactions of the D-galacturonic pathway in plants are yet insufficiently studied and require further biochemical, physiological, and genetic studies. Homologs of the *GalUR* gene have been annotated in the genomes of the sunflower (*H. annuus*) and tobacco (*N. attenuata*); their lengths are 2671 and 12 453 bp, respectively; and they comprise four exons and three introns.

Enzymes involved in AsA recycling

Dehydroascorbate reductase (DHAR1) and monodehydroascorbate reductase (MDHAR). MDHAR enzyme activity is tightly correlated with the AsA accumulation in tomatoes at a decreased temperature, suggesting its significant role in the regulation of AsA synthesis under stress.

Identification of the *MDHAR* and *DHAR* genes and their further functional analysis have shown that an overexpression of the *DHAR* gene provides a 1.6-fold increase in the AsA content in the tomato fruits grown at a relatively low illumination. A study of expression levels of two *MDHAR* isoforms demonstrates that an increase in the transcription of this gene is negatively correlated with an increase in the AsA level during tomato fruit ripening (Li et al., 2013). However, it is assumed that *MDHAR* is an important determinant of the changes in AsA level under stress (Ioannidi et al., 2009). In particular, the MDHAR activity considerably elevates the AsA content in fruits in the case of cold (Li et al., 2013) and oxidative (Gest et al., 2013) stresses.

The role of MDHAR in the increase of AsA content has been unambiguously demonstrated for tomatoes by QTL mapping (Sauvage et al., 2014) and by expression and enzyme activity profiling during fruit ripening (Mellidou et al., 2012). Expression of this gene also correlates with the vitamin C accumulation in blueberries (Liu et al., 2015). The suppression of *MDHAR* in tomato fruits decreases the AsA content, thereby suggesting that the recycling control via an increase in MDHAR activity may be an efficient way for increasing the vitamin C content (Truffault et al., 2017). Moreover, it has been shown using siRNA that a decrease in this enzyme activity makes tomato plants unable to resist cold stress (El Airaj et al., 2013).

Expression of the *DHAR* gene correlates with the AsA accumulation in the chestnut rose (Huang et al., 2014) and blueberry (Liu et al., 2015) fruits.

Three paralogous genes – *DHAR1*, *DHAR2*, and *DHAR3* – are known in plants. The paralogous genes display a low degree of homology. The *DHAR1* gene has a length of approximately 6000 bp and comprises six exons and five introns.

Among the horticultural crops, *DHAR* genes are known in the grape vine (*V. vinifera*), tomato (*S. lycopersicum*), sweet cherry (*P. avium*), pepper (*C. annuum*), and apple (*M. domestica*).

The *MDHAR* paralogs also display a low degree of homology. The number of exons in these genes varies from nine to 17. These genes have been found in the tomato (*S. lycopersicum*), arabidopsis (*A. thaliana*), and pepper (*C. annuum*).

Thus, the activities of the enzymes involved in AsA recycling needs further studies, the more so since the enzymes of this pathway may be directly associated with the resistance

to cold and oxidative stress, which has an important applied value.

Conclusion

This paper briefs the main pathways of L-AsA biosynthesis and recycling in plant tissues. The key genes involved in the AsA biosynthesis and accumulation in fruits are considered. A huge volume of data on this issue demonstrates that the most significant role in the AsA biosynthesis, accumulation, and recycling is played by a synergistic interaction of all these components. Most likely, the insight into these interactions will form the background for the research into the vitamin C metabolism in plants during the next decade.

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ORCID ID

E.Z. Kochieva orcid.org/0000-0002-6091-0765

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Изучение посткриогенного регенерационного потенциала сортов картофеля в разных условиях культивирования

Е.С. Беспалова[1], Ю.В. Ухатова[1], Н.Н. Волкова[1], Е.В. Овэс[2], Н.А. Гаитова[2], Т.А. Гавриленко[1, 3]

[1] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
[2] Всероссийский научно-исследовательский институт картофельного хозяйства им. А.Г. Лорха, Кусково, Москва, Россия
[3] Санкт-Петербургский государственный университет, биологический факультет, Санкт-Петербург, Россия
e-mail: tatjana9972@yandex.ru

Криоконсервация обеспечивает долгосрочное хранение генофонда селекционных сортов картофеля в криобанках при сверхнизких температурах. В настоящее время для криоконсервации сортов картофеля наиболее широко используется метод дроплет-витрификации, который постоянно совершенствуется с целью повышения регенерационной способности сохраняемого растительного материала. В ведущих мировых генбанках картофеля используются различные модификации этого метода. В данной работе представлены результаты изучения влияния условий культивирования после замораживания–оттаивания апексов побегов и пазушных почек *in vitro* растений на их способность к посткриогенному восстановлению. Для криоконсервации был использован метод дроплет-витрификации, модифицированный в ВИР. Фактор «длительная темновая инкубация эксплантов» не оказывал существенного влияния на частоту посткриогенной регенерации изученных сортов, за исключением одного сорта (Крепыш), для которого отмечено достоверное ($p < 0.05$) увеличение частоты регенерации в варианте культивирования апексов микропобегов в темноте по сравнению с вариантом культивирования при фотопериоде 16/8 ч (свет/темнота). Фактически у всех сортов частота посткриогенной регенерации апексов микропобегов была выше, чем у пазушных почек, однако достоверное превышение ($p < 0.05$) данного показателя для апексов побегов отмечено только в двух случаях: для сорта Удача – культивирование эксплантов при фотопериоде 16/8 ч и для сорта Крепыш – культивирование в условиях темновой инкубации. Результаты двухфакторного дисперсионного анализа указывают на отсутствие значимого эффекта совместного действия двух факторов (темновая инкубация и тип экспланта) на способность сортов к посткриогенному восстановлению. С учетом полученных результатов дальнейшую криоконсервацию расширенной выборки из девяти селекционных сортов проводили с использованием только одного типа эксплантов – апексов микропобегов, которые после замораживания–оттаивания культивировали при фотопериоде 16/8 ч. Частота посткриогенной регенерации этих сортов варьировала от 30 до 60 %. Установлено достоверное влияние генотипа на регенерационную способность сортов после замораживания–оттаивания. Способность сортов к посткриогенному восстановлению не связана со значениями морфогенетических показателей *in vitro* растений, которые используются в оригинальном семеноводстве картофеля. Возраст мериклона (2–4 года) не оказывал существенного влияния ни на показатели морфогенеза, ни на частоту посткриогенной регенерации сортов.

Ключевые слова: картофель; *Solanum tuberosum*; длительное хранение; криоконсервация; условия *in vitro* культивирования; морфогенез *in vitro* растений.

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Investigation of the post-cryogenic regeneration ability of potato varieties under different cultivation conditions

E.S. Bespalova[1], Yu.V. Ukhatova[1], N.N. Volkova[1], E.V. Oves[2], N.A. Gaitova[2], T.A. Gavrilenko[1, 3]

[1] Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
[2] Lorch Potato Research Institute, Kuskovo, Moscow, Russia
[3] Saint Petersburg State University, Biological Faculty, St. Petersburg, Russia
e-mail: tatjana9972@yandex.ru

Cryopreservation provides long-term storage of the gene pool of potato varieties in cryobanks at extremely low temperatures. Currently, droplet vitrification is the most widely used method for cryopreservation of potato varieties, which is constantly improving to increase the regeneration rates of the stored plant material. Different modifications of this method are used in the world's leading potato genebanks. This paper presents the results of studying the effect of cultivation conditions after plunging into liquid nitrogen and thawing of shoots tips and axillary buds of *in vitro* plants on their post-cryogenic recovery. The droplet-vitrification method modified at VIR was used for cryopreservation. The factor "prolonged dark incubation of explants" did not have a significant effect on the frequency of post-cryogenic regeneration of the studied varieties except for one variety (Krepysh), for which a significant increase in the regeneration rate was observed for the shoot tips cultivated in the darkness compared to the cultivation under the photoperiod 16/8 hours (light/darkness). The

frequency of post-cryogenic regeneration of shoot tips was higher than that of the axillary buds for all varieties; however, these differences were significant ($p < 0.05$) only in two cases: for the variety Udacha (a photoperiod of 16/8 hours) and for the variety Krepysh (the dark incubation). The results of two-factor analysis of variance indicate that there is no effect of interaction of factor 1 (prolonged dark incubation) and factor 2 (explant type) on the ability of varieties to post-cryogenic recovery. Taking into account the obtained results, the further cryopreservation of an extended subset of 9 varieties was carried out using shoot tips, which, after freezing-thawing, were cultivated under the photoperiod of 16/8 hours. The frequency of post-cryogenic regeneration of these varieties varied from 30 to 60 %. A significant effect of genotype on post-cryogenic recovery has been established. The ability of varieties to regenerate shoots after freezing and thawing was not related to the values of morphogenic indices of *in vitro* plants. The age of the meriklons (2–4 years) did not significantly affect either the morphogenic indices or the frequency of post-cryogenic regeneration.
Key words: potato; *Solanum tuberosum*; long-term preservation; cryoconservation; *in vitro* cultivation conditions; *in vitro* morphogenesis.

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Введение

Важнейшей вегетативно размножаемой продовольственной культурой является возделываемый картофель *Solanum tuberosum*. Генофонд селекционных сортов картофеля сохраняется в полевых коллекциях, а также в дублетных – *in vitro* и криоколлекциях. Гарантированное сохранение обеспечивается при наличии в генбанке всех трех типов коллекций (Гавриленко и др., 2007; Филипенко, 2007; FAO, 2014; Niino, Valle Arizaga, 2015). Долгосрочное хранение генофонда селекционных сортов картофеля в контролируемых условиях проводят при сверхнизких температурах в криобанках.

Для криоконсервации картофеля используют различные методы: витрификации, дроплет-замораживания, cryoplate, дроплет-витрификации (Kaczmarczyk et al., 2011; Niino, Valle Arizaga, 2015; Ухатова, Гавриленко, 2018). В настоящее время наиболее часто применяется метод дроплет-витрификации, разработанный Panis et al. (2005) для криоконсервации образцов банана. Данный метод был многократно модифицирован в разных лабораториях и используется в крупнейших мировых генбанках для создания криоколлекций картофеля (Kim et al., 2006; Panta et al., 2015; Vollmer et al., 2016, 2017; Ухатова и др., 2017; Jenderek, Reed, 2017; Гавриленко и др., 2019). Подробное сравнение различных модификаций метода дроплет-витрификации проведено в нашей другой работе (Гавриленко и др., 2019).

Наиболее крупная по численности (1533 образца) криоколлекция находится в Международном центре картофеля (CIP) в Перу (Vollmer et al., 2017). Первая в России криоколлекция селекционных и аборигенных сортов картофеля, насчитывающая 230 образцов, сохраняется в криобанке ВИР. Эта коллекция создается с использованием модифицированного в 2011 г. метода дроплет-витрификации (Дунаева и др., 2011; Shvachko, Gavrilenko, 2011).

Метод дроплет-витрификации, модифицированный в CIP, включает этап длительного (в течение одной недели) культивирования в темноте апексов *in vitro* растений после их замораживания–оттаивания. В генбанке NAC, NAAS, Кореи для криоконсервации используются и пазушные почки микрорастений. В модифицированном в ВИР методе этап длительной темновой инкубации не применяется, культивирование эксплантов с момента размораживания–оттаивания до получения полностью сформированных регенерантов проходит при фотопериоде 16/8 ч (свет/темнота) (Дунаева и др., 2011; Гавриленко и др., 2019). Отметим, что работ по изучению влияния темновой инкубации на индукцию и эффективность посткриогенной регенерации картофеля в доступной нам литературе нет.

Цель настоящей работы заключалась в изучении влияния длительной темновой инкубации на эффективность посткриогенной регенерации различных типов эксплантов (апексов и пазушных почек *in vitro* растений). С учетом полученных результатов в дальнейшем проводились эксперименты по криоконсервации расширенной выборки селекционных сортов картофеля.

Материалы и методы

В качестве материала были использованы *in vitro* растения 13 селекционных российских сортов картофеля, полученных из Банка здоровых сортов картофеля (БЗСК) Всероссийского НИИ картофельного хозяйства им. А.Г. Лорха: Антонина, Василек, Гулливер, Ильинский, Ирбитский, Крепыш, Кузнечанка, Любава, Накра, Удача, Тулеевский, Фрителла, Югана. Исходные *in vitro* растения выращивали три-четыре недели на питательной среде MS без гормонов для получения выровненных по физиологическому состоянию микрорастений.

Для изучения влияния длительной темновой инкубации на посткриогенное восстановление эксплантов отобрали четыре сорта картофеля (Ильинский, Крепыш, Накра, Удача), контрастных по способности к посткриогенному восстановлению (Ухатова и др., 2017). У микрорастений этих четырех сортов вычленяли как апексы микропобегов, так и пазушные почки (из верхних двух междоузлий).

Криоконсервацию сортов картофеля проводили в отделе биотехнологии ВИР с использованием модифицированного метода дроплет-витрификации (Дунаева и др., 2011), детальное описание которого приведено в работе (Гавриленко и др., 2019). Изолированные экспланты помещали в жидкую среду MS, затем переносили в жидкую среду LS (MS с 0.4M сахарозой и 2M глицеролом) на 20 мин, после чего экспланты помещали в раствор PVS2 с криопротекторами (MS с добавлением 3.26 M глицерола, 2.42 M этиленгликоля, 1.9 M ДМСО и 0.4 M сахарозы) и оставляли на 30 мин на льду. Экспланты, погруженные в капли раствора PVS2, переносили в заполненные жидким азотом криопробирки, которые помещали на один час в

сосуд Дьюара с жидким азотом. Оттаивание материала проводили при комнатной температуре в жидкой среде RS (MS с добавлением 1.2 М сахарозы) в течение 15 мин. Затем экспланты переносили в чашки Петри со средой MSTo (MS с добавлением 0.5 мг/л зеатин рибозида, 0.5 мг/л ИУК, 0.2 мг/л ГК, 20 г/л сахарозы, 7 г/л агар-агара) и культивировали при фотопериоде 16/8 ч (свет/темнота) (Гавриленко и др., 2019).

В вариантах опыта с темновой инкубацией экспланты после замораживания–оттаивания переносили в чашки Петри со средой MSTo, которые плотно заворачивали алюминиевой фольгой и оставляли на тех же светоустановках на 7 дней. Через одну неделю фольгу снимали и продолжали культивирование эксплантов при фотопериоде 16/8 ч (свет/темнота).

В конце восьмой недели культивирования после замораживания–оттаивания учитывали регенерационную способность эксплантов в каждом варианте опыта (число эксплантов, сформировавших побеги). Данные представляли в процентах к общему числу криоконсервированных эксплантов. Эксперименты выполняли в трех повторностях.

После получения результатов изучения влияния темновой инкубации и типа эксплантов на посткриогенное восстановление была проведена еще одна серия экспериментов по криоконсервации девяти дополнительных сортов (Антонина, Любава, Тулеевский, Фрителла, Югана, Ирбитский, Василек, Гулливер, Кузнечанка). В этой серии экспериментов в каждой повторности опыта изолировали по 20 эксплантов на сорт для контроля посткриогенной регенерации и дополнительно еще 30 эксплантов с последующей их закладкой на длительное криохранение в биокриокомплекс ВИР.

Данные посткриогенного восстановления девяти сортов сравнивали с четырьмя показателями морфогенеза растений в культуре *in vitro* (Овэс и др., 2018): 1) продолжительностью периода от черенкования до формирования микрорастениями двух-трех междоузлий; 2) продолжительностью периода от черенкования до формирования микрорастениями четырех-шести междоузлий; 3) продолжительностью периода активного роста микрорастений; 4) продолжительностью всего вегетационного периода микрорастений – от черенкования до формирования ими микроклубней. Кроме того, учитывали «возраст мериклона» – общую продолжительность пребывания данного клона в культуре *in vitro*.

Обработку полученных результатов и оценку достоверности различий между вариантами опытов проводили с помощью методов вариационной статистики (*t*-критерий Стьюдента, коэффициент корреляции), а также компьютерной программы STATISTICA 6.0 (модули одно- и двухфакторного анализа).

Результаты

Изучение влияния условий культивирования различных типов эксплантов на частоту их посткриогенной регенерации. Уже на третьей неделе после замораживания–оттаивания наблюдали появление первых регенерантов и на апексах микропобегов, и на пазушных почках в двух вариантах опыта – культивирование эксплантов в темноте и при фотопериоде 16/8 ч (см. рисунок). К концу восьмой недели культивирования, когда проводился учет частоты посткриогенного восстановления, число регенерирующих эксплантов, как правило, возрастало.

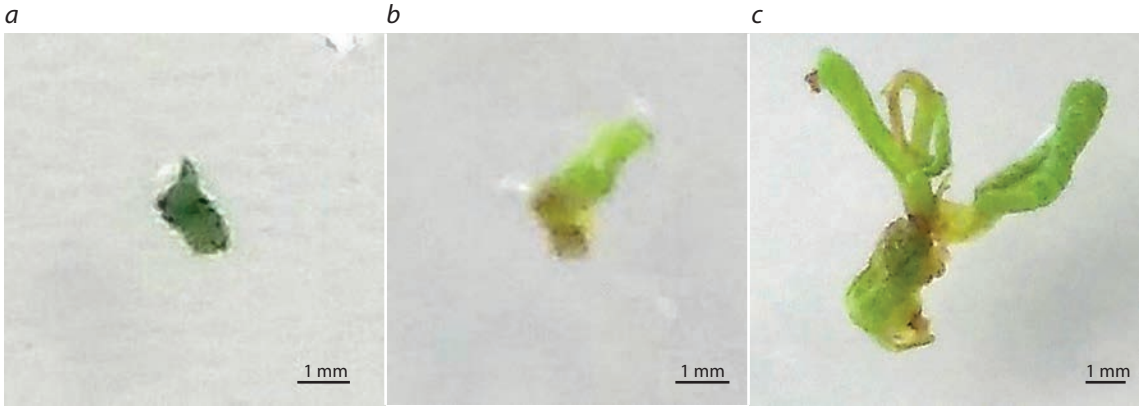
В табл. 1 представлены результаты изучения влияния условий культивирования после замораживания–оттаивания апексов микропобегов и пазушных почек *in vitro* растений на их способность к посткриогенному восстановлению. Длительная темновая инкубация эксплантов не оказывала существенного влияния на частоту посткриогенной регенерации изученных сортов, за исключением сорта Крепыш, для которого отмечено достоверное увеличение частоты регенерации в варианте культивирования апексов микропобегов в темноте по сравнению с вариантом культивирования при фотопериоде 16/8 ч (свет/темнота) ($t_{st} = 2.8$, $p < 0.05$) (см. табл. 1). В среднем по суммарным данным, полученным для всех четырех сортов, существенного влияния темновой инкубации на посткриогенное восстановление как апексов микропобегов, так и пазушных почек выявлено не было ($t_{st} = 0.85$, $p > 0.05$). Результаты однофакторного анализа подтвердили отсутствие достоверного влияния фактора «темновая инкубация» на уровень посткриогенной регенерации изученных сортов ($p = 0.154$).

Влияние типа экспланта на уровень регенерации после оттаивания четырех сортов было статистически значимым ($p = 0.002$). Частота посткриогенной регенерации апексов микропобегов была достоверно выше ($p < 0.05$), чем у пазушных почек, у сорта Удача (культивирование при фотопериоде 16/8 ч) и у сорта Крепыш (культивирование в условиях темновой инкубации) (см. табл. 1). Результаты двухфакторного дисперсионного анализа указывают на отсутствие значимого эффекта совместного действия двух факторов на способность сортов к посткриогенному восстановлению. Отмечено статистически значимое влияние генотипа ($p = 0.039$) на частоту посткриогенной регенерации.

С учетом полученных результатов дальнейшую криоконсервацию расширенной выборки из девяти селекционных сортов проводили с использованием апексов микропобегов, которые после замораживания–оттаивания культивировали при фотопериоде 16/8 ч (свет/темнота).

Изучение показателей морфогенеза *in vitro* и способности к посткриогенному восстановлению у селекционных сортов картофеля. В табл. 2 представлены данные по регенерационной способности апексов микропобегов девяти сортов картофеля после замораживания–оттаивания. По уровню посткриогенной регенерации изученные сорта можно разделить на две группы: образцы с регенерационной способностью менее 40 % (сорта Любава, Тулеевский, Фрителла), и сорта, регенерационная способность которых была выше 40 % (Антонина, Ирбитский, Василек, Гулливер, Кузнечанка). Полученные результаты указывают на существенное ($p < 0.05$) влияние генотипа на показатель посткриогенной регенерации, что отмечается и в большинстве работ по криоконсервации разных видов растений, включая картофель (Bamberg et al., 2016; Volk et al., 2016; Ухатова и др., 2017; Vollmer et al., 2017).

В нашей работе была изучена связь между различными морфогенетическими признаками – способностью сортов к посткриогенному восстановлению и показателями фаз



Formation of postcryogenic regenerants in Krepysh variety during the cultivation of shoot tips on MSTo nutrient medium, lighting schedule 16L:8D.

(*a*) Explant in the first week after thawing; (*b*) emergence of a regenerant in the third week; (*c*) regenerant development in the eighth week of cultivation.

Table 1. Frequencies of postcryogenic regeneration in different conditions of cultivation of two types of explants (shoot tips and axillary buds) of potato varieties

Variety	Variants of experiments			
	16L:8D		Long dark incubation (1 week)	
	Shoot tips	Axillary buds	Shoot tips	Axillary buds
Il'inskiy	33.3 ± 22.0 b	16.7 ± 11.0 bc	40.8 ± 15.8 ab	12.5 ± 7.2 bc
Krepysh	43.3 ± 6.7 b	36.7 ± 12.0 b	62.5 ± 1.4 a	30.0 ± 11.5 bc
Nakra	20.0 ± 10.0 bc	20.0 ± 10.0 bc	35.0 ± 5.0 b	17.5 ± 7.5 bc
Udacha	30.0 ± 5.8 b	14.2 ± 3.0 c	46.7 ± 16.7 ab	17.5 ± 6.3 bc
$X \pm m_x$	31.7 ± 4.8 b	21.9 ± 5.9 bc	46.3 ± 5.9 b	19.4 ± 3.7 bc

Note: Differences between values marked with different letters are significant at $p < 0.05$.

Table 2. Frequencies of postcryogenic regeneration of potato varieties and their morphogenetic indicators in micropropagation

Variety	Frequency of postcryogenic regeneration, %	Morphogenetic parameters of microplants (the timing of development phases, days))				Age of the original mericlone
		Phase of intense growth of microplants		3	4	
		1	2			
Lyubava	30.0 ± 15.3	12–15	30–35	30–45	45–85	3
Tuleevskiy	35.0 ± 5.0	12–15	30–35	30–45	45–75	4
Fritella	35.0 ± 5.0	20–23	38–45	45–55	55–80	3
Yugana	40.0 ± 10.0	12–15	25–30	25–45	45–80	4
Antonina	41.7 ± 8.4	12–15	25–30	25–45	45–80	4
Irbitskiy	45.0 ± 5.0	20–23	38–45	45–55	55–80	4
Vasilek	45.0 ± 5.0	12–15	30–35	30–45	45–65	3
Гулливер	50.0 ± 16.7	12–15	25–30	25–45	45–80	2
Kuznechanka	60.0 ± 10.0	15–20	30–35	30–65	65–90	4

Notes: Designations of morphogenetic parameters: 1, time from grafting to the formation of two or three internodes in the plants; 2, time from grafting to the formation of four to six internodes; 3, time of intense growth; 4, duration of the whole vegetation period of microplants, from grafting to microtuber formation.

роста и развития *in vitro* растений в процессе их микроразмножения (см. табл. 2). Данные морфогенетические показатели применяются при выращивании микрорастений с целью дальнейшего получения мини-клубней и в настоящее время начинают использоваться в коммерческих компаниях, производящих микрорастения картофеля в больших объемах (Овэс и др., 2018).

Три сорта из девяти (Гулливер, Югана, Антонина) характеризовались ускоренным протеканием фаз интенсивного роста микрорастений (показатели 1 и 2), поэтому период достижения микрорастениями этих сортов стандартных размеров (4–6 междоузлий) не превышал одного календарного месяца (см. табл. 2). Эти три сорта выделялись также наиболее короткой продолжительностью периода активного роста *in vitro* растений (показатель 3). Поздний срок наступления фазы интенсивного роста микрорастений отмечен для сортов Ирбитский и Фрителла. Наибольшая продолжительность всего вегетационного периода в культуре *in vitro* отмечена для микрорастений сорта Кузнечанка (см. табл. 2). Наличие статистически значимой положительной корреляции отмечено для морфогенетических показателей 1 и 2 ($r = 0.90$), 1 и 3 ($r = 0.96$), 2 и 3 ($r = 0.88$), 3 и 4 ($r = 0.93$). В то же время статистически достоверной корреляции между способностью изученных девяти сортов к ускоренному морфогенезу в условиях *in vitro* и частотой их посткриогенной регенерации не выявлено. Возраст мериклона не оказывал существенного влияния ни на показатели морфогенеза, ни на частоту посткриогенной регенерации сортов (см. табл. 2).

Заключение

Результаты изучения посткриогенного регенерационного потенциала сортов картофеля в разных условиях культивирования указывают на отсутствие существенного влияния длительной темновой инкубации эксплантов и значительный эффект типа экспланта: фактически у всех сортов частота посткриогенной регенерации апексов микропобегов была выше, чем у пазушных почек. Установлено достоверное влияние генотипа на регенерационную способность сортов после замораживания–оттаивания. Способность сортов к посткриогенному восстановлению не связана со значениями морфогенетических показателей *in vitro* растений; возраст мериклона также не оказывал существенного влияния ни на показатели морфогенеза, ни на частоту посткриогенной регенерации сортов. В практическом плане для дальнейшего пополнения криоколлекции сортов картофеля можно рекомендовать модифицированный в ВИР метод дроплет-витрификации. При его применении следует использовать апексы микропобегов, регенерация которых проводится при стандартных для этого метода условиях (Гавриленко и др., 2019). Данный метод эффективен для криоконсервации сортов различного происхождения, контрастных по морфогенетическому потенциалу в культуре *in vitro*.

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ORCID ID

T.A. Gavrilenko orcid.org/0000-0002-2605-6569

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Polymorphism of ITS sequences in 35S rRNA genes in *Elymus dahuricus* aggregate species: two cryptic species?

A.V. Rodionov[1, 2] ✉, K.S. Dobryakova[1], N.N. Nosov[1], A.A. Gnutikov[1, 3], E.O. Punina[1], A.A. Kriukov[4], V.S. Shneyer[1]

[1] Komarov Botanical Institute, RAS, St. Petersburg, Russia
[2] Saint-Petersburg State University, Faculty of Biology, St. Petersburg, Russia
[3] Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
[4] All-Russia Research Institute for Agricultural Microbiology, Pushkin, Saint-Petersburg, Russia
✉ e-mail: avrodionov@mail.ru

Nuclear ribosomal internal transcribed spacer (ITS) sequences were sequenced for 23 species and subspecies of *Elymus sensu lato* collected in Russia. The Neighbor-Net analysis of ITS sequences suggested that there are four ribotypes called Core Northern St-rDNA, Core Southern St-rDNA, Northern dahuricus St-rDNA and Southern dahuricus St-rDNA. The Core Southern variant of St-rDNA is closely related to rDNA of diploid *Pseudoroegneria stipifolia* (PI 313960) and *P. spicata* (PI 547161). The Core Northern St-rDNA is closely related to rDNA of *P. cognata* (PI 531720), a diploid species of Kyrgyzstan carrying St^Y variant of the St genome. The Core Northern St-rDNA is widespread among the *Elymus* species of Siberia and the Far East, including Yakutia and Chukotka. The Core Southern St-ribotype is typical of southern *Elymus* and *Pseudoroegneria* of the South Caucasus, Primorye, Pakistan, and South Korea. The Northern dahuricus St-ribotype and Southern dahuricus St-ribotype are derivatives of the Core Northern and Core Southern St-ribotypes, correspondingly. Both of them were found in all four studied species of the *E. dahuricus* aggregate: *E. dahuricus* Turcz. ex Griseb., *E. franchetii* Kitag., *E. excelsus* Turcz. ex Griseb. and Himalayan *E. tangutorum* (Nevski) Hand.-Mazz. In other words, there are at least two population groups (two races) of the *Elymus dahuricus* aggregate species that consistently differ in their ITS-sequences in Siberia, the Far East and Northern China. Each contains all morphological forms, which taxonomists now attribute either to different species of *E. dahuricus* aggr. (*E. dahuricus sensu stricto*, *E. franchetii*, *E. tangutorum*, *E. excelsus*) or subspecies of *Campeiostachys dahurica* (Turcz. ex Griseb.) B.R. Baum, J.L. Yang et C.C. Yen. At the moment it is unknown if there are any morphological differences between plants carrying either Northern or Southern dahuricus rDNA. Probably, they are cryptic species, but it is certain that if differences in morphology between the two races exist, they are not associated with signs that are now considered taxonomically significant and are used to separate *E. dahuricus* s. s., *E. franchetii*, *E. tangutorum*, and *E. excelsus*.
Key words: *Elymus dahuricus* aggr.; interspecific hybridization; rDNA; 35S rRNA; Triticeae.

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Полиморфизм ITS-последовательностей генов 35S рРНК у видов *Elymus dahuricus* aggr.: два криптических вида?

А.В. Родионов[1, 2] ✉, К.С. Добрякова[1], Н.Н. Носов[1], А.А. Гнутиков[1, 3], Е.О. Пунина[1], А.А. Крюков[4], В.С. Шнеер[1]

[1] Ботанический институт им. В.Л. Комарова Российской академии наук, Санкт-Петербург, Россия
[2] Санкт-Петербургский государственный университет, биологический факультет, Санкт-Петербург, Россия
[3] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
[4] Всероссийский научно-исследовательский институт сельскохозяйственной микробиологии, Пушкин, Санкт-Петербург, Россия
✉ e-mail: avrodionov@mail.ru

Секвенированы последовательности внутренних транскрибируемых спейсеров (ITS) 23 видов и подвидов *Elymus sensu lato*. При анализе молекулярно-филогенетической сети Neighbor-Net все последовательности ITS образцов *Elymus* s. l. были разделены на четыре типа: основной северный Elymus-риботип, основной южный Elymus-риботип, северный dahuricus-риботип и южный dahuricus-риботип. рДНК основного южного риботипа родственна рДНК диплоидного вида *Pseudoroegneria stipifolia* (PI 313960) и *P. spicata* (PI 547161). рДНК основного северного риботипа родственна рДНК *P. cognata* (PI 531720), диплоидного вида из Казахстана, несущего St^Y – вариант St-генома. Основной северный риботип широко распространен у видов *Elymus* Сибири и Дальнего Востока, включая Якутию и Чукотку. Основной южный St-риботип характерен для относительно южных популяций *Elymus* и *Pseudoroegneria*, включая Закавказье, Приморье, Пакистан, Южную Корею. Отметим, что северный Elymus dahuricus-риботип и южный Elymus dahuricus-риботип были обнаружены у всех четырех видов группы родства *E. dahuricus* aggr.: *E. dahuricus* Turcz. ex Griseb., *E. franchetii* Kitag., *E. excelsus* Turcz. ex Griseb. и у гималайского вида *E. tangutorum* (Nevski) Hand.-Mazz. Иными словами, молекулярно-филогенетические исследования образцов, относимых к *E. dahuricus* aggr., говорят о том, что в Сибири, на Дальнем Востоке и в Северном Китае существуют по крайней мере две группы популяций (две расы), надежно различающиеся

по ITS-последовательностям, в каждой из которых представлены все морфологические формы, относимые сейчас одними систематиками к четырем разным видам *E. dahuricus* aggr. (*E. dahuricus sensu stricto*, *E. franchetii*, *E. tangutorum*, *E. excelsus*), а другими – к одному виду *Campeiostachys dahurica* (Turcz. ex Griseb.) B.R. Baum, J.L. Yang et C.C. Yen. Имеются ли между этими группами морфологические различия, или это криптические виды (подвиды) – неизвестно, но с уверенностью можно сказать, что если различия в морфологии между этими двумя расами есть, то они не связаны с признаками, которые сейчас считаются таксономически значимыми и используются для разделения *E. dahuricus* s. s., *E. franchetii*, *E. excelsus*, *E. tangutorum*.
Ключевые слова: *Elymus dahuricus* aggr.; межвидовая гибридизация; 35S рРНК; Triticeae.

Introduction

The beginning of the 21 century was marked by very wide using of DNA sequencing in systematics and phylogeny of animals and plants. Remarkable result of this was an exponential rise in the discovery of cryptic species in different groups of animals (Bickford et al., 2007). However, such discoveries are much rarer in plants, especially in angiosperms (Shneyer, Kotseruba, 2015). We suggest that cryptic species may exist in particular, in the genus *Elymus* L. (Triticeae).

Now it is considered the genus *Elymus* is represented in Russia by 53 species (Tzvelev, Probatova, 2010). All these species are allopolyploids with St, Y, H subgenomes and haplomes StY (2*n* = 28), StH (2*n* = 28) and StYH (2*n* = 42), whereas primary diploids (2*n* = 14) are absent in the genus (Agafonov et al., 2001; Agafonov, 2007). Based on the results of interspecies hybridization, DNA sequencing and GISH, it has been suggested that all the *Elymus* species share a common St subgenome originated from the genus *Pseudoroegneria* (Nevski) Á. Löve species and H subgenome from an ancestor of the genus *Hordeum* L. (Dewey, 1984; Sun, Zhang, 2011; Yan et al., 2011; Mason-Gamer, 2013). It was suggested that North American perennial bunchgrass *Pseudoroegneria spicata* (Pursh) A. Löve was most likely donor of the Y subgenome, although Asiatic species *P. cognata* (Hackel) A. Löve (syn.: *Agropyron ferganense* Drobow) and *P. libanotica* (Hack.) D.R. Dewey also could not be excluded (Okito et al., 2009), particularly for the Asiatic *Elymus* species with StY and StHY genome compositions.

C. Yen et al. (2005) divided the genus *Elymus* s. l., strictly in accordance with their genomic constitution, into six genera: *Douglasdeweya* C. Yen, J.L. Yang et B.R. Baum (StStPP); *Roegneria* C. Koch (StStYY); *Anthosachne* Steudel (StSt WWYY); *Kengylia* C. Yen et J.L. Yang (StStPPYY); allohexaploid species with the StStYYHH karyotypes (Yen et al., 2005; Baum et al., 2011) were referred to the genus *Campeiostachys* Drobov, and *Elymus* L. in this treatment included only the species with the StStHH/StStHHHH/StStStStHH karyotypes (Yen et al., 2005; Yen, Yang, 2009). Though the separation of species into genera based on the karyotype constitution is attractive from a genetic point of view (Dewey, 1984; Tzvelev, 1991; Agafonov, 2007), it should be noted that the division species into genera only based on their genome composition does not always correlate with morphological criteria by which species and genera defined and delimited (Jensen, Chen, 1992; Baum et al., 2011). Internal transcribed spacers ITS1 and ITS2 of the nuclear genes 35S rRNA were widely employed in molecular phylogenetic studies of *Elymus* of China and North America (Liu et al., 2006; Wang et al., 2009; Mason-Gamer, 2013; Rabey, 2014; Gao et al., 2015; and others).

The main objective of our study is an assessment of interspecial ITS-polymorphism of *Elymus* of Siberian and the Far Eastern flora. This is interesting from the genetic point of view because, the phenomenon of interspecific and introgressive hybridization is widespread among Siberian and Far Eastern populations/natural races of *Elymus* (Agafonov, 1997; Wu et al., 2015). East Eurasian species of *Elymus* have all the features of a syngameon (Lotsy, 1925). It was necessary to ascertain how this fact effects on genetic distances between the Siberian *Elymus* 'varieties' which taxonomists delimitate as several morphologically discret species. Also, the aim of our study was to study relationships in *Elymus dahuricus* aggr. to which Tzvelev, Probatova (2010) referred four species, also treated as subspecies of species *Campeiostachys dahurica* (Turcz. ex Griseb.) B.R. Baum, J.L. Yang et C.C. Yen (Baum et al., 2011).

Material and methods

Nuclear ribosomal internal transcribed spacer sequences (ITS) were sequenced from 34 accessions belonging to 23 species and subspecies of *Elymus* s. l. (Table 1). The plant samples were collected in the Altai Krai and Altai Republic, Khakassia, the Kemerovo Oblast, Yakutia and the Northern Caucasus from 2004 to 2013. Herbarium specimens are stored in the herbarium of the Laboratory of Biosystematics and Cytology and in the Herbarium LE of the Komarov Botanical Institute.

Total genomic DNA was isolated using the CTAB method (Doyle J.J., Doyle J.L., 1987), with minor modifications described previously (Rodionov et al., 2008). Amplification of the ITS region was performed using primers ITS 1P (Ridgway et al., 2003) and ITS 4 (White et al., 1990). The PCR reaction was carried out in a total volume of 50 μL containing 1× SE-buffer AS (SibEnzyme, Russia), 2.5 mM Mg^{2+}, 2 mM each of dATP, dTTP, dCTP, dGTP) (Helicon, Russia), 0.01 μM of each primer (Beagle, Russia), 1–2 μL total DNA, 5 units of Taq-polymerase (SibEnzyme, Russia) and distilled water to the final volume. PCR amplification was done also using 1× Maxima Hot Start Taq buffer (Thermo Scientific, Sweden), 2.5 mM Mg^{2+} (Thermo Scientific, Sweden), 2 mM dATP, dTTP, dCTP, dGTP (Helicon, Russia), 0.01 μM of each primer, 1–2 μL total DNA, 5 units of Maxima Hot Start Taq polymerase (Thermo Scientific, Sweden), and distilled water. Amplification *parameters*: primary denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR products were electrophoresed in 1 % agarose gel. The QiaGen Extraction Kit (Qiagen, Germany) was used to extract the DNA from the gel. Sanger sequencing was performed in The Core Facilities Center "Cell and Molecular Technologies in Plant Science" at the Komarov Botanical Institute of the Russian Academy of Sciences. The PCR products were sequenced in both directions on ABI

Table 1. Types of ITS sequences in species of *Elymus* and *Pseudoroegneria*, our data

No.	Species	Genome/Haplome	Origin	GenBank	Ribotype
	Section Turczaninovia (Nevski) Tzvel. (syn. genus *Campeiostachys* Drobov)				
1	*E. dahuricus* Turcz. ex Griseb.	StHY	RF: Khakassia	KJ540222	Southern dahuricus St-rDNA
2	*E. dahuricus* Turcz. ex Griseb.	StHY	RF: Altai Republic	KJ540223	
	Section Goulardia (Husn.) Tzvel.				
3	*E. amurensis* (Drob.) Czer.	StY	RF: Primorsky Krai	KM871828	Southern dahuricus St-rDNA
4	*E. caninus* (L.) L.	StH	RF: Altai Republic	KJ561233	Northern St-rDNA
5	*E. caninus* (L.) L.	StH	RF: North Caucasus	KJ561234	
6	*E. ciliaris* (Trin.) Tzvel.	StY	RF: Primorsky Krai	KM871829	Southern St-rDNA
7	*E. fibrosus* (Schrenk) Tzvel.	StH	RF: Altai Republic	KM363383	Northern St-rDNA
8	*E. fibrosus* (Schrenk) Tzvel.	StH	Finland	KM871830	
9	*E. gmelinii* (Ledeb.) Tzvel.	StY	RF: Kemerovo Oblast	KJ755831	
10	*E. gmelinii* (Ledeb.) Tzvel.	StY	RF: Altai Republic	KM363382	
11	*E. jacutensis* (Drob.) Tzvel.	Unknown	RF: Yakutia	KM363381	
12	*E. jacutensis* (Drob.) Tzvel.	Unknown	RF: Altai Republic	KM575844	
13	*E. komarovii* (Nevski) Tzvel.	StH		KJ561236	
14	*E. macrourus* (Turcz.) Tzvel.	StH		KM379150	
15	*E. macrourus* (Turcz.) Tzvel.	StH	RF: Yakutia	KM502299	
16	*E. mutabilis* (Drob.) Tzvel.	StH	RF: Altai Republic	KM871827	
17	*E. nevskii* Tzvel.	StY	RF: Altai Krai	KJ540224	
18	*E. probatovae* Tzvel.	Unknown	RF: Chukotka	KM871831	
19	*E. sajanensis* (Nevski) Tzvel.	StH	RF: Altai Republic	KM502300	
20	*E. sajanensis* (Nevski) Tzvel.	StH	RF: Tuva	KM871825	
21	*E. scandicus* (Nevski) Tzvel.	StH	RF: Altai Republic	KJ561237	
22	*E. subfibrosus* (Tzvel.) Tzvel.	StH	RF: Yakutia	KM975705	
23	*E. trachycaulus* (Link) Gould et Shinners	StH	RF: Primorsky Krai	KM975706	
24	*E. transbaicalensis* (Nevski) Tzvel.	StH	RF: Altai Republic	KJ561235	
25	*E. transbaicalensis* (Nevski) Tzvel.	StH		KM363385	
26	*E. transbaicalensis* (Nevski) Tzvel.	StH		KM575845	
27	*E. vernicosus* (Nevski ex Grub.) Tzvel.	StY		KJ540221	
28	*E. vernicosus* (Nevski ex Grub.) Tzvel.	StY		KM871821	Northern dahuricus St-rDNA
	Section Elymus				
29	*E. peschkovae* Tzvel.	StH	RF: Yakutia	KM871824	Northern St-rDNA
30	*E. schrenkianus* (Fisch. et C.A. Mey.) Tzvel.	StHY	RF: Altai Republic	KM502297	
31	*E. schrenkianus* (Fisch. et C.A. Mey.) Tzvel.	StHY	RF: Tuva	KM502298	
32	*E. schrenkianus* (Fisch. et C.A. Mey.) Tzvel.	StHY	RF: Altai Republic	KM502301	
33	*E. sibiricus* L.	StH		KJ540220	
	The hybrid				
34	*Elymus* sp.	Unknown	RF: Altai Republic	KJ561239	Northern St-rDNA
	Genus *Pseudoroegneria* (Nevski) A. Löve				
35	*P. geniculata* (Trin.) A. Löve (syn. *Elytrigia geniculata* (Trin.) Nevski)	StSt	RF: Khakassia	KJ561242	Northern St-rDNA

Prism 3130 (Applied Biosystems, USA). All sequences were submitted to the GenBank (NCBI) database. The sequences were aligned with ClustalW using the MEGA 6 (Tamura et al., 2013) software package with subsequent visual verification. The SplitsTree4 algorithm Neighbor-Net (Huson, Bryant, 2006), proposed for the study of network evolution (Bryant, Moulton, 2004; Huson, Bryant, 2006) was used.

Results

We studied variability of ITS-sequences of *E. dahuricus*, *E. excelsus*, *E. franchetii*, *E. tangutorum*, species that constitute *Elymus dahuricus* aggr. (Tzvelev, Probatova, 2010). ITS-sequences of these species, as well as of some other species of *Elymus*, *Elytrigia*. *Pseudoroegneria* and *Hordeum* are provided in Table 1 and Table 2.

Table 2. Types of ITS sequences in species of the genus *Elymus*, *Elytrigia*, *Pseudoroegneria* and *Hordeum*. ITS1-genes 5.8S rRNA-ITS2 sequences from the international database GenBank used in our work

No.	Species	Genome	Origin	GenBank	Ribotype
	Section Turczaninovia (Nevski) Tzvel. (syn. Genus *Campeiostachys* Drobov)				
36	*E. dahuricus* Turcz. ex Griseb.	StHY	South Korea	HQ600520 (Kim Y.D. et al., unpubl.)	Southern dahuricus St-rDNA
37	*E. dahuricus* Turcz. ex Griseb.	StHY	China?	JN009816	Northern St-rDNA
38	*E. dahuricus* Turcz. ex Griseb.	StHY	South Korea: Taeangun, Choongcheongnam-do	KF713222 (Lee J. et al., unpubl.)	Southern dahuricus St-rDNA
39	*E. dahuricus* Turcz. ex Griseb.	StHY	China: Shandan, Gansu	KF905152 (Song et al., 2015)	Northern dahuricus St-rDNA
40	*E. dahuricus* Turcz. ex Griseb.	StHY	China: Yuzhong, Gansu	KF905178 (Song et al., 2015)	
41	*E. dahuricus* Turcz. ex Griseb.	StHY	China	KJ526338 (Gao et al., 2015)	Southern dahuricus St-rDNA
42	*E. excelsus* Turcz. ex Griseb.	StHY	China: Neimenggu	KJ526341	
43	*E. excelsus* Turcz. ex Griseb.	StHY		KJ526342	Northern dahuricus St-rDNA
44	*E. excelsus* Turcz. ex Griseb.	StHY		KJ526343	
45	*E. excelsus* Turcz. ex Griseb.	StHY	China	JN009803 (Li X. et al., unpubl.)	Southern dahuricus St-rDNA
46	*E. excelsus* Turcz. ex Griseb.	StHY		JN009809 (Li X. et al., unpubl.)	
47	*E. franchetii* Kitag. (*E. dahuricus* var. *cylindricus* Franch.)	StHY	China?	JN009805 (Li X. et al., unpubl.)	
48	*E. franchetii* Kitag.	StHY	China: Haiyuan	KF905180 (Song et al., 2015)	Northern dahuricus St-rDNA
49	*E. franchetii* Kitag.	StHY	China: Xinjiang	KJ526336 (Gao et al., 2015)	Southern dahuricus St-rDNA
50	*E. franchetii* Kitag.	StHY		KJ526337 (Gao et al., 2015)	
51	*E. tangutorum* (Nevski) Hand.-Mazz.	StHY		KJ526351 (Gao et al., 2015)	
52	*E. tangutorum* (Nevski) Hand.-Mazz.	StHY		KJ526352 (Gao et al., 2015)	Northern dahuricus St-rDNA
	Section Goulardia (Husn.) Tzvel.				
53	*E. caninus* (L.) L.	StH	China: Nei Monggol, Xilinhot	KJ526335 (Dong et al., 2015)	Northern St-rDNA
54	*E. dolichatherus* (Keng) S.L. Chen	StY	China	EU617242 (Liu Q. et al., unpubl.)	Southern dahuricus St-rDNA
55	*E. dolichatherus* (Keng) S.L. Chen	StY		EU617245 (Liu Q. et al., unpubl.)	Northern St-rDNA
56	*E. fedtschenkoi* Tzvel.	StY	China: Xinjiang, Habahe	AY740838 (Liu et al., 2006)	
57	*E. gmelinii* (Ledeb.) Tzvel.	StY	China: Xinjiang, Altay	AY740842 (Liu et al., 2006)	Northern dahuricus St-rDNA
	Section Clinelymopsis (Nevski) Tzvel.				
58	*E. caucasicus* (K. Koch) Tzvel.	StY	Armenia: Dilidjan	AY740808 (Liu et al., 2006)	Southern St-rDNA
	Section Elymus				
59	*E. confusus* (Roshev.) Tzvelev	StH	Mongolia	FJ040160 (Wang et al., 2009)	Northern St-rDNA
60	*E. sibiricus* L.	StH	China: Gansu, Hezuo	EF396962 (Wang et al., 2009)	
	Gen. *Elytrygia* Desv.				
61	*E. repens* (L.) Nevski	StStH	South Korea: Yungyanggun	KF713228 (Lee J. et al., unpubl.)	Southern St-rDNA
62	*E. repens* (L.) Nevski	StStH	China	MF893161 (Yang et al., 2017)	
	Pseudoroegneria (Nevski) A. Löve				
63	*P. cognata* (Hackel) A. Löve	St	Kyrgyzstan: Osh	EF014226 (Yu et al., 2008)	Northern St-rDNA
64	*P. elytrigioides* (C. Yen & J.L. Yang) B.R. Lu	StSt	China: Tibet, Changdu	AY740798 (Liu et al., 2006)	
65	*P. geniculata* (Trin.) A. Löve	StSt	RF: Altai Republic	EF014228 (Yu et al., 2008)	
66	*P. geniculata* (Trin.) A. Löve	StSt		EU617141 (Liu Q. et al., unpubl.)	
67	*P. kosanini* (Nabelek) A. Löve	Unknown ($2n = 56$)	Turkey	EF014235 (Yu et al., 2008)	Southern St-rDNA

Table 2 (end)

No.	Species	Genome	Origin	GenBank	Ribotype
68	*P. kosanini* (Nabelek) A. Löve	Unknown (2*n* = 56)	Turkey	EF014236 (Yu et al., 2008)	Northern St-rDNA
69	*P. sosnowskyi* (Hack.) A. Löve	St		GQ365150 (Dizkirici et al., 2010)	
70	*P. sosnowskyi* (Hack.) A. Löve	St		GQ365151 (Dizkirici et al., 2010)	
71	*P. spicata* (Pursh) A. Löve	St and StX	USA: Oregon	AY740793 (Liu et al., 2006)	Southern St-rDNA
72	*P. spicata* (Pursh) A. Löve	St and StX	USA: Wyoming, Half Moon Lake	EF014239 (Yu et al., 2008)	Northern St-rDNA
73	*P. stipifolia* (Czern. ex Nevski) A. Löve	St	RF: Stavropol Botanical Garden	EF014240 (Yu et al., 2008)	
74	*P. stipifolia* (Czern. ex Nevski) A. Löve	St		EU617041 (Liu Q. et al., unpubl.)	Southern St-rDNA
75	*P. strigosa* (Bieb.) A. Löve	St? 2*n* = 28	Crimea, Ai-Petri	EF014241 (Yu et al., 2008)	
76	*P. tauri* (Boiss. & Bal.) A. Löve	StP	Iran	EU617155 (Liu Q. et al., unpubl.)	Northern St-rDNA
77	*P. tauri* (Boiss. & Bal.) A. Löve	StP		EU617173 (Liu Q. et al., unpubl.)	Southern St-rDNA
			Gen. *Hordeum*		
78	*H. bogdanii* Wilensky	H	China	AY740876 (Liu et al., 2006)	*Hordeum* spp.
79	*H. murinum* ssp. *leporinum* (Link) Arcang.	HH	Iran: Tehran	KP126672 (Makhoul M.T. et al., unpubl.)	
80	*H. murinum* L. ssp. *murinum* (Hack.) H. Scholz et Raus	HH	Germany	KC193786 (Rabey, 2014)	
81	*H. vulgare* L.	H		FJ593180 (Daniel C. and Knoess W., unpubl.)	
82	*H. vulgare* var. *distichon* (L.) Hook. f.	H	Egypt	KC193783 (Rabey, 2014)	
83	*H. vulgare* subsp. *spontaneum* K. Koch	H	Afghanistan	KM217265 (Georgiev O. et al., unpubl.)	

Traditional evolution models, implying a gradual accumulation of mutations followed by dichotomous branching of phylogenetic trees, are ill-suited for describing species divergence in these taxa (Dobryakova, Nosov, 2015; Rodionov et al., 2017, 2018). Therefore, the results of ITS sequencing results were processed with the Neighbor-Net algorithm by the program SplitsTree4, suggested for reconstruction of reticulate evolution (Huson, Bryant, 2006). The Neighbor-Net algorithm builds a network called a split graph. The split graph (Fig. 1) shows several possible ways of grouping DNA sequences with varying degrees of probability, known as "splits", and reflects the presence of *homoplasy* in the data.

Fig. 1 shows that all species carrying St genomes, *Elymus*, *Pseudoroegneria* and *Elytrigia*, are distributed between two main clusters. We called them according their geographical location, respectively, "Nothern" and "Southern" (Fig. 2). Each of these clusters then split into two separated ribotypes groups called "Core Northern St-ribotype"/"Northern dahuricus St-ribotype" and "Core Southern St-ribotype"/"Southern dahuricus St-ribotype", respectively. The Core Northern Elymus ribotype is widespread among the *Elymus* taxa of Eurasia, including Yakutia, Mountain Altai and Northern and High Mountain China (Tibet, Nei Mongol, Xinjiang, Gansu). It was found also in Finland, the Far East of the Russian Federation, and Mongolia. The Core Southern St-ribotype is typical mostly for more southern populations, including the Caucasus, Primorsky Krai (RF), Pakistan, South Korea, a part of China and Turkey. The Core Southern rDNA was found in *Elytrigia repens* and in diploid *Pseudoroegneria strigosa*, as well as in some other *Pseudoroegneria* species: *P. spicata* (haplome St or StX – Wang et al., 1986), and *P. sosnovskyi* (haplome St – Assadi, 1994). On the other hand, the Core Northern St-ribotype is characteristic feature of *P. cognata* (2*n* = 14 – Lu et al., 1991) and *P. spicata* (PI 232134, 2*n* = 14 – Okino et al., 2009), both carry the haplome St^Y (Okino et al., 2009).

The "Northern dahuricus" St-rDNA and "Southern dahuricus" St-rDNA (ribotypes) are derivatives of these two base types of rDNA, "Core Southern" and "Core Northern". There are 6 SNPs and one deletion that delimited consensus sequences of the "Core Southern" and "Core Northern" ribotypes (Fig. 3). The consensus sequence "Southern dahuricus" St carries 5 SNPs and one deletion that differ from that of "Core Southern" St-ribotype. Differences between consensus "Core Northern" St-ribotype and consensus "Northern dahuricus" St-ribotype consist of 5 SNPs. As results, consensus sequences of the "Northern dahuricus" St-ribotype and the "Southern dahuricus" St-ribotype differ in 11 STPs and two indels.

It should be noted that two different variants of rDNA were found in many species. For example, one of the plants *P. tauri* belongs to the "Core Northern" ribotype, another – to the "Core Southern" ribotype (see Fig. 1, Table 2). One can see the same phenomenon in *P. stipifolia* from Stavropol, *P. spicata* of USA, *P. kosanini* of Turkey, *E. dolichatherus* of China (see Table 2). It appears that this can be correlated with the allopolyploid karyotypes of these tetraploid species.

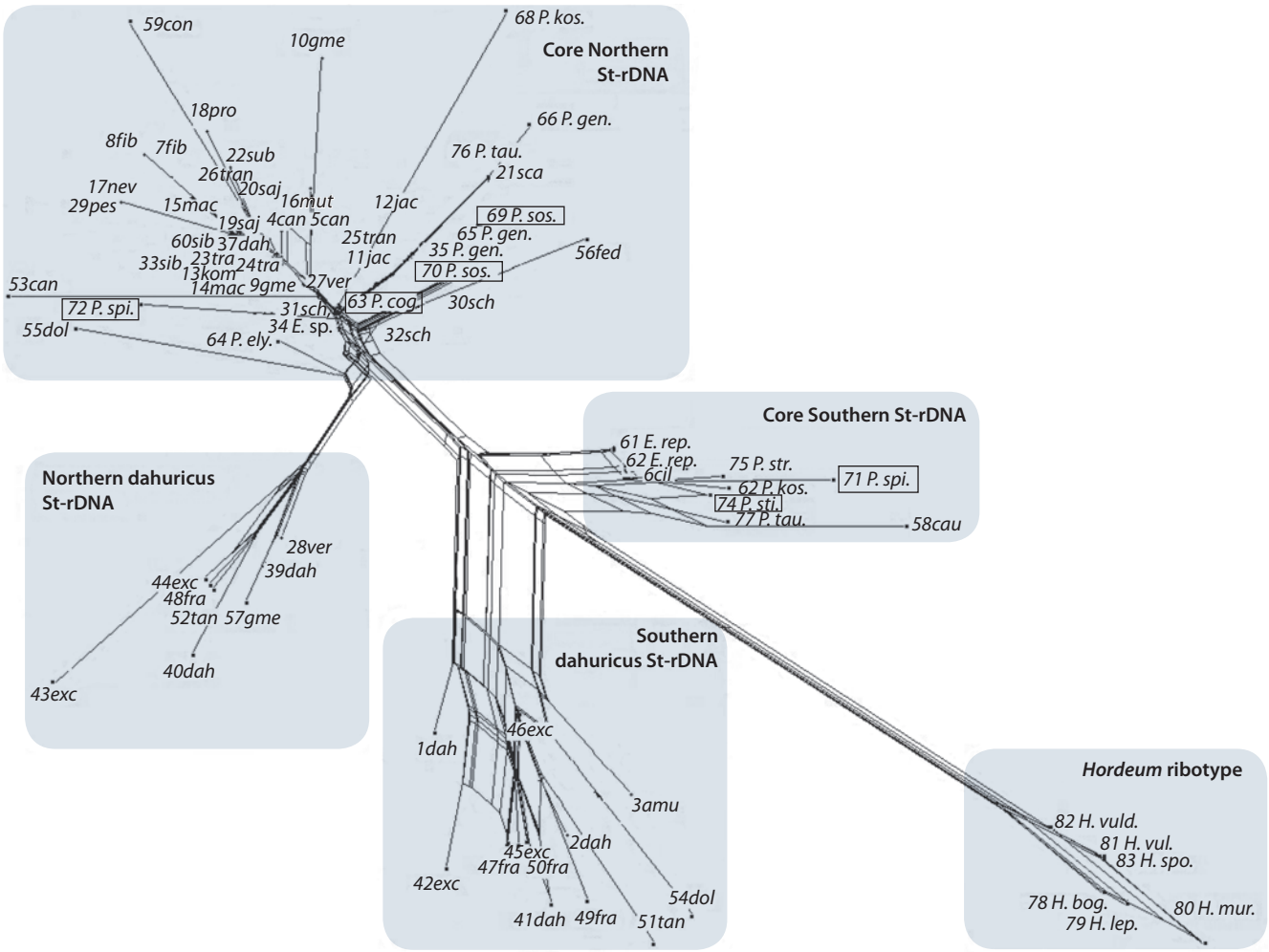

Fig. 1. Split graph for the ITS sequences of *Elymus* species and some other species of the tribe Triticeae generated by SplitsTree4 (Huson, Bryant, 2006).

Species names and numbers of accessions see Tables 1 and 2. Positions of the diploid species with St genome are indicated by boxes. *amu* – *Elymus amurensis*, *can* – *E. caninus*, *cau* – *E. caucasicus*, *cil* – *E. ciliaris*, *con* – *E. confuses*, *dah* – *E. dahuricus*, *dol* – *E. dolichatherus*, *exc* – *E. excelsus*, *fed* – *E. fedtschenkoi*, *fib* – *E. fibrosus*, *fra* – *E. franchetii*, *gme* – *E. gmelinii*, *jac* – *E. jacutensis*, *kom* – *E. komarovii*, *mac* – *E. macrourus*, *mut* – *E. mutabilis*, *nev* – *E. nevskii*, *pes* – *E. peschkovae*, *pro* – *E. probatovae*, *saj* – *E. sajanensis*, *sca* – *E. scandicus*, *sch* – *E. schrenkianus*, *sib* – *E. sibiricus*, *sub* – *E. subfibrosus*, *tan* – *E. tangutorum*, *tra* – *E. trachycaulus*, *tran* – *E. transbaicalensis*, *ver* – *E. vernicosus*, and *E.* sp. – *Elymus* sp., *E. rep*. – *Elytrigia repens*, *H. bog.* – *Hordeum bogdanii*, *H. lep.* – *H. murinum* ssp. *leporinum*, *H. mur.* – *H. murinum* L. ssp. *murinum*, *H. spo.* – *H. vulgare* subsp. *spontaneum*, *H. vul.* – *H. vulgare*, *H. vuld.* – *H. vulgare* var. *distichon*, *P. cog.* – *Pseudoroegneria cognata*, *P. ely.* – *P. elytrigioides*, *P. gen.* – *P. geniculate*, *P. kos.* – *P. kosanini*, *P. sos.* – *P. sosnowskyi*, *P. spi.* – *P. spicata*, *P. sti.* – *P. stipifolia*, *P. str.* – *P. strigose*, *P. tau.* – *P. tauri.*

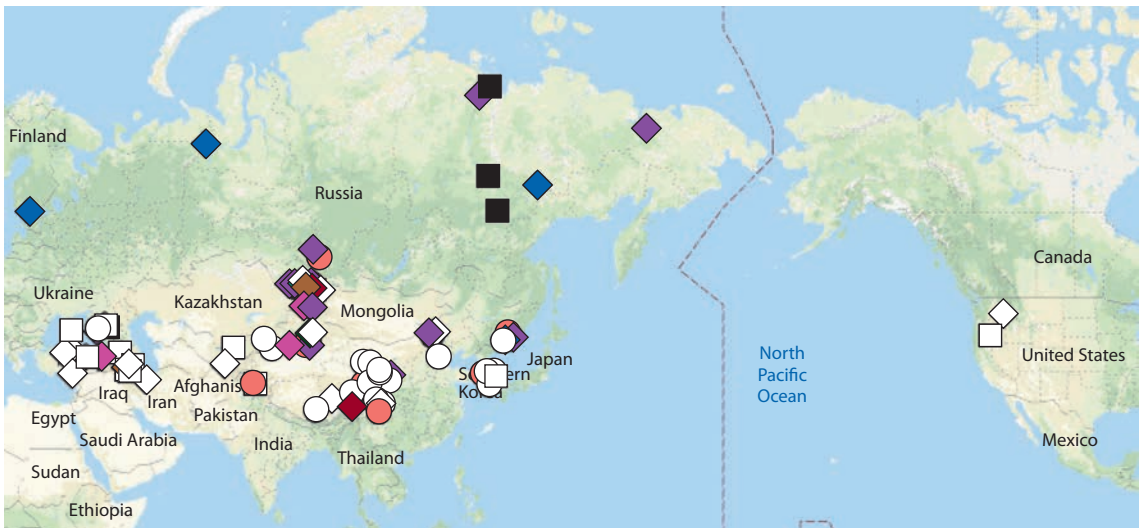

Fig. 2. Distribution of species *Elymus* s. l. with Northern (squares) and Southern (cycles) ribotypes.

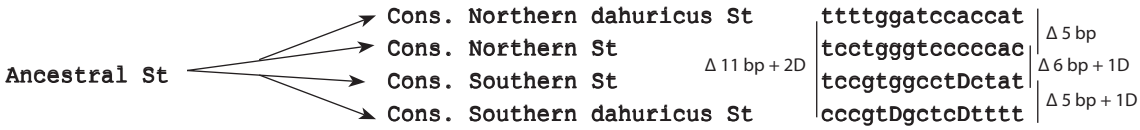

Fig. 3. The origin of St-ribotypes of the genus *Elymus*.

In the figure are shown only positions that are different in the consensus sequences of "Core Northern", "Core Southern", "Northern dahuricus" and "Southern dahuricus" ribotypes. D – deletion.

Discussion

In the present study we have shown that all species of *Elymus* in eastern Eurasia can be divided, according to their ITS sequences, into two families of ribotypes, conventionally called by us as the "Northern" and the "Southern" variants of St-rDNA. Each of these families is reliably divided into two subfamilies, the main, or "Core" variant of St-rDNA, and a modification of the St-ribotype distributed mainly between species *Elymus dahuricus* aggr. that we called "Northern dahuricus" and the "Southern dahuricus" ribotypes. Comparison of ITS sequences of *Elymus* and of *Pseudoroegneria* species showed that the "Core Northern" St-ribotype is close to rDNA of diploid *Pseudoroegneria cognata* with St genomes, accession PI 531720, collected in Kyrgyzstan (Dewey, 1990a; Yu et al., 2008). The "Southern" rDNA variant is closely related to that of *Elytrigia strigosa* PI 531752 (Dewey, 1990b; Yu et al., 2008) of Crimea and of *P. stipifolia* PI 313960 of Stavropol (Hyland, 1969).

The fact that there are *Pseudoroegneria* species with different St genomes have been shown earlier by Yan and co-workers (Yan et al., 2011) that studied nuclear genes *RPB2* and *EF-G*. They shown that *P. libanotica* and *P. tauri* St genomes are separated from the St genome of other *Pseudoroegneria* species, in particular *P. spicata* and *P. strigosa*.

The existence of significant uncertainty in the genome composition of the studied *Pseudoroegneria* species makes it difficult to interpret the results of the comparison between rDNA of *Pseudoroegneria* and *Elymus*. Thus, *P. strigosa* studied by Petrova (1967) was diploid with $2n = 14$. However, Dewey (1990a) observed $2n = 28$ in his sample of this species. In both cases the plants were from Crimea. Later, Khuat and co-workers studied *P. strigosa* from Mongolia and China and showed that they are hexaploids ($2n = 42$) (Khuat et al., 2015). Another *Pseudoroegneria*, *P. spicata* can be diploids ($2n = 14$) and tetraploids ($2n = 28$) (Wang et al., 1996; Khuat et al., 2015). Meiotic analysis and GISH showed that second genome of tetraploid *P. spicata* and second and third genomes of hexaploid *P. strigosa* are not St genomes (Wang et al., 1996; Khuat et al., 2015). So, according to genomic concept of the genus, these tetraploids and hexaploids should not be classified as *Pseudoroegneria*.

The occurrence in eastern Asia of plants of *E. dahuricus* with two different variants of rDNA Northern dahuricus and Southern dahuricus ribotypes implies that these two variants have a common pattern of morphological characters, some *E. dahuricus* syndrome, but they are reproductively isolated. This suggestion can be confirmed by the results of hybridological experiments performed earlier by Agafonov and coauthors (Agafonov et al., 2001; Savchkova et al., 2003). These authors revealed that seed fertility in crossings with various combinations of *E. dahuricus* aggr. parents does not depend primarily on the combination determined by the taxa morphology. It is important that some combinations of seed and pollen parents, delimitated by their morphological characters as the same species, were almost sterile: *E. dahuricus* MES-8709 (Primorye, near Posyet) × *E. dahuricus* CHI-8635 (Siberia, Chitinskij region) – only 4.8 % seed fertility, for comparison: *E. dahuricus* POP-8403 (Primorye, Popov island) × *E. woroschilowii* VLA-8642 (Primorye, Vladivostok) – 69 % seed fertility (Agafonov et al., 2001).

We suggest that there are probably not five different species but only two species in the *E. dahuricus* aggr. in Siberia and Northern China, one of them with the "Northern dahuricus" ribotype and another with the "Southern dahuricus" ribotype. Very likely, they are completely or almost completely genetically isolated from each other. It is unknown if there are any morphological distinctions between plants with different ribotypes or if these are cryptic species. However, it can be said with certainty that if there are differences in morphology, they are not connected with characters that are considered to be taxonomically significant to delimitation of the current species of *E. dahuricus* aggr.

It is appeared that morphological characters currently used for differential diagnosis of *Elymus dahuricus* aggr. species, do not allow to delimit plants with different ribotypes and even current traditional species because the diagnostic characters are weak. For example, various authors indicate curved glumes awns and the thicker stems of *E. excelsus* as diagnostic characters, delimiting *E. dahuricus sensu stricto* from *E. excelsus* (Tzvelev, Probatova, 2010). However, Savchkova et al. (2003) showed that hybrids have an intermediate state between direct and curved awns of lemma (inheritance type is unknown). F2 hybrids are more likely to show curved awns. This character is manifested in varying degrees at the plants' different stages of maturity: as spicules ripen, the awns of lemmas become more curved. Specimens with non-curved awns (an *E. dahuricus* diagnostic character) were collected among the Far Eastern populations, usually considered as *E. excelsus* populations, while examples with curved awns were found among the Altai populations of *E. dahuricus sensu stricto* (Savchkova et al., 2003). Similarly, the differences between *E. franchetii* and *E. excelsus* are insignificant, the first exhibit leaf blade widths of 3–8 mm and the second 8–18 mm (Tzvelev, Probatova, 2010).

Conclusion

In conclusion, we suppose that it is important to determine distribution areas of *Elymus* with the "Northern dahuricus" and the "Southern dahuricus" St-rDNA genomes (ribotypes). After this, it is necessary to reconsider the system of taxonomically

significant characters and try to find unique morphological characteristics appropriate only for plants with the "Northern dahuricus" St-rDNA or with the "Southern dahuricus" St-rDNA ribotypes. There is reason to believe that within this complex, there are at least two different, probably reproductively isolated, cryptic species or two reproductively isolated groups of species and these species (groups of species) may have different origin.

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ORCID ID

A.V. Rodionov orcid.org/0000-0003-1146-1622
K.S. Dobryakova orcid.org/0000-0002-5468-6883
N.N. Nosov orcid.org/0000-0002-7096-9242
A.A. Gnutikov orcid.org/0000-0002-5264-5594
E.O. Punina orcid.org/0000-0001-9745-4604
A.A. Kriukov orcid.org/0000-0002-8715-6723
V.S. Shneyer orcid.org/0000-0002-6122-6770

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СЕЛЕКЦИЯ РАСТЕНИЙ НА ИММУНИТЕТ И ПРОДУКТИВНОСТЬ
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Molecular identification of the stem rust resistance genes in the introgression lines of spring bread wheat

O.A. Baranova[1] ✉, S.N. Sibikeev[2], A.E. Druzhin[2]

[1] All-Russian Institute of Plant Protection, Pushkin, St. Petersburg, Russia
[2] Agricultural Research Institute of the South-East Region, Saratov, Russia
✉ e-mail: baranova_oa@mail.ru

A total of 57 introgression lines and 11 cultivars of spring bread wheat developed by All-Russian Institute of Plant Protection and cultivated in the Volga Region were analyzed. The lines were obtained with the participation of CIMMYT synthetics, durum wheat cultivars, direct crossing with *Agropyron elongatum* (CI-7-57) and have introgressions from related species of bread wheat, namely translocations from *Ag. elongatum* (7DS-7DL-7Ae#1L), *Aegilops speltoides* (2D-2S), *Ae. ventricosum* (2AL-2AS-2MV#1), *Secale cereale* (1BL-1R#1S), 6Ag^{i} (6D) substitution from *Ag. intermedium* and triticale Satu. Cultivars and lines were assessed for resistance to Saratov, Lysogorsk, Derbent and Omsk stem rust pathogen populations (*Puccinia graminis* f. sp. *tritici*), and analyzed for the presence of the known *Sr* resistance genes using molecular markers. The analysis of the cultivars' and lines' resistance to the Saratov pathogen population in the field, as well as to Omsk, Derbent and Lysogorsk populations at the seedling stage, showed the loss of efficiency of the *Sr25* and *Sr6Ag^{i}* genes. The *Sr31* gene remained effective. Thirty one wheat lines out of 57 (54.4 % of samples) were resistant to all pathogen populations taken into analysis. The *Sr31*/*Lr26*, *Sr25*/*Lr19*, *Sr28*, *Sr57*/*Lr34* and *Sr38*/*Lr37* genes were identified in the introgression lines. The *Sr31*/*Lr26* gene was identified in 19 lines (33.3 % of samples). All lines carrying the 1RS.1BL translocation (*Sr31*/*Lr26*) were resistant to all pathogen populations taken into analysis. The *Sr25*/*Lr19* gene was identified in 49 lines (86 %). The gene combination *Sr31*/*Lr26* + *Sr25*/*Lr19* was identified in 15 lines (26.3 %). The gene combinations *Sr38*/*Lr37* + *Sr25*/*Lr19*, *Sr57*/*Lr34* + *Sr25*/*Lr19* and *Sr31*/*Lr26* + *Sr25*/*Lr19* + *Sr28* were identified in 3 introgression lines. These three lines were characterized by resistance to the pathogen populations studied in this work. The *Sr2*, *Sr24*, *Sr26*, *Sr32*, *Sr36* and *Sr39* genes were not detected in the analyzed wheat lines.
Key words: spring bread wheat; introgression lines; *Puccinia graminis* f. sp. *tritici*; *Sr* genes.

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Молекулярная идентификация генов устойчивости к стеблевой ржавчине в интрогрессивных линиях яровой мягкой пшеницы

О.А. Баранова[1] ✉, С.Н. Сибикеев[2], А.Е. Дружин[2]

[1] Всероссийский научно-исследовательский институт защиты растений, Пушкин, Санкт-Петербург, Россия
[2] Научно-исследовательский институт сельского хозяйства Юго-Востока, Саратов, Россия
✉ e-mail: baranova_oa@mail.ru

Проанализированы 57 интрогрессивных линий и 11 сортов яровой мягкой пшеницы селекции НИИ сельского хозяйства Юго-Востока, возделываемых на территории Поволжья. Линии получены с участием синтетиков селекции CIMMYT, сортов твердой пшеницы, прямого скрещивания с *Agropyron elongatum* (CI-7-57) и имеют интрогрессии от родственных видов мягкой пшеницы, а именно транслокации от *Ag. elongatum* (7DS-7DL-7Ae#1L), *Aegilops speltoides* (2D-2S), *Ae. ventricosum* (2AL-2AS-2MV#1), ржи культурной (1BL-1R#1S), замещения от *Ag. intermedium* 6Ag^{i} (6D) и тритикале Satu. Сорта и линии были оценены на устойчивость к саратовской, лысогорской, дербентской и омской популяциям возбудителя стеблевой ржавчины, а также проанализированы на наличие идентифицированных *Sr* генов устойчивости с использованием известных молекулярных маркеров. Анализ устойчивости сортов и линий к саратовской популяции патогена в полевых условиях, а также к омской, дербентской и лысогорской популяциям *Puccinia graminis* f. sp. *tritici* на стадии проростков показал потерю эффективности генов *Sr25* и *Sr6Ag^{i}*. Ген *Sr31* пока сохраняет свою эффективность. Ко всем взятым в анализ популяциям патогена была устойчива 31 линия пшеницы из 57 (54.4 % образцов). У исследуемых интрогрессивных линий идентифицированы гены *Sr31*/*Lr26*, *Sr25*/*Lr19*, *Sr28*, *Sr57*/*Lr34* и *Sr38*/*Lr37*. Ген *Sr31*/*Lr26* определен у 19 линий (33.3 % образцов). Все линии, несущие транслокацию 1RS.1BL (*Sr31*/*Lr26*), были устойчивы ко всем взятым в анализ популяциям патогена. Ген *Sr25*/*Lr19* идентифицирован у 49 линий

(86 % образцов). Сочетание генов *Sr31/Lr26+Sr25/Lr19* идентифицировано у 15 линий (26.3 %). У одной линии идентифицировано сочетание генов *Sr38/Lr37+Sr25/Lr19*, у другой линии – сочетание генов *Sr57/Lr34+ +Sr25/Lr19*, и еще у одной – *Sr31/Lr26+Sr25/Lr19+Sr28*. Все они были устойчивы ко всем взятым в анализ популяциям патогена. Гены *Sr2*, *Sr24*, *Sr26*, *Sr32*, *Sr36*, *Sr39* у анализируемых линий обнаружены не были.
Ключевые слова: яровая мягкая пшеница; интрогрессивные линии; *Puccinia graminis* f. sp. *tritici*; гены *Sr*.

Introduction

In recent years, the increasing severity of wheat stem rust (caused by the biotrophic fungus *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn.) both on the territory of Africa and America, and countries of the Eurasian continent is observed. The emergence of the new highly aggressive stem rust race in Uganda in 1999 – Ug99 (TTKSK), infecting wheat cultivars which contain gene *Sr31* (Pretorius et al., 2000), caused great concern of world wheat producers, because with epiphytotic development of this pathogen on susceptible cultivars yield losses can reach 100 % (Hailu et al., 2015). Currently, there are 13 biotypes of this race (http://rusttracker.cimmyt.org/?page_id=22). The Ug99 race is common in the countries of Africa and the Middle East, it spread in the direction of Central and Southeast Asia and it is possible to spread it into the territory of the Russian Federation.

On the other hand, new aggressive races of stem rust that differ from Ug99 have appeared in the world. So, in 2012 in Ethiopia, a new race TKTTF was identified and called Digalu, after the name of the wheat cultivar that it overcome. Crop losses of this cultivar in 2013–2014 reached almost 100 %. In Germany during 2013, 6 races: TKTTF, TKKTF, TKPTF, TKKTP, PKPTF and MMMTF, which are not part of the Ug99 racial group, were allocated from 48 isolates of *P. graminis* (Olivera et al., 2017). German isolates of race TKTTF differed from the Ethiopian race TKTTF in virulence to the lines with genes *Sr7a*, *Sr45* and *SrTt-3*. Another isolated race, TKKTP, turned out to be virulent to the lines with the genes *Sr24*, *SrTmp* and *Sr1RSAmigo*. It is known that 55 % of North American and international cultivars and breeding lines resistant to the TTKSK race (Ug99) are susceptible to the TKKTP race (Olivera et al., 2017). The TKTTF race is found in the countries of the Middle East and Europe (www.wheatrust.org/stem-rust-tools-maps-and-charts/race-frequency-map), including the UK (Lewis et al., 2018). In 2016, the new race of stem rust (TTTTF) injured several thousand hectares of durum wheat in Sicily. It was the largest epiphytoty of stem rust in Europe in recent decades. The race TTTTF is virulent to the lines with the genes *Sr9e*, *Sr13* and avirulent to the genes *Sr31*, *Sr24* and *Sr25* (Bhattacharya, 2017). Also in 2016 in Ecuador, the race RRTTF virulent to genes *Sr38* and *Sr13* was discovered which became a real threat to wheat production in North and South America, since much of the modern commercial cultivars turned out to be susceptible to it. The origin of the race RRTTF in Ecuador is unknown; earlier race RRTTF was identified in Asia (Pakistan), East Africa (Ethiopia) and the Middle East (Yemen) (http://rusttracker.cimmyt.org/?p=7143). In the summer of 2017 in Sweden, the epiphytotic development of stem rust was observed in wheat fields of Almunge, Uppland. Moreover, the last major epiphytotics of stem rust in Sweden was in 1951. In 2015, 2016, epiphytotic development of stem rust was observed in northern Kazakhstan (Koyshybaev, 2017).

On the territory of the Russian Federation, the epiphytotic development of the disease was noted in 2015, 2016 and 2017 in Western Siberia, the Central Region of the European part of the Russian Federation and in Lower Volga Region. In 2016, race TTTTF different from the Sicilian race was identified in Western Siberia (according to Global Rust Reference Center http://wheatrust.org/fileadmin/www.grcc.au.dk/International_Services/Pathotype_SR_Results/Country_report_Russia_-_August2017.pdf). In the Lower Volga Region on the territory of the Saratov Oblast, the strong epiphytotics of stem rust was observed in 2004 and 2006, the severity of disease development was 50–60 %, in 2013–2014 the severity of disease was moderate, up to 30 % (Markelova, 2015). However, in 2016 in the Saratov Oblast, especially in the Right-Bank part of Volga river, epiphytotic of stem rust began during the milky ripeness of the grain (early July) and continued until full maturity and harvest. The degree of development reached 80 %, the affected cultivars reduced the yield by 50 %, and the mass of 1000 seeds was at the level of 18–19 grams (Sibikeev et al., 2017b; Sibikeev, unpubl. data). Also in 2016, the spread of stem rust on spring bread wheat in the period of earing was noted throughout the territory of the Republic of Tatarstan. All wheat cultivars, recommended for cultivation in Tatarstan, were susceptible, except for the Tulaikovskaya 5 and Belka cultivars, as well as samples of *Triticum dicoccum* (Vasilova et al., 2017).

It should be noted that often there is an alternation of years with epiphytoties of stem rust with the years of epiphytotic development of leaf rust. So in the Saratov region, as already noted, in 2016 the strong stem rust epiphytotics, and in 2017 the strong leaf rust epiphytotics were observed. Thus, in order to prevent economically significant losses, the cultivars and promising lines with resistance to both diseases are necessary, that complicates the breeding work and may lead to the narrowing of genetic diversity. Complexes (linkages) of genes that determine resistance to stem, leaf and yellow rust and powdery mildew of various origins, including introgressed from related species, take on special significance.

Wild relatives of wheat are the valuable reservoir of genetic diversity (Wulff, Moscou, 2014). Studies of introgression lines and the use of their genetic potential in the breeding process are carried out both in Russia (Salina et al., 2015; Baranova et al., 2016; Lapochkina et al., 2017; Leonova, Budashkina, 2017; Sibikeev et al., 2017a) and abroad (Wulff, Moscou, 2014; Ali et al., 2016; Rakszegi et al., 2017). Molecular markers are widely used to identify alien introgression and loci for economically valuable traits, including resistance to disease genes (Miedaner, Korzun, 2012; Leonova, 2013; Yaniv et al., 2015; Ali et al., 2016).

The aim of this work was to evaluate the spring bread wheat cultivars and introgression lines by ARISER breeding and widespread in the Lower Volga Region, for resistance to

leaf and stem rust, and to identify effective *Sr* genes using molecular markers.

Materials and methods

Phytopathological analysis. Breeding material from the ARISER (57 introgression lines) and 11 cultivars of spring bread wheat which cultivated in the Volga Region and related to the analyzed lines were used in this work. All lines were obtained with the participation of genetic material from related species of bread wheat, namely, translocation from tall wheatgrass *Agropyron elongatum* (7DS-7DL-7Ae#1L), substitution from intermediate wheatgrass *Ag. intermedium* ($6Ag^{i}$ (6D)), rye *Secale cereale* (1BL-1R#1S), and also with the participation of synthetic lines from CIMMYT, cultivars of durum wheat, translocations from *Aegilops speltoides* (2D-2S), *Ae. ventricisum* (2AL-2AS-2MV#1), triticale Satu and direct crossing with tall wheatgrass *Ag. elongatum* (2*n* = 70) CI-7-57.

The Omsk, Derbent, and Lysogorsk populations of the pathogen, collected in 2017 from the spring bread wheat cultivar Favorit, which carries the $6Ag^{i}$ (6D) substitution, were used for laboratory evaluation of resistance at the seedling stage. Virulence of populations was evaluated using the set of 20 differentiators (North American differential set) (Cereal Disease Laboratory) and additional near isogenic lines (21 lines) (Suppl. Material 4)[1]. The cultivars Avrora (gene *Sr31*) and Hakasskaya (susceptible control) were also used in the analysis. Inoculation of plants was carried out in accordance with the methods adopted in world practice (Jin et al., 2007) (http://www.fao.org/fileadmin/templates/rust/img/race_analysis_web.pdf).

The analyzed samples were grown in plastic cuvettes of size 11 × 15 × 6 cm filled with "Terra Vita" peat soil, using grow light systems, at 21–23 °C with a 14-hour photoperiod. Eleven samples (three plants per sample) plus susceptible control – wheat cultivar Hakasskaya, were placed in a cuvette. Ten-day old seedlings with the first leaf fully unfolded were inoculated with the urediniospores suspension of pathogen populations collected from the cultivar Favorit in 2017. Infectious load was 1 mg of spores in 1 ml (Singh et al., 2008). 5 ml of spore suspension was used per cuvette (12 samples = 36 plants), which was approximately 0.14 mg spores per plant. Inoculated plants were placed in the dark moist chambers for 16 hours at 21–23 °C and a relative humidity of 100 %, and then returned to the grow light system with same temperature. The reaction of seedlings to inoculation (IT) with the spores suspension of stem rust pathogen was taken into account on the 10–12th day after infection on the standard scale (Stackman et al., 1962) (Suppl. Material 5). The resistance or susceptibility of the sample was judged based on the types of reaction (IT) in two replications. Plants with ITs "0", "0;", "1", "2", "X" were considered as resistant and as susceptible with IT = "3", "4". As criteria for evaluation of adult plants resistance were the ITs and the degree of plant damage on the scale recommended by CIMMYT (Roelfs et al., 1992): R – resistance to infection; MR – medium resistance; MS – medium susceptibility; S – susceptible reaction.

DNA isolation and PCR analysis. DNA was isolated from five-day wheat seedlings using cetyltrimethylammonium bromide (CTAB) (Murray, Thompson, 1980). The composition of the reaction mixtures and the conditions of PCR are presented in the Suppl. Material 2.

DNA markers recommended for marker-assisted selection (MAS) were used to identify resistance genes (*Sr2*, *Sr24*, *Sr25*, *Sr26*, *Sr28*, *Sr31*, *Sr32*, *Sr36*, *Sr38* and *Sr39*) (Suppl. Material 1). Amplification was performed on C1000 Thermal Cycler amplifiers (BioRad), separation of PCR products was carried out in 2 % agarose gels with addition of ethidium bromide. Near isogenic lines and cultivars with *Sr* genes served as positive control (Suppl. Material 3), susceptible cultivar Hakasskaya served as negative control. GeneRulerTM 50 bp DNA Ladder (Fermentas) was used as a molecular weight marker. The amplification products were visualized using the ChemiDoc™ XRS+ imaging system (Bio-Rad). PCR was carried out in 2 replicates.

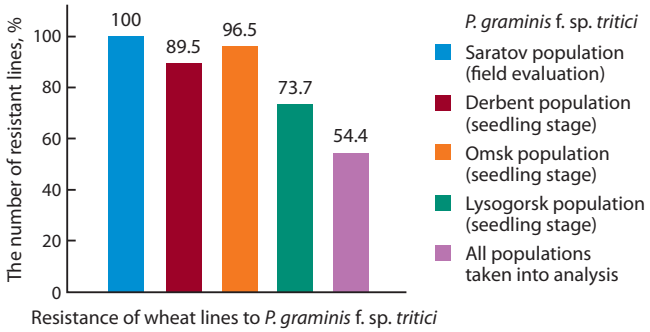
Results

Phytopathological analysis of resistance to causative agent of stem rust

At the first stage of work, 57 lines and 11 wheat cultivars were evaluated for resistance to the Saratov population of stem rust under field conditions and for resistance to the Derbent, Omsk and Lysogorsk pathogen populations at the seedling stage under laboratory conditions. The stem rust populations were previously evaluated for virulence. Against the Omsk population of 2017, the genes *Sr11*, *Sr31*, *Sr13*, *Sr25*, *Sr26*, *Sr32*, *Sr35* and the combinations of genes *Sr26* + *Sr9g*, *Sr31* + *Sr36* and *Sr31* + *Sr24* were effective; against Lysogorsk population – *Sr13*, *Sr17*, *Sr31*, *Sr26*, *Sr32*, *Sr35*, *Sr24* + *Sr31*, *Sr36* + *Sr31*, *Sr24* + *Sr36* and *Sr26* + *Sr9g*; against Derbent population – genes *Sr13*, *Sr24*, *Sr25*, *Sr26*, *Sr31*, *Sr35*, *Sr40* and the combinations of genes *Sr24* + *Sr31*, *Sr36* + *Sr31*, *Sr24* + *Sr36*, *Sr26* + *Sr9g*.

All lines were resistant to the Saratov population of stem rust in the field conditions. During evaluation at the seedling stage, 6 lines susceptible to the Derbent pathogen population, 2 lines susceptible to the Omsk pathogen population and 15 lines susceptible to the Lysogorsk pathogen population were identified. Thirty one lines (54.4 %) were resistant to all the populations taken in the analysis (see the Figure).

From the 11 analyzed cultivars almost all were susceptible in the field – namely: cultivars Saratovskaya 55, Saratovskaya 68, Saratovskaya 70, Saratovskaya 73, Albidum 32, Favorit, Voevoda and Lebyodushka. The cultivars Prokho-



Results of the phytopathological analysis of introgression lines.

[1] Supplementary Materials 1–8 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx5.pdf

rovka, Yugo-Vostochnaya 2 and Dobrynya were heterogeneous for resistance, the susceptible plants were observed among resistants. During laboratory analysis of cultivars the following results were obtained: Prokhorovka, Yugo-Vostochnaya 2 and Dobrynya cultivars are resistant to the Derbent population of stem rust; the cultivars Prokhorovka, Yugo-Vostochnaya 2, Dobrynya and Saratovskaya 55 are resistant to the Lysogorsk population, the cultivar Saratovskaya 73 turned out to be heterogeneous. Almost all cultivars are resistant to the Omsk pathogen population, Albidum 32, Dobrynya, and Voyevoda are heterogeneous. Heterogeneity for the genes of resistance to stem rust can be explained by the fact that in the breeding process there was no selection for resistance to this disease, which allows for the presence of several biotypes in the cultivar.

Identification of resistance genes

The gene *Sr31* was identified as gene ineffective to Ug99, but effective against local stem rust populations. The scm9 marker, developed for rye translocation 1BL.1RS carrying the gene complex resistant to stem (*Sr31*), leaf (*Lr26*) and yellow (*Yr9*) rust and powdery mildew (*Pm8*), was used to identify it. The translocation 1BL.1RS (gene *Sr31*) was identified in 19 lines out of 57 (33.3 %) (Suppl. Material 8). All samples carrying the 1BL.1RS translocation were resistant to the Saratov population of stem rust during the field evaluation and to all analyzed pathogen populations during evaluation of resistance at the seedling stage. The gene *Sr31* was identified in the Prokhorovka and Yugo-Vostochnaya 2 cultivars. However, during evaluation of resistance to the Saratov population of pathogen, susceptible plants of these cultivars were identified. A question arose about the possible appearance of fungal pathotypes in the Saratov population of pathogen that virulent to the gene *Sr31*. DNA from 10 individual grains of the infected cultivar Prokhorovka was tested for the presence of amplification fragments using the primer scm9 to confirm the present or absence of 1BL.1RS translocation in susceptible plants. Diagnostic fragment 207 bp was obtained only from the control cultivar Avrora (carrier of *Sr31*) that means the absence of 1RS.1BL (*Sr31*) translocation in analyzed susceptible plants. According to morphological characteristics (plant height, type of ear) admixture of another plant is unlikely. Moreover, this fact was observed (IT = 0 and 3) at different seed sources, including the original ones, the heterogeneity is most likely as the loss of 1RS.1BL translocation. It was also concluded that the gene *Sr31* still retains its effectiveness.

The gene *Sr25*, which effective for Ug99, was identified in 49 lines (86 %). For identification this gene was used the Gb marker recommended for the marker-assisted selection. The *Sr25* gene was also identified in the cultivars Dobrynya and the Lebyodushka. The gene *Sr38* was detected only in one line (Tselinnaya20/Dobr//Dobr/3/DobrLr9/4/Milan/Prinia*//4Dobr) using the VENTRIUP-LN2 primers. In this line, the combination of genes *Sr25/Lr19* + *Sr38/Lr37* was identified (Suppl. Material 7). In one line (L505*2//Croc/Ae.squar(224)//Yaco) using the csLV34 marker in addition to the *Sr25* gene, the gene of adult resistance *Sr57/Lr34* was identified. Both of these lines were resistant to all analyzed populations of pathogen.

In addition, using markers wPt-7004-PCR and Xwmc332, the gene *Sr28* was identified in one of the lines; however, this fact should be verified during further work using other markers. In this line, the combination of the *Sr31/Lr26* + *Sr25/Lr19* + *Sr28* genes provided resistance to all analyzed populations of the pathogen. The CAPS marker csSr2 was used to identify the *adult plant resistance* gene – *Sr2*. Amplification product with primers csSr2F/R was observed in almost all analyzed samples. After restriction of amplification product by restriction endonuclease *BspHI*, the diagnostic fragment 172 bp was observed only in control cultivars Arthur and Oasis. The genes from *Ae. speltoides* were not found in the wheat accessions, although the presence of introgressions from this species in the pedigree of lines suggested that genes such as *Sr32* and *Sr39/Lr35* could be present. However, it is likely that specific markers, other than markers for *Sr32* and *Sr39*, are needed for identification the *Sr* genes of the studied lines.

Thus, the genes *Sr31/Lr26* and *Sr25/Lr19* were mainly identified in the introgression lines. The combination of these genes – *Sr31* + *Sr25* was found in 15 lines (26.3 %). The genes *Sr2*, *Sr24*, *Sr26*, *Sr32*, *Sr36*, *Sr39* were not detected in the analyzed lines.

Discussion

In the Volga region of Russia, one of the factors limiting the grain yield of wheat are the epiphytotics of rust fungi – leaf and stem rust, and the harmfulness of stem rust in recent years has increased significantly. The success of anticipatory breeding for resistance to these pathogens is largely determined by the diversity of used resistance genes. For targeted wheat breeding, the identification of resistance genes in cultivars, identification of their effectiveness and join its in combinations is necessary.

The analyzed in this research cultivars of spring bread wheat occupy over 95 % of the area in the Saratov Oblast and Volgograd Oblast and are also widespread in the Middle Volga Region, Ural Region and partially in the Central Black Soil Region of Russia. Thus, it is their genotypes for rust resistance genes that determine (inhibit or not) the development of the epiphytoties of these pathogens. It should be noted that the substitution of $6Ag^{i}$ (6D) from *Ag. intermedium* was previously identified in the cultivars Favorit and Voevoda, while the Lebyodushka cultivar had the genes *Sr6Ag*^{i} + *Lr19/Sr25* (combination of substitution $6Ag^{i}$ (6D) and translocation 7DS-7DL-7Ae#1L) (Sibikeev et al., 2017a), and the translocation 7DS-7DL-7Ae#1L from tall wheatgrass with gene *Sr25/Lr19* was identified in the Dobrynya cultivar (Badaeva et al., 2018). The presence of the gene *Sr25* in the Dobrynya and Lebyodushka cultivars is confirmed in this work (Table 1).

It is known that the gene *Sr25* (cultivars L503, L505, Dobrynya) showed the susceptible type of reaction and the percentage of lesion was 15–20 % in 2006, and in 2016 the IT = 2, 3 and degree of lesion was 15–25 %. For the gene(s) *Sr6Ag*^{i} (cultivars Belyanka, Favorit, Voevoda) in 2006 IT = 3 and severity 50–60 %, in 2016 – 70–80 %. For cultivars with gene *Sr31* (Prokhorovka), already in 2006, 0/3 single pustules reaction was observed (Sibikeev et al., 2008, 2009, 2017b). The fact that the field evaluation of these cultivars in 2016 and 2017 showed their susceptibility to the Saratov population of the pathogen, as well as to the Derbent and Lysogorsk populations of *P. graminis* in the laboratory conditions (see Table 1) shows the loss of efficiency of both the gene *Sr6Ag*^{i}

Table 1. Results of phytopathological analysis and identification of *Sr* genes in the cultivars

Cultivar	Resistance to *P. graminis*				Identified genes
	Saratov population (field evaluation)*	Laboratory evaluation to *P. graminis* populations at the seedling stage			
		I	II	III	
Saratovskaya 55	70S	3–	2	2	–
Saratovskaya 68	60S	3	3	X	–
Saratovskaya 70	70S	3	3	2–	–
Saratovskaya 73	60S	3	2+3	2–	–
Albidum 32	70S	3	4	2+4	–
Prokhorovka	R and 3S	1	1;	1;2+	*Sr31*
Yugo-Vostochnaya 2	R and 3S	0	0;	1–	*Sr31*
Dobrynya	MS, 25S	2	2–	2+3+	*Sr25*
Favorit	75S	3	3	0	*Sr6Ag*^{i}**
Voevoda	75S	3=	3+	2 3–	*Sr6Ag*^{i}**
Lebyodushka	70S	4	3–	2–	*Sr25* + *Sr6Ag*^{i}**

Notes: I – Derbent pathogen population; II – Lysogorsk pathogen population (from cultivar Favorit); III – Omsk pathogen population; * degree of development in 2016, type of reaction; ** according to (Sibikeev et al., 2017a).

Table 2. Results of phytopathological analysis and identification of *Sr* genes (susceptible lines)

Pedigree	Resistance to *P. graminis* populations				Identified genes
	Saratov population (field)	at the seedling stage			
		I	II	III	
Dobr*5//Milan/Prinia L653	15MR	2+	3–	1	*Sr25*
Dobr*5//Milan/Prinia L654	15MR	0	3–	2	*Sr25*
S55/Agr.el*4//S29/3/L1015 ?Ag^{e}(3B)/?Ag^{e}(3D)	R, 10MR	0	3–	2+	*Sr25*
L505*2//L503*2/Kukushka L195	10MR	4	3	4	–
Dobr/Prokh//Dobr	R	2	3–	1	*Sr25*
S55/Agr. el *6//S29	10MR	2	2+	3	*Sr25*
Dobr*4/Nik	R	0	3–	X	*Sr25*
Dobr*4/3/Croc/Ae.squar(205)//Weaver	20MR	2	3	2–	*Sr25*
Croc/Ae.squar(205)//Weaver/3/L505/4/Dobr*Lr25*	20MR	2++	3	2	*Sr25*
S55/Agr. el *5//S29(? Ag^{e}-7D)/3/S68	15MR	X	3–	2+	*Sr25*
S55/Agr. el *5//S29(? Ag^{e}-7D)/3/S68	15MR	2	3	X	*Sr25*
Bel/3/Altar84/Ae.squar(224)//Pgo/4/S68(L481/16)	20MR	2++	3+	1	*Sr25*
Dobr//6R/Agis181(L426/16)	R	1	3–	1	–
Dobr*Lr25*/Bel//L505	20MR	3=	3	2–	*Sr25*
Dobr/Zol.volna//Dobr/3/Dobr	R	4	2	2–	*Sr25*
S55/Agr. el *6//S29	R	3–	2+	1	*Sr25*
Prokh*2/L164*2//L164	R	3–	2+	1	*Sr25*
Croc/Ae.squar//Weaver/3/*4L505/4/S73	R	2;	3–	2	*Sr25*
S70*4/3/Croc/Ae.squar(224)//Yaco	R	3	3	2	*Sr25*
S55/Agr. el *6//S29	15MR	2+	2 3	2	*Sr25*
Lyud//S55*2/T.dic-s//S.zol./L164//S55	10MR	2	2+3	2+	*Sr25*
L528//S55*4/T.dic-s	R	2++	2–3	1	*Sr25*
S74/*T.dicoccum k*7507*3//S73	R	0	3 2	X	*Sr25*
LC-SR25-ARS#	–	12	3–	12	*Sr25*

Notes: I – Derbent pathogen population; II – Lysogorsk pathogen population (inoculum from cultivar Favorit); III – Omsk pathogen population; # control line with *Sr25*.

Table 3. Identification of *Sr* genes in resistant lines

Lineage	*Sr* genes	Lineage	*Sr* genes
L2032*6/Curinda87	*Sr25* + *Sr31*	Sar.zol/T.dic-s//S58/3/*2 Bel/4/Voevoda	*Sr25* + *Sr31*
Dobrynya*4/TsLr25	*Sr25*	L503Lr26/Ottan(Rl1,Rl2)//Revansh	*Sr25*
L503Lr19Lr26	*Sr25* + *Sr31*	L18(L503Lr26)/S68//Revansh	*Sr25* + *Sr31*
L505//L503//L583/Kukushka//L505L200	*Sr25*	Tulaykovskaya10//Agis181/S29+Agis181/S58	*Sr25* + *Sr31* + *Sr28*
S55*3/T.dic-s//L2032	*Sr31*	ThatcherLr37*4/L503	*Sr25*
L2032*5/Seri82	*Sr25* + *Sr31*	L503Lr26/Ottan(Rl1,Rl2)//Revansh	*Sr25* + *Sr31*
L505*2//Croc/Ae.squar(224)//Yaco	*Sr25* + *Sr57*	Yu-V-2/L505//L503Lr26/3/L505/4/S68	*Sr25* + *Sr31*
L505/3/Croc/Ae.squar(205)//Weaver/4/L505/5/S68	*Sr25* + *Sr31*	Milan/Prinia//*4Dobr/3/Favorit	*Sr25*
L505/3/Croc/Ae.squar(205)//Weaver/4/L505/5/L505	*Sr25* + *Sr31*	Tselinnaya20/Dobr//Dobr/3/DobrLr9/4/Milan/Prinia//*4Dobr	*Sr25* + *Sr38*
Bel/3/Croc/Ae.squar(205)//Weaver/4/Bel	*Sr31*	Dobr*5//Milan/Prinia/3/Belyanka/4/S68	*Sr25*
L12(DobrLr24)/S68//S68	*Sr25*	L503/Sr35//L503/3/L503	*Sr25*
L505*2/Prokh//Bel(L496/16)	*Sr31*	Satu/S70//S74/3/S74	–
S55*3/T.dic-s//L2032(L501/16)	*Sr31*	Sar.zol/T.dic-s//S58/3/*2Bel/4/Voevoda	*Sr25* + *Sr31*
Dobr/Zol.volna//DobrLr24/3/Dobrynya	*Sr25*	L503Lr26/Ottan(Rl1,Rl2)//Revansh	*Sr25*
Prokh/MultiLr6R//S68/3/Dobr	*Sr25*	L18(L503Lr26)/S68//Revansh	*Sr25* + *Sr31*
L505/S42/4/L505*3//Prokh//L505/3/S70/4/DobrLr24	*Sr25* + *Sr31*	Tulaykovskaya10//Agis181/S29+Agis181/S58	*Sr25* + *Sr31* + *Sr28*
L505/L164/4/L503//Trap#1/Bow/3/L503/5/L505/6/S68	*Sr25* + *Sr31*	ThatcherLr37*4/L503	*Sr25*
Yu-V-2/L505//L503Lr26/3/L505/4/S68	*Sr25* + *Sr31*	L503Lr26/Ottan(Rl1,Rl2)//Revansh	*Sr25* + *Sr31*
Croc/Ae.squar(205)//Weaver/3/L505/4/DobrLr25	*Sr25*	Yu-V-2/L505//L503Lr26/3/L505/4/S68	*Sr25* + *Sr31*
Croc/Ae.squar(205)//Weaver/3/L505/4/Bel	*Sr25* + *Sr31*	Milan/Prinia//*4Dobr/3/Favorit	*Sr25*
Dobr*5/TcLr9//L505//L503*3/TRAP≠BOW//Prokh/S55	*Sr25* + *Sr31*	Tselinnaya20/Dobr//Dobr/3/DobrLr9/4/Milan/Prinia//*4Dobr	*Sr25* + *Sr38*
Dobr*5//Milan/Prinia/3/Belyanka/4/S68	*Sr25*	L503/Sr35//L503/3/L503	*Sr25*

from the *Ag. intermedium* and gene *Sr25* on the territory of the Volga Region. This is consistent with the data of Vasilova and co-authors on the loss of efficiency of the genes *Sr6Ag*^{i} and *Sr25* during stem rust epiphytoties in 2016 in Tatarstan (Vasilova et al., 2017). However, under stem rust epiphytoties in 2013–2015 in the Novosibirsk region, the gene *Sr6Ag*^{i} (*SrBel*) inhibited the development of the disease in cultivar Favorit (type of reaction to the pathogen R) (Sochalova, 2016). This agrees with the evaluation data of the cultivar Favorit resistance to the Omsk population of the pathogen at the seedling stage (see Table 1). According to our data, gene *Sr31* still retains its effectiveness.

The whole set of introgressive lines, taken in the analysis, was previously evaluated for resistance to the causative agent of stem rust under strong epiphytoties conditions in 2016 in the experimental field of the Laboratory of Genetics ARISER, Saratov, and in the phytonurseries, located in the South-West of the Saratov oblast. As already noted, the degree of disease development in cultivars Favorit and Voevoda reached 80 %, which led to significant yield losses (Sibikeev, unpubl. data). The IT in the studied set of lines mainly ranged from 0 to 2 (R, MR). In 2017 during the field evaluation, the lines were also resistant to pathogen, however, during the laboratory evaluation, 15 lines showed susceptible type of reaction (3) to inoculums of the Lysogorsk pathogen population taken from the cultivar Favorit (Table 2). Perhaps this is due to the fact that the pathogen population from the cultivar Favorit is 100 % virulent to *Sr6Ag*^{i}.

As can be seen from the table, only the gene *Sr25* was identified in all susceptible lines, which indicates a loss in the efficiency of this gene. However, the combination of gene *Sr25* with the genes *Sr31/Lr26*, *Sr38/Lr37* and the adult resistant gene *Sr57/Lr34* provided resistance to all populations of *P. graminis* taken in the analysis.

Lines resistant to all populations of the pathogen, with the identified *Sr* genes are presented in Table 3. The phytopathological evaluation of these lines is provided in the Suppl. Material 6.

Of interest are the spring bread wheat lines shown in Table 3, resistant to all populations of the pathogen, but with an identified *Sr25* gene. The analysis of these lines lineages suggests that they contain other resistance genes. Thus, the cultivars Prinia and Weaver carry the 1RS.1BL translocation (*Sr31*) according to the GRIS database (Genetic Resources

Information System for Wheat and Triticale). It is possible that the gene *Sr31* is present in the lines with the participation of these cultivars, but they are heterogeneous according to this gene. The cultivar Dobrynya, participating in the lineage of resistant lines in which only *Sr25* was identified, was heterogeneous in resistance to the Saratov (field) and Omsk populations of the pathogen and resistant to the Derbent and Lysogorsk populations (see Table 1). Probably, besides *Sr25*, there are other genes in it that influence the manifestation of the trait. In addition, the lines Dobrynya *Lr24*, Dobrynya *Lr25*, L503 *Lr26* (participating in lineages) and Thatcher *Lr37**4/L503 were obtained from crosses with the corresponding isogenic lines of the Thatcher cultivar, which carries the gene complex *Sr5* + *Sr9g* + *Sr12* + *Sr16* (McIntosh et al., 2013). The possibility of transferring these genes or their combination in a hybrid progeny is not excluded. As can be seen from the lineage, in the line (L503/Sr35//L503/3/L503) the presence of the *Sr35* gene is possible, it will be clarified in the further research.

Conclusion

Thus, genes *Sr31* and *Sr25* were mainly identified in the analyzed introgression lines. The combination of these genes – *Sr31/Lr26* + *Sr25/Lr19* was identified in 15 lines (26.3 %). The combination of genes *Sr38/Lr37* + *Sr25/Lr19* was identified in one line; *Sr31/Lr26* + *Sr25/Lr19* + *Sr28* – in one line; gene *Sr25/Lr19* with the adult resistant gene *Sr57/Lr34* – in one line. These lines were resistant to all analyzed populations of the pathogen, which makes them promising for further use in breeding. The genes *Sr2*, *Sr24*, *Sr26*, *Sr32*, *Sr36*, *Sr39* were not detected in the analyzed lines. It is also necessary to note the promising use of triticale cultivar Satu, which carries the genes linkage *LrSatu/SrSatu*, in protection against stem rust pathogen (McIntosh et al., 1995).

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ORCID ID
O.A. Baranova orcid.org/0000-0001-9439-2102
S.N. Sibikeev orcid.org/0000-0001-8324-9765
A.E. Druzhin orcid.org/0000-0002-3968-2470

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Potato mosaic viruses which infect plants of tuber-bearing *Solanum* spp. growing in the VIR field gene bank

E.V. Rogozina[1] ✉, N.V. Mironenko[2], N.A. Chalaya[1], Yu. Matsushita[3], H. Yanagisawa[4]

[1] The N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
[2] All-Russian Institute of Plant Protection, Pushkin, St. Petersburg, Russia
[3] Institute of Vegetable and Floriculture Science, National Agriculture and Food Research Organization, Tsukuba, Japan
[4] Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Tsukuba, Japan
✉ e-mail: erogozina@vir.nw.ru

Potato crop is particularly affected by virus diseases, and potato virus Y (PVY) currently considered the most important pathogen distributed worldwide as a diversity of strains. Wild and cultivated tuber-bearing species of the genus *Solanum* L., stored in the VIR collection, are used as the initial material in creation domestic potato varieties (*Solanum tuberosum* L.) resistant to virus diseases. The preservation and rational utilization of the potato collection is based on regular phytosanitary monitoring, including quarantine objects, foremost PSTVd (potato spindle tuber viroid). The aim of the work is to examine plants of tuber-bearing *Solanum* species in the field gene bank of VIR for the presence of PSTVd and PVX (potato virus X), PVS (potato virus S), PVM (potato virus M) and PVY, which are the most common viruses on potatoes in the North-West District of Russia. We examined clonal plants of 137 genotypes representing 31 species of the section Petota of the genus *Solanum* L. A diagnostic was carried out using ELISA, RT-PCR and indicator plants. No PSTVd was found in the studied plants, but a plural infestation by mosaic viruses was detected, more than half of the tested clones are infected with two or more viruses. In the studied samples, only 17 genotypes (12 %) are not infected by PVX, PVS, PVM and PVY according to the ELISA test. There are statistically significant differences in the virus infestation of *Solanum* species with different origins, according to Pearson's chi-squared test. Among the studied genotypes of wild relatives of potatoes, the proportion of those affected by PVY was significantly higher in the South American than in the North American species ($\chi^2 = 4.56$, $p = 0.03$); the proportion of genotypes affected by PVX was significantly higher in the North American species ($\chi^2 = 8.81$, $p = 0.003$), the critical value was $\chi^2 = 3.841$. PVY strains were identified by multiplex RT-PCR in 37 genotypes of *Solanum* spp. We found that 27 genotypes are infected by a common PVY^O strain, two genotypes are infected by PVY^NW (A) and PVY^NW (B) strains, respectively, seven genotypes are infected by a mixture of $PVY^O + PVY^{NW}$ (A) strains, and one is infected by a mixture of $PVY^O + PVY^{NTN-NW}$ (SYRI) + SYRIII strains. The recombinant strains of PVY are detected in the North-West District of Russia for the first time. Coherency of the results of PVY strains detection by various (immunological, molecular and biological) methods is discussed.
Key words: wild tuber-bearing *Solanum* spp.; potato spindle tuber viroid; potato mosaic viruses; PVY strains; recombinant strains; ELISA; RT-PCR; indicator plant; mixed infection.

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Мозаичные вирусы картофеля, поражающие растения клубненосных видов рода *Solanum* L. в полевом генном банке ВИР

Е.В. Рогозина[1] ✉, Н.В. Мироненко[2], Н.А. Чалая[1], Ю. Мацухита[3], Х. Янагисава[4]

[1] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
[2] Всероссийский научно-исследовательский институт защиты растений, Пушкин, Санкт-Петербург, Россия
[3] Институт овощеводства и цветоводства Национальной организации сельского хозяйства и исследований пищевых продуктов, Цукуба, Япония
[4] Центральный сельскохозяйственный научно-исследовательский центр Национальной организации сельского хозяйства и исследований пищевых продуктов, Цукуба, Япония
✉ e-mail: erogozina@vir.nw.ru

Вирусные болезни наносят большой ущерб картофелеводству, и особую проблему повсеместно представляет вирус картофеля Y (potato virus Y – PVY), отличающийся разнообразием штаммового состава. Для создания отечественных сортов картофеля (*Solanum tuberosum* L.), устойчивых к вирусным болезням, исходным материалом служат дикие и культурные клубнеобразующие виды рода *Solanum* L., сохраняемые в коллекции генетических ресурсов картофеля ВИР. Сохранение и рациональное использование коллекции основано на регулярном фитосанитарном мониторинге, в том числе карантинных объектов, в первую очередь – вироида веретеновидности клубней картофеля (potato spindle tuber viroid – PSTVd). Цель работы – обследование растений клубненосных видов *Solanum* L. в полевом генном банке ВИР на наличие PSTVd и

мозаичных вирусов PVX (potato virus X), PVS (potato virus S), PVM (potato virus M) и PVY (potato virus Y), наиболее распространенных на картофеле в Северо-Западном регионе Российской Федерации. Обследованы клоновые растения 137 генотипов, представляющие 31 вид секции Petota рода *Solanum* L. Диагностика проведена методами ELISA, ОТ-ПЦР и растений-индикаторов. Среди изученных растений PSTVd не обнаружен, но диагностировано массовое поражение мозаичными вирусами, более половины тестированных клонов инфицировано двумя и более вирусами. Выявлено 17 генотипов (12 %) с отрицательной реакцией ELISA на PVX, PVS, PVM и PVY. Различия в поражении мозаичными вирусами растений *Solanum* spp., относящихся к разным филогенетическим группам, статистически значимы (по критерию χ^2 Пирсона). Среди исследованных генотипов южноамериканских видов доля пораженных PVY достоверно больше, чем среди генотипов североамериканских видов ($\chi^2 = 4.56$, $p = 0.03$), PVX, напротив, чаще детектирован у генотипов из группы североамериканских видов ($\chi^2 = 8.81$, $p = 0.003$). Штаммы PVY идентифицировали у 37 генотипов *Solanum* spp. методом мультиплексной ОТ-ПЦР. Выявлено 27 генотипов, пораженных обычным штаммом PVY^O, по одному генотипу – пораженные штаммами PVY^NW (A) и PVY^NW (B), семь генотипов, пораженных смесью штаммов $PVY^O + PVY^NW$ (A), и один – смесью штаммов $PVY^O + PVY^{NTN-NW}$ (SYRI) и SYRIII. Рекомбинантные штаммы PVY^NW (A), PVY^{NTN-NW} (SYRI) и SYRIII впервые обнаружены в Северо-Западном регионе Российской Федерации. Обсуждается согласованность результатов диагностики штаммов PVY разными (иммунологический, молекулярный и биологический) методами.

Ключевые слова: дикие клубненосные *Solanum* spp.; вироид веретеновидности клубней картофеля; мозаичные вирусы картофеля; штаммы PVY; рекомбинантные изоляты; ELISA; ОТ-ПЦР; растение-индикатор; смешанная инфекция.

Introduction

In a changing climate, sustainable agricultural production of sufficient volumes of diverse high-quality food products is necessary to provide the population of the world with food.

One of the leading crops in global agriculture is potato, a vegetatively propagated crop, vulnerable to virus infections. No less than 40 potato-infecting virus species are known (Potato Biology..., 2007), of which six are the most harmful and widespread: namely, the potato leafroll virus (PLRV) and the mosaic viruses: potato virus X (PVX), potato virus S (PVS), potato virus M (PVM), potato virus A (PVA) and potato virus Y (PVY). Virus diseases, especially those that are caused by a mixture of PVY and other viruses of the mosaic group, lead to significant losses in production of the commodity in question and impede seed production. PVY is distinguished by a variety of strains, among which there are five non-recombinant and more than three dozens of recombinant ones (Green et al., 2018). In recent years, in many countries where potatoes are cultivated, including the Russian Federation, there has been a significant spread, and sometimes dominance, of recombinant PVY strains (Karasev, Gray, 2013; Uskov et al., 2016; Green et al., 2017). PVY recombinant isolates represent a particular problem for the potato industry, since many of them cause necrotic lesions or potato tubers fissuring.

An ecologically safe and effective strategy of protecting potatoes from virus infections is based on the development of resistant varieties and their introduction into cultivation. The initial material for potato breeding for resistance to virus diseases is represented by wild and cultivated tuber-bearing species of the genus *Solanum* L. In comparison to other agricultural plants, potatoes have the largest number of tuber-bearing wild relative species (Vincent et al., 2013). According to FAO, at present extensive *ex situ* collections of potatoes totaling around 98000 accessions are maintained in 30 countries around the world (Machida-Hirano, 2015). The VIR collection of potato genetic resources is among the most representative ones, with about 8000 accessions of wild, primitive and cultivated species, varieties and breeding clones of potato. An important aspect of the work on the conservation and regeneration of the cultivated forms and wild relatives of potato is the phytosanitary monitoring of the collection and control over the non-proliferation of the quarantine objects, first of all, of the Potato spindle tuber viroid (PSTVd). It is transmitted by contact, with the sap of infected plants or botanical seeds, which become infected if they form in plants with infected pollen or ovules. In addition to potato varieties, PSTVd is of high threat to the stolon- and tuber-forming Solanum spp., accessions of which are conserved in genebank collections (Jeffries, 1998). The present study was aimed at monitoring PSTVd, as well as PVX, PVS, PVM and PVY (most common mosaic potato viruses in North-West District of Russia in plants of wild *Solanum* tuber-bearing species in the field genebank of VIR.

Materials and methods

The study involved 137 plants representing 31 species of wild tuber-bearing *Solanum* spp. belonging to such series in the North American group as Demissa Buk. (*S. iopetalum*), Longipedicellata Buk. (*S. fendleri*, *S. hjertingii*, *S. papita*, *S. polytrichon*, *S. stoloniferum*), Pinnatisecta Rydb. (*S. jamesii*, *S. pinnatisectum*), Cardiophylla Buk. (*S. cardiophyllum*, *S. ehrenbergii*), and such series in the South American group as Acaulia Juz. (*S. acaule*), Yungasensia Corr. (*S. arnezii*), Glabrescentia Buk. (*S. chacoense*), Bukasoviana Gorbat. (*S. alandiae*, *S. avilesii*, *S. gourlayi*, *S. hondelmannii*, *S. kurtzianum*, *S. leptophyes*, *S. okadae*, *S. oplocense*, *S. sparsipilum*, *S. spegazzinii*, *S. venturii*, *S. vernei*), Tarijensia Corr. (*S. berthaultii*, *S. neocardenasii*, *S. tarjiense*), Simpliciora (Buk.) Gorbat. (*S. microdontum*, *S. simplicifolium*) and Maglia Bitt. (*S. molinae*). Names of the series and the species are given in accordance with classifications by S.M. Bukasov (1978) and L.E. Gorbatenko (1990).

The species were represented by 2 to 16 genotypes conserved as clonal plants. The studied *Solanum* spp. genotypes either constituted a trait-specific collection of wild potato relatives characterized for their resistance to phytopathogens and the presence of the corresponding *R* gene markers (Rogozina et al., 2014), or were a part of the working collection of geno-

Table 1. Classification of potato virus Y (PVY) strains, according to the multiplex PCR (Chikh Ali et al., 2010)

Products in the multiplex PCR, bp	Identified PVY strain
853 + 532	PVY^{O}
1307 + 633 + 398	PVY^{N}
1307	NA-PVY^{N}
853 + 633 + 441	$PVY^{N}W$ (A)
853 + 441	$PVY^{N}W$ (B)
1307 + 633 + 441	PVY^{NTN} (A)
1307 + 441	PVY^{NTN} (B)
1076 + 633 + 441	PVY^{NTN-NW} (SYRI)
1076 + 441	PVY^{NTN-NW} (SYRII)
1076 + 441 + 278	SYRIII

types studied for a set of traits of breeding importance. The plants tested in field conditions were grown from the tubers reproduced annually in protected soil.

Viroid detection. The total RNA was isolated from 100 mg of fresh leaves of wild *Solanum* spp. using the RNeasy Plant Mini Kit (QIAGEN, Germany), following the manufacturer recommendations. The RNA was used to detect PSTVd viroid by RT-PCR, according to the protocol (Yanagisawa, et al., 2017) envisaging the use of the 6Pospi-F/R primer set. A plant of potato cultivar 'Osen' infected with PSTVd viroid and conserved in the collection of the A.G. Lorch Potato Research Institute (Korenevo, Moscow Province), was used as a positive control.

Virus detection. An ELISA double sandwich method (Clark, Adams, 1977) was used to detect PVX, PVS, PVM and PVY viruses in the leaves of wild *Solanum* spp. The used diagnostic kits were produced by the "Biotechnology" Scientific Production Association at the A.G. Lorch Potato Research Institute.

For additional detection of PVY, RT-PCR with specific primers was used (San et al., 2009). The PVY strain composition was determined by multiplex RT-PCR using a set of 12 primers (Lorenzen et al., 2006), which allowed identification of 10 individual strains: PVY^{O}, PVY^{N}, NA-PVY^{N}, $PVY^{N}W$ (two genotypes), PVY^{NTN} (two genotypes), PVY^{NTN-NW} (two genotypes) and SYRIII (Table 1), as well as the cases of mixed infection with these strains (Chikh Ali et al., 2010). The Prime Script One Step RT-PCR kit (TaKaRa, Japan) was used for RT-PCR; the PCR products were separated on agarose gels and stained with ethidium bromide.

Nicotiana tabacum L. indicator plants ('Samsun' variety) were tested to verify the RT-PCR results for diagnosing and determining the PVY strain composition (Jeffries, 1998).

Results

PSTVd detection. RT-PCR assay of 137 genotypes of 31 wild tuber-bearing *Solanum* spp. did not reveal PSTVd in the tested leaf tissue samples, with the exception for the positive control. The obtained results are consistent with the previous testing of other genotypes of wild *Solanum* species from the VIR collection for PSTVd presence (T.B. Kastaljeva, pers. commun.) and indicate the absence of PSTVd in accessions of wild potato species in the VIR field genebank.

Potato mosaic viruses detection. Mass infection of plants of wild *Solanum* species with PVX, PVS, PVM and PVY (Table 2) has been detected by ELISA assay.

Only 17 genotypes (12 % of the tested) were not infected with mosaic viruses. They belong to the species *S. acaule* (k-23004), *S. cardiophyllum* (k-16827, k-16828), *S. gourlayi* (k-11446, k-12416), *S. hjertingii* (k-23366), *S. hondelmannii* (k-20023), *S. leptophyes* (k-5764), *S. polytrichon* (k-19164, k-24410), *S. sparsipilum* (k-9798, k-19344), *S. spegazzinii* (k-11422, k-11975, k-12688), *S. stoloniferum* (k-24420), and *S. vernei* (k-11447). It should be noted that in other genotypes of *S. acaule* (k-23004) and *S. vernei* (k-11447), PVY and PVY/PVS mixed infections were detected, respectively. Clones of *Solanum* species selected as sources of resistance to late blight or golden nematode, were found to be infected with a monoinfection or a mixture of PVX, PVS and PVY.

The greatest occurrence in plants of a set of studied tuber-forming *Solanum* spp. was demonstrated by PVY, as 58 % of the tested genotypes were infected with this virus. The infection with PVY was detected in 22 % of genotypes, and 36–37 % of genotypes were found to be infected with PVM and PVS (See Table 1). Statistically significant differences between the two groups of potato species were found: among the studied genotypes of *Solanum* spp. from the South American group, the proportion of those infected with PVY was significantly higher than among the species from the North American group (chi-square value $\chi^2 = 4.56$ > critical chi-square value $\chi^2 = 3.84$ at .05 probability level), while PVX was more often detected in the genotypes of potato species from the North American group (chi-square value $\chi^2 = 8.81$ > critical chi-square value $\chi^2 = 3.84$).

More than half of the genotypes in the studied set of tuber-forming *Solanum* spp. were infected with two or more mosaic viruses. A mixed infection of all four mosaic viruses was found in plants of nine genotypes belonging to the species *S. alandiae*, *S. fendleri*, *S. microdontum*, *S. papita*, *S. polytrichon*, *S. simplicifolium* and *S. stoloniferum*. A complex of three viruses was detected in 15 genotypes belonging to the species *S. chacoense*, *S. kurtzianum*, *S. microdontum*, *S. molinae*, *S. pinnatisectum*, *S. polytrichon*, *S. simplicifolium* and *S. stoloniferum*.

PVY strains identification. Plants of 40 genotypes representing the species *S. alandiae*, *S. avilesii*, *S. cardiophyllum*, *S. chacoense*, *S. ehrenbergii*, *S. fendleri*, *S. hjertingii*, *S. iopetalum*, *S. jamesii*, *S. kurtzianum*, *S. leptophyes*, *S. neocardenasii*, *S. pinnatisectum*, *S. polytrichon*, *S. simplicifolium*, *S. spegazzinii* and *S. stoloniferum* were additionally tested for the presence of PVY by RT-PCR (Sun et al., 2009). PVY was not found in plants of *S. leptophyes* (k-5764) and *S. neocardenasii* (k-24612), which is consistent with ELISA results. The PVY strains were identified by the presence of diagnostic amplification products of various sizes, obtained as a result of multiplex RT-PCR (Chikh Ali et al., 2010). Fig. 1 shows samples of PVY strains identification.

It should be noted that the results of multiplex RT-PCR do not always allow the unambiguous identification of PVY strains. For example, there is an 'extra' amplification product

Table 2. Potato mosaic virus infection in plants of tuber-bearing *Solanum* spp. (St. Petersburg, Pushkin, 2016–2017)

Series (number of species)	No. of tested genotypes	No. of genotypes with positive ELISA reaction to mosaic viruses			
		PVY	PVX	PVS	PVM
North-American group					
Demissa (1)	2	2	0	2	0
Longipedicellata (5)	40	23	17	19	21
Pinnatisecta (2)	11	2	2	1	5
Cardiophylla (2)	8	2	2	1	1
Total	61	29[a]	21[b]	23	27
South-American group					
Yungasensia (1)	2	2	0	1	0
Glabrescentia (1)	10	5	1	8	5
Acaulia (1)	2	1	0	0	0
Bukasoviana (12)	46	30	4	9	6
Tarijensia (3)	4	3	0	2	2
Simpliciora (2)	10	8	4	6	9
Maglia (1)	2	2	0	2	1
Total	76	51[a]	9[b]	28	23
Total genotypes (%)	137 (100)	80 (58)	30 (22)	51 (37)	50 (36)

Note: The statistically significant differences at a significance level of α = 0.05 are marked with letters.

of 278 bp in size in lanes 3 and 4 (see Fig. 1); and in lane 6, in addition to the diagnosed strains NA-PVY^{N} (1307 bp) and PVY^{O} (853 and 532 bp), fragments of 1076, 633 and 278 bp are clearly seen, though a 441 bp fragment is lacking for the identification of other recombinant strains.

A total of 27 genotypes were found to be infected with the common PVY^{O} strain, one genotype infected with recombinant strains of PVY^{N}W (A) and PVY^{N}W (B), seven genotypes showed infection with a mixture of PVY^{O} + PVY^{N}W (A) strains, and one was infected with a mixture of PVY^{O}, + PVY^{NTN-NW} (SYRI) and SYRIII strains. The results of PVY diagnosing by immunological and molecular methods coincided in 68 % of cases. A discrepancy was observed in the results of the diagnosis of 13 genotypes belonging to *S. cardiophyllum*, *S. fendleri*, *S. hjertingii*, *S. kurtzianum*, *S. polytrichon*, *S. stoloniferum* and *S. pinnatisectum*, which had a negative ELISA, but a positive RT-PCR result.

Indicator plant tests. To verify the results of immunological and molecular analyses, 21 genotypes of *Solanum* spp. were tested for infection with PVY in a biological test using *N. tabacum* L. indicator plants. The plants of *N. tabacum* L. 'Samsun' variety inoculated with sap from plants of 13 *Solanum* spp. genotypes, in which PVY had been detected, showed symptoms of infection on the 7th day. One group of tobacco plants had an interveinal clearing, followed by mottle, the symptoms of which persisted a month later and kept appearing gradually on new leaves as they grew and developed. These symptoms evidence the infection of tobacco plants with the common PVY^{O} strain. In other plants of *N. tabacum*, a severe veinal necrosis and puckering of the leaf tissue were observed, which were followed by chlorosis of the entire leaf and stunting of the plant growth (Fig. 2). These symptoms evidence tobacco infection with a necrotic strain of PVY^{N}. The visible

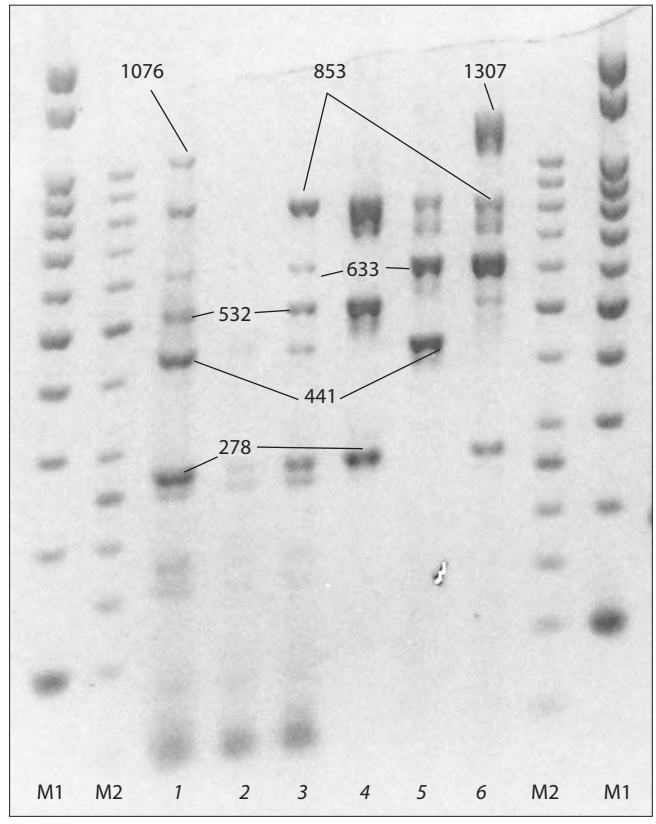

Fig. 1. PVY strains identification in samples of wild *Solanum* species by multiplex RT-PCR.

DNA molecular size markers M1 and M2 – GeneRuler^{TM} 1 kb and 50 bp (Fermentas DNA Ladder, respectively). Accessions of wild *Solanum* species: *1 – S. cardiophyllum*; *2 – S. jamesii*; *3 – S. polytrichon*; *4 – S. avilesii*; *5 – S. iopetalum*; *6 – S. jamesii*. Numbers indicate diagnostic amplification product size, in bp. The identified PVY strains are presented in Table 3.

Table 3. PVY strains identification in *Solanum* spp. accessions according to the amplified products presence in the multiplex RT-PCR (Chikh Ali et al., 2010) (see Fig. 1)

No.	*Solanum* spp.	VIR Catalog number	Size, bp	PVY strain
1	*S. cardiophyllum*	24375	1076, 853, 633, 532, 441, 278	PVY^{O}, PVY^{NTN-NW} (SYRI) and SYRIII
2	*S. jamesii*	24920	Small amount of RNA	Not detected
3	*S. polytrichon*	24410	853, 633, 532, 441, 278	PVY^{O} and $PVY^{N}W(A)$
4	*S. avilesii*	20884	853, 532, 278	PVY^{O}
5	*S. iopetalum*	24393	853, 633, 441	$PVY^{N}W(A)$
6	*S. jamesii*	24920	1307, 1076, 853, 633, 532, 278	PVY^{O} и NA-PVY^{N}

Fig. 2. Symptoms of PVY infection in *N. tabacum* after inoculation with sap from *Solanum* spp. plants.

Top – systemic veinal necrosis (PVY^{N} necrotic strain); bottom – veinal clearing symptoms (PVY^{O} common strain).

symptoms of virus infection in *N. tabacum* plants clearly differed depending on the genotypes of *Solanum* species used as sources of the virus inoculum. In accordance with the local and systemic response observed on the leaves of the indicator plants after inoculation with the sap of the corresponding wild potato plant, infection of eight *Solanum* spp. genotypes with the common strain and of five *Solanum* spp. genotypes with the necrotic strain of virus Y has been detected (Table 4).

No symptoms of virus infection were found on tobacco plants after inoculation with the sap of *S. leptophyes* and *S. neocardenasii* plants, which, according to ELISA and multiplex RT-PCR, were PVY-free. The results of PVY detection by the immunological and biological methods coincided for 19 (90 % of the tested) genotypes of *Solanum* spp. Inoculation with the sap from plants of five genotypes, that is *S. fendleri*, *S. hjertingii*, *S. pinnatisectum* (two genotypes), *S. cardiophyllum* (PVY-infected according to multiplex RT-PCR) and *S. simplicifolium* (with a positive ELISA and RT-PCR reaction) have not caused visible effects in *N. tabacum* plants. The results of indicator plants tests and of RT-PCR for virus Y detection coincided for 15 (71 % of the tested) genotypes, and the identification of virus Y strain composition coincided for 9 (62 % of the tested) genotypes of *Solanum* species.

The *N. tabacum* plants inoculated with sap from *Solanum* spp. plants were tested for the presence of PVY by RT-PCR. An analysis of 12 phenotypically different tobacco plants confirmed the presence of the virus in 10 test samples (See Table 4). The results of the strain composition determination from the symptoms and by RT-PCR of indicator plants coincided (or partially coincided) for seven genotypes: *S. avilesii* (k-20884 and k-20158), *S. iopetalum* (k-24393), *S. jamesii* (k-24920) (221), *S. polytrichon* (k-18142, k-24410 and k-24462). For the genotypes of *S. chacoense* (k-21321) and *S. kurtzianum* (k-20038), the virus Y necrotic strain identified by the response of the indicator plant, did not match the results of RT-PCR. For *S. cardiophyllum* (k-24375) infected with a mixture of virus Y strains, the results of the indicator plant testing did not confirm the RT-PCR data.

Discussion

Conservation, study and reproduction of wild relatives of the tuber-bearing *Solanum* spp. is carried out in the field gene bank of VIR located on the territory of the Science and Production Base "Pushkin and Pavlovsk Laboratories of VIR" in Pushkin town. For over 40 years, the reproduction and study of the collection of varieties, breeding clones and species belonging to section Petota of the genus *Solanum* L., as well as the production of TPS of tuber-bearing *Solanum* species has been carried out here. A characteristic feature of the agrocenosis that had formed here is the local concentration of genetic diversity of cultivated forms and wild relatives of potatoes, which is favorable for the manifestation of diseases and the development of pests. Probably, the populations of

Table 4. PVY detection in *Solanum* and *N. tabacum* plants by a complex of methods

Species	VIR Catalog number (genotype)	Detection of PVY in *Solanum* spp.		*N. tabacum* (symptoms)	RT-PCR of *N. tabacum*
		ELISA	RT-PCR		
S. alandiae	21240	PVY	PVY^{O}	Mosaic	–
S. avilesii	20158	PVY	PVY^{O}	»	PVY^{O}
S. avilesii	20884	PVY	PVY^{O}	»	PVY^{O}
S. cardiophyllum	24375	n.d.	PVY^{O}, PVY^{NTN-NW} (SYRI) и SYRIII	No	PVY^{O}, PVY^{NTN-NW}
S. chacoense	21321	PVY	PVY^{O}	Veinal necrosis	PVY^{O}
S. chacoense	22687	PVY	PVY^{O}	»	–
S. fendleri	5751	n.d.	PVY^{O}	No	–
S. hjertingii	15194	n.d.	PVY^{O}, $PVY^{N}W(A)$	»	–
S. iopetalum	24393	PVY	PVY^{O}, $PVY^{N}W(A)$	Mosaic	$PVY^{N}W(A)$
S. jamesii	24920 (221)	PVY	PVY^{O}	Veinal necrosis	PVY^{O}, PVY^{N}
S. jamesii	24920 (223)	PVY ?	PVY^{O}, $PVY^{N}W(A)$	Mosaic	n.d.
S. kurtzianum	20038	PVY	PVY^{O}	Veinal necrosis	PVY^{O}
S. leptophyes	5764	n.d.	n.d.	No	–
S. neocardenasii	24612	n.d.	n.d.	»	–
S. pinnatisectum	21955 (387)	n.d.	PVY^{O} ?	»	–
S. pinnatisectum	21955 (401)	n.d.	PVY^{O}, $PVY^{N}W(A)$	»	–
S. polytrichon	18142	PVY	PVY^{O}	Mosaic	PVY^{O}
S. polytrichon	24410	n.d.	PVY^{O}, $PVY^{N}W(A)$	»	PVY^{O}, $PVY^{N}W(A)$
S. polytrichon	24462	PVY	PVY^{O}	»	PVY^{O}
S. simplicifolium	12658	PVY	PVY^{O}	No	n.d.
S. spegazzinii	11431	PVY	PVY^{O}	Veinal necrosis	–

Note: PVY? – weak reaction; n.d. – PVY not detected; "–" no PCR performed.

phytopathogens attacking potato collection plantings are highly polymorphic, which ensures survival of the parasites in interaction with the population of the host plant. This assumption is confirmed by the results of a comparative analysis of *Phytophthora infestans* isolates (late blight pathogen) collected from the leaves of potato accessions in the VIR field genebank and of the commercial plantings of potato varieties in the Leningrad Province (Kuznetsova et al., 2016; Sokolova et al., 2017).

The authors have discovered a diversity of virus Y isolates, including those of the recombinant type, in plants of different *Solanum* species. For the first time, PVY-$^{N}W(A)$, PVY^{NTN-NW} (SYRI) and SYR III isolates were found in plants of potato relatives in the North-West District of Russia. Earlier, isolates of recombinant PVY^{NTN} and $PVY^{N:O}$ strains were found in seed potatoes from the central regions of Russia and Belarus (Uskov et al., 2016).

When testing some plants of *Solanum* spp. (genotypes of *S. cardiophyllum*, *S. chacoense*, *S. hjertingii*, *S. fendleri*, *S. kurtzianum*, *S. pinnatisectum*, *S. simplicifolium* and *S. spegazzinii*) for PVY presence by a complex of methods (immunological, molecular and biological), contradictory results have been obtained.

The multiplex PCR for the identification of 10 PVY strains, including rare recombinant ones, has been developed with the ability to detect mixed infections (Chikh Ali et al., 2010). However, the authors noted the impossibility in some cases of mixed infection to identify the genotype of each strain. In our research, we also came across examples of insufficient match between the obtained amplification products with the diagnostic fragments mentioned in the paper by Chikh Ali et al. (2010) to make accurate identification of all genotypes of PVY strains. Obviously, the quantitative ratio of different genotypes of virus strains in the infected plant can play an important role in multiplex PCR for mixed infections, which can lead to a 'deficiency' or, on the contrary, an "excess" of some diagnostic amplification products for accurate identification of strains. In the biological sense, it can be assumed that strains with different genotypes differ in competitiveness in different species of host plants. This circumstance may explain the discrepancy in the identification of virus strains in potatoes and tobacco after inoculation of the latter.

The controversial results of virus Y strains detection in plants of *Solanum* spp. and *N. tabacum* L. may be due to genetic differences between wild relatives and potato varieties (*Solanum tuberosum* L.), which are used for developing and testing diagnostic methods. At present, a classification of PVY strains infecting potatoes will primarily consider the response of varieties with hypersensitivity genes to certain virus strains and the molecular characterization of the virus isolate, while the appearance of necrosis on *N. tabacum* plants will be considered as a secondary symptom (Karasev, Gray, 2013). Probably, changes in the biological or immunological properties of individual virus Y isolates occur at the interaction with a host plant with a different genetic basis, that is, with wild relatives of potatoes, representing other *Solanum* species. The disturbed structure of the virus shell protein in recombinant strains, for example, prevents ELIZA assay.

The diversity of PVY strains, especially of recombinant ones, is extensively studied using immunological and molecular genetic methods, whereas biological properties have been studied only for a limited number of isolates (Karasev, Gray, 2013; Green et al., 2017). PVY is considered as an interesting model for studying the evolution of a virus, which is influenced by selection when interacting with genetically different host plants and in different environmental conditions. By evolving through mutations and recombinations between different strains, PVY is able to overcome the resistance of potato varieties with *N*-genes. It has been established that PVY^{N}-W or $PVY^{N:O}$ strains that have spread in potatoes recently, have resulted from the recombination of isolates belonging to common PVY^{O} and necrotic PVY^{N} strains. More than 30 recombinant strain variants have been discovered, the appearance of which is explained by recombination between isolates of different subgroups of strains, including the recombinant isolates that had appeared previously (Green et al., 2018). The PVY isolates with atypical characters found in plants of *S. cardiophyllum*, *S. chacoense* and *S. kurtzianum*, are of particular interest for further research.

Conclusions

PSTVd was not detected in plants of tuber-bearing *Solanum* species in the VIR field genebank, however potato mosaic viruses were found to be widespread. A great part of clones of wild relatives of potato (*Solanum* species) selected for late blight and golden nematode resistance, are susceptible to viruses Y, S, M, X.

In the studied set of plants of tuber-bearing *Solanum* species infected with virus Y, the usual PVY^{O} strain prevails, while the second most commonly distributed is the recombinant $PVY^{N}W$ (A) strain. Virus Y isolates with different biological and immunological properties were found in plants of *Solanum* spp.

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ORCID ID

E.V. Rogozina 0000-0002-2743-068X

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Prioritization of potato genes involved in the formation of agronomically valuable traits using the SOLANUM TUBEROSUM knowledge base

P.S. Demenkov[1, 2] ✉, O.V. Saik[1], T.V. Ivanisenko[1], N.A. Kolchanov[1], A.V. Kochetov[1], V.A. Ivanisenko[1]

[1] Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
✉ e-mail: demps@bionet.nsc.ru

The development of highly efficient technologies in genomics, transcriptomics, proteomics and metabolomics, as well as new technologies in agriculture has led to an "information explosion" in plant biology and crop production, including potato production. Only a small part of the information reaches formalized databases (for example, Uniprot, NCBI Gene, BioGRID, IntAct, etc.). One of the main sources of reliable biological data is the scientific literature. The well-known PubMed database contains more than 18 thousand abstracts of articles on potato. The effective use of knowledge presented in such a number of non-formalized documents in natural language requires the use of modern intellectual methods of analysis. However, in the literature, there is no evidence of a widespread use of intelligent methods for automatically extracting knowledge from scientific publications on cultures such as potatoes. Earlier we developed the SOLANUM TUBEROSUM knowledge base (http://www-bionet.sysbio.cytogen.ru/and/plant/). Integrated into the knowledge base information about the molecular genetic mechanisms underlying the selection of significant traits helps to accelerate the identification of candidate genes for the breeding characteristics of potatoes and the development of diagnostic markers for breeding. The article searches for new potential participants of the molecular genetic mechanisms of resistance to adverse factors in plants. Prioritizing candidate genes has shown that the *PHYA*, *GF14*, *CNIH1*, *RCI1A*, *ABI5*, *CPK1*, *RGS1*, *NHL3*, *GRF8*, and *CYP21-4* genes are the most promising for further testing of their relationships with resistance to adverse factors. As a result of the analysis, it was shown that the molecular genetic relationships responsible for the formation of significant agricultural traits are complex and include many direct and indirect interactions. The construction of associative gene networks and their analysis using the SOLANUM TUBEROSUM knowledge base is the basis for searching for target genes for targeted mutagenesis and marker-oriented selection of potato varieties with valuable agricultural characteristics.
Key words: potato; *Solanum tuberosum*; ANDSystem; text mining; knowledge base; automatic extraction of knowledge from texts; prioritization of genes; associative gene networks.

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Приоритизация генов картофеля, вовлеченных в формирование селекционно-значимых признаков, с использованием базы знаний SOLANUM TUBEROSUM

П.С. Деменков[1, 2] ✉, О.В. Сайк[1], Т.В. Иванисенко[1], Н.А. Колчанов[1], А.В. Кочетов[1], В.А. Иванисенко[1]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
✉ e-mail: demps@bionet.nsc.ru

Развитие высокоэффективных технологий в геномике, транскриптомике, протеомике и метаболомике, а также новых технологий в сельском хозяйстве привело к «информационному взрыву» в биологии растений и растениеводстве, включая производство картофеля. Лишь небольшая часть информации попадает в формализованные базы данных (например, Uniprot, NCBI Gene, BioGRID, IntAct и др.). Один из основных источников достоверных биологических данных – научная литература. Объем литературы велик, так, известная база данных PubMed содержит более 18 тыс. тезисов статей, посвященных картофелю. Эффективное получение знаний, представленных в таком количестве неформализованных текстовых документов, требует применения современных интеллектуальных методов анализа. Однако в литературе нет свидетельств широкого применения интеллектуальных методов автоматического извлечения знаний из научных публика-

ций по таким культурам, как картофель. В рамках настоящей работы использовалась разработанная нами ранее база знаний SOLANUM TUBEROSUM (http://www-bionet.sysbio.cytogen.ru/and/plant/). Интегрированная в базе знаний информация о молекулярно-генетических механизмах, лежащих в основе селекционно-значимых признаков, способствует ускорению идентификации генов-кандидатов для селекционно-значимых характеристик картофеля и разработки диагностических маркеров для селекции. В статье выполнен поиск новых потенциальных участников молекулярно-генетических механизмов устойчивости к неблагоприятным факторам у растений. Приоритизация генов-кандидатов показала, что гены *PHYA*, *GF14*, *CNIH1*, *RCI1A*, *ABI5*, *CPK1*, *RGS1*, *NHL3*, *GRF8* и *CYP21-4* наиболее перспективны для дальнейшей проверки их связей с устойчивостью к неблагоприятным факторам. В результате проведенного анализа выявлено, что молекулярно-генетические взаимоотношения, ответственные за формирование значимых сельскохозяйственных признаков, являются комплексными и включают множество как прямых, так и опосредованных взаимодействий. Построение ассоциативных генных сетей и их анализ с использованием базы знаний SOLANUM TUBEROSUM – это основа поиска генов-мишеней для направленного мутагенеза и маркер-ориентированной селекции сортов картофеля, обладающих ценными сельскохозяйственными признаками.
Ключевые слова: картофель; *Solanum tuberosum*; ANDSystem; анализ текстов; база данных; методы автоматического извлечения знаний из текстов; приоритизация генов; ассоциативные генные сети.

Introduction

Potatoes (*Solanum tuberosum* L.) have high nutritional, technical, feed value and are one of the most important crops. The high nutritional value of potatoes is achieved due to the high content of carbohydrates, ascorbic acid, salts of potassium, calcium, magnesium and other trace elements, as well as good digestibility of proteins. Potato starch is a raw material used in the production of alcohol, molasses, dextrins, glucose, maltose, as well as many other products for the chemical industry (Khlestkin et al., 2018). Potato tuber starch is widely used in the paper, textile and other industries (Kraak, 1992; Ellis et al., 1998; Jobling, 2004).

The development of high-performance technologies in the field of genomics, transcriptomics, proteomics, and metabolomics, as well as in agriculture led to an "information explosion" in the plant biology. At the same time, only a small amount of information gets into the formalized factographic databases (for example, NCBI Gene, UniProt, IntAct, BioGRID, etc.). One of the main sources of reliable biological data is scientific literature. The well-known PubMed database contains more than 18000 abstracts of articles devoted to potatoes, which makes the manual analysis of such data extremely difficult for researchers.

The lack of unified resources that integrate all available information leads to a strong complication of tasks related to the identification of relationships between the data sets that describing the important and practically useful properties of plants, their structure and the processes on the molecular level (Khlestkin et al., 2017). Thus, the efficiency of the use of obtained results decreases as well.

The problem of the processing of large and extra-large amounts of data is becoming increasingly common in various areas of human activity (Kilicoglu, 2017), making the methods of automated text analysis (text-mining) more and more popular. These methods can be divided into two main groups: methods based on manually created semantic rules and templates, and methods which are using machine learning approaches. Methods based on semantic rules and templates normally have a high level of accuracy, but the completeness of the extracted information often is relatively low (Aggarwal, 2012). Another approach for automatic information retrieval is the use of machine learning techniques. These methods do not require manually created rules and are widely used recently. At the same time, one of the main disadvantages of such methods is the need for extensive training sets, which are often impossible to obtain without manual analysis.

The text-mining methods found a wide use in the dealing with various problems of biomedicine, systems and integrative biology (Friedman et al., 1999; Meystre et al., 2008; Cao et al., 2011; Shetty, Dalal, 2011; Rebholz-Schuhmann et al., 2012; Li et al., 2013; Wei et al., 2013; Sarker et al., 2015).

At the same time, most of the published scientific literature contains information regarding the application of text-mining approaches only to the model plants. For example, the PLAN2L system (Krallinger et al., 2009) contains results of the automatic extraction of information from protein-protein interactions and genetic regulation from the full-text articles dedicated to *Arabidopsis thaliana*, as well as some data describing associations of genes with some cellular and developmental processes (flower, root, etc.). Da Costa and colleagues (2018) developed an interactive system that allows identification of pests and diseases of rice based on information obtained from farmers in the form of short textual messages (SMS).

Previously, we developed a computer platform for integrated intellectual analysis of scientific publications in the field of potato growing – the SOLANUM TUBEROSUM knowledge base, available at http://www-bionet.sysbio.cytogen.ru/and/plant/ (Saik et al., 2017; Ivanisenko et al., 2018). The software of this platform provides the automatic extraction and formalized representation of information in the base of knowledge, including the genetics data, DNA markers, breeding, seed production, diagnosis of diseases, methods of protection and potato storage technologies. The developed graphical interface to the SOLANUM TUBEROSUM knowledge base provides user access to the data, execution of user-specified queries and visualization of obtained results. Automated analysis of texts was carried out by using the adapted methods of the ANDSystem tool (Demenkov et al., 2012; Ivanisenko et al., 2015; Saik et al., 2016).

The integration of knowledge about the molecular-genetic mechanisms underlying inside the significant for breeding traits can help to accelerate the identification of candidate genes essential for the important breeding characteristics of potatoes, as well as the development of diagnostic markers for breeding.

At present, prioritization methods are widely used in bioinformatics to identify candidate-genes which are potentially involved in the trait and/or biological process

(Chen et al., 2009). Analysis of gene networks is one of such approaches. Previously, we developed criteria for the prioritization of genes, based on the analysis of the structure of the associative gene networks of ANDSystem (Saik et al., 2018; Yankina et al., 2018). In this work, the prioritization of genes was aimed at identifying promising candidates to study their relationship with resistance to adverse factors.

Materials and methods

The SOLANUM TUBEROSUM knowledge base is available at http://www-bionet.sscc.ru/and/plant/. The base consists of three main modules.

The text-mining module is used for the extraction of information about the interactions between objects from the texts of scientific publications. The module is based on the ANDSystem software tool (Ivanisenko et al., 2015). The ANDSystem provides a multi-stage text analysis, consisting of preprocessing of texts, retrieval of information describing the relationships between the objects based on the semantic-linguistic templates, and the presenting of the results in a formalized form. The current version of ANDSystem works only with English texts. In addition to the text analysis tools, ANDSystem also contains tools for the collecting and integrating of information from the external factographic databases.

The module of the SOLANUM TUBEROSUM database consists of the two sections: Dictionary (dictionaries of objects and terms) and Associative networks (information about the relationships between objects and terms).

The Dictionary section includes:

• molecular genetic data for potatoes and model plants (genes, proteins, metabolites, miRNA, biological processes);
• genetic biomarkers;
• potato varieties;
• properties significant for breeding, economically valuable traits and consumer properties of potatoes and model plants;
• physiological, phenotypic traits and diseases of potatoes;
• molecular genetic data on pathogens and potato pests (genes, proteins, metabolites, biological processes);
• genetic markers of resistance to plant protection products;
• molecular targets for plant protection chemicals;
• biotic environmental factors;
• abiotic environmental factors (soil, humidity, temperature, light, air, climate, and microclimate, etc.);
• methods and technologies:
– breeding;
– diagnosis of diseas;
– protection against diseases;
– cultivation, processing, and storage of potatoes.

The Associative networks section contains:

• physical interactions (molecular complexes protein/protein, protein/ligand, protein/DNA);
• chemical interactions (catalytic reactions and processes) such as a substrate-enzyme-product;
• regulatory interactions and associations (regulation of gene expression, regulation of protein activity, gene/traits association, etc.);
• the interactions between the terms of breeding, phenomics and seed production, diseases, diagnostic techniques and methods of protection.

The module of visualization and bioinformatics is used for interactive construction of associative gene networks and their analysis using bioinformatics methods.

Associative gene networks were reconstructed using the ANDVisio program (Demenkov et al., 2012) using information from the SOLANUM TUBEROSUM knowledge base (Saik et al., 2017; Ivanisenko et al., 2018).

Gene prioritization was carried out on the basis of the cross-talk centrality index (CTC), calculated using the ANDVisio program's Intelligent Filtration function using the formula:

$$CTC_j = N_j/M,$$

where N_j is the number of links of the j-th gene/protein with the participants of the associative gene network; M is the number of vertices of the associative gene network (Yankina et al., 2018). When ranking candidate genes, the sorting was performed in descending order of the CTC value. Thus, the genes with the highest CTC score receive the highest priority.

Results

Using the information from the SOLANUM TUBEROSUM knowledge base, we performed the reconstruction and analysis of associative gene networks describing biological processes involved in the formation of selective agricultural traits, such as resistance to adverse environmental factors, response to various stresses (excess salt, cold, drought, high temperature). The reconstructed associative gene network of resistance to adverse factors is provided in Figure. The network includes 542 genes, 544 proteins, 34 biological processes and 2406 interactions between them.

Table 1 contains the list of biological processes that are responsible for resistance to adverse factors in potatoes. From the table, it can be seen that the largest number of genes and proteins is associated with the "response to oxidative stress" Gene Ontology process (Gene Ontology identifier – GO:0006979). In plants, the oxidative stress is observed under the majority of unfavorable environmental factors, including the cold, drought, soil salinization, high temperatures and pathogens (Mittler, 2002; Ramirez et al., 2018).

A number of studies for potatoes discussing the possibilities for creating the plant lines resistant to various adverse environmental conditions, which can be obtained by modifying the biological processes presented in Table 1 have already been conducted (Jones et al., 2014; Kikuchi et al., 2015). For example, it was shown that transgenic potatoes in which the Cu- and Zn-superoxide dismutase genes of tomato were expressed had increased resistance to oxidative (Perl et al., 1993), as well as to cold and salt (Shafi et al., 2017) stresses. P. Monneveux et al. (2013) discussed the relationship of 14 potato genes to drought tolerance and the possibilities of their use for the development of transgenic plants. The relationship between the *ACS4*, *ACS5* potato genes and the response to biotic stress has been studied by C.D. Schlagnhaufer (1997). The creation of transgenic potato lines with different levels of expression of the *PHYB* gene can open up the possibilities to study the mechanisms of resistance of potatoes to the effects of elevated temperatures (Trapero-Mozos et al., 2018).

The prioritization of genes carried out using the CTC (cross-talk centrality) index, allowed to identify candidate genes which are most promising for further study of their relationship

with resistance to adverse environmental factors, as well as response to various stresses. Table 2 contains the top 10 of such candidate genes, ranked according to the values of the CTC indicator, which reflects the degree of gene connectivity in the gene network presented in Figure.

From Table 2 it can be seen that the first place belongs to the *PHYA* gene that encodes the photoreceptor phytochrome A participating in various biological processes, including the control of the circadian rhythm, flowering and leaf movements in response to exposure to light with different wavelengths (Yanovsky et al., 2000). R.J. Sawers et al. (2005) discussed in their paper the use of phytochromes in crop breeding programs for developing varieties resistant to negative growth factors under thickened sowing conditions. Other examples of studies of the effects of phytochrome mutations on plant phenotypes are the works performed by (Chen et al., 2013; Zhang et al., 2013). Thus, J. Zhang et al. (2013) demonstrated the effect of *phyB* mutations in *A. thaliana* on a number of plant phenotypic traits, while J. Chen et al. (2013) showed that loss of PHYC functional activity in wheat could lead to changes in the circadian rhythm and a sharp delay in flowering during the long daylight hours.

The second, fourth and ninth places belong to the genes from the 14-3-3-like proteins family (*GF14*, *RCI1A*, and *GRF8*, respectively). These proteins regulate the cell cycle, apoptosis, immune processes, nitrogen and carbon metabolism, and are involved in the regulation of starch synthesis, ATP production, detoxification by peroxide and in some other biochemical pathways. Also, the plant development and seed germination are controlled by factors which are activated by interacting with 14-3-3-like proteins (Fulgosi et al., 2002). Świędrych et al. (2002) showed that decrease in the level of GF14 protein leads to an increase in calcium, starch and an increase in the ratio of soluble sugars to starch in potato tubers, as well as to the significant increase of methionine, proline, and arginine in potato cells. It was shown that the suppression of *GF14e* gene expression by the RNA interference method could lead to the increased resistance of rice to the virulent strain of the *Xanthomonas oryzae* pv. *oryzae* (Xoo) bacterial phytopathogen (Manosalva et al., 2011). It is known that plants with mutations in the *RCI1A* and *GRF8* genes are having increased resistance to the low temperatures (Catalá et al., 2014; Liu et al., 2017).

Table 1. Top 10 biological processes that provide resistance to adverse environmental factors, ordered according to the number of associated genes

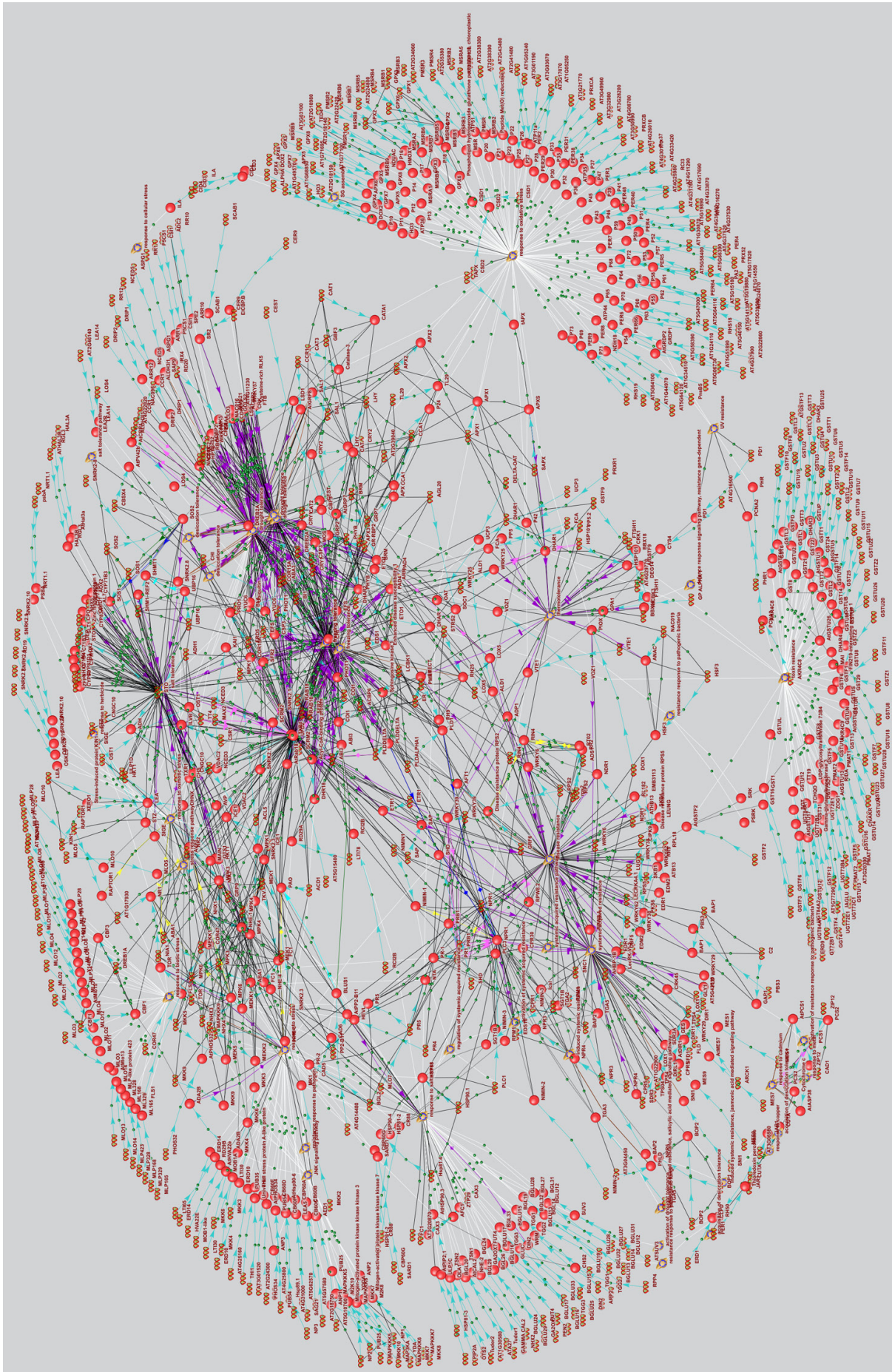
Name of the Gene Ontology biological process	Gene Ontology ID number	Genes/ proteins
Response to oxidative stress	GO:0006979	118
Response to biotic stimulus	GO:0009607	71
Response to toxic substance	GO:0009636	68
Obsolete drought tolerance	GO:0009633	62
Response to salt stress	GO:0009651	49
Obsolete disease resistance	GO:0009614	39
Response to cold	GO:0009409	29
Systemic acquired resistance	GO:0009627	26
Response to osmotic stress	GO:0006970	23
Heat acclimation	GO:0010286	14

The third place was given to the *CNIH1* gene, which encodes the plants protein interacting with the sodium transporter HKT1 and providing the correct location of the transporter on the Golgi apparatus membrane (Rosas-Santiago et al., 2017). In the work of M.M. Wudick et al. (2018), the effect of mutations in the *CNIH1* gene on pollen and calcium homeostasis in *A. thaliana* was studied. It is interesting to note that potato cultivar Yubiley Zhukova, which has enhanced salt and drought tolerance, was obtained due to the overexpression of the vacuolar Na^+/H^+ antiporter NHX2 (Belyaev et al., 2011).

The homolog of the transcription factor ABI5 bZIP-type (*ABI5*) was ranked fifth. ABI5 plays an important role in seed germination, which is regulated by abscisic acid

Table 2. The top 10 high-priority candidate genes potentially involved in resistance to adverse factors

Rank	Gene	Name of protein	Cross-talk centrality
1	*PHYA*	Phytochrome A	0.011514615
2	*GF14*	14-3-3-like protein	0.011514615
3	*CNIH1*	Antiporter CNIH1	0.011514615
4	*RCI1A*	14-3-3-like protein GF14 psi	0.010628875
5	*ABI5*	ABI5-bZIP transcription factor ABI5 homolog	0.009743136
6	*CPK1*	Calcium-dependent protein kinase 1	0.008857396
7	*RGS1*	Regulator of G-protein signaling 1	0.008857396
8	*NHL3*	NDR1/HIN1-like protein 3	0.008857396
9	*GRF8*	14-3-3-like protein GF14 kappa	0.008857396
10	*CYP21-4*	Peptidyl-prolyl cis-trans isomerase CYP21-4	0.007971656



Associative gene network of resistance to adverse factors in potato.
Genes are presented by spirals, proteins by red balls, biological processes by brown ovals, and the interaction by lines.

(Finkelstein, 1994; Lopez-Molina et al., 2002). The ABI5 bZIP-type transcription factor is involved in the activation of genes responsible for the accumulation of proteins during seed development. It is known that the reduction of the expression of the *ABI5* gene activates the meristem growth (Lopez-Molina et al., 2002). Mutations of the *ABI5* gene in *A. thaliana* are associated with reduced sensitivity to abscisic acid, as well as to salt and osmotic stress during the germination (Finkelstein, Lynch, 2000; Carles et al., 2002; Tezuka et al., 2013).

In sixth place was the *CPK1* gene, it encodes a calcium-dependent protein kinase C, which is involved in the immune response, resistance to fungal diseases and pathogens (Gravino et al., 2015). Mutations of *CPK1* gene in the *A. thaliana* are known to cause hypersensitivity to salt stress and drought, while transgenic plant lines with increased expression of *CPK1* showed significant resistance to salt stress and drought (Huang et al., 2018).

The seventh place was taken by the *RGS1* gene, which encodes the negative regulator of the signaling pathway of G-protein type 1. It is known that the expression of this gene decreases in response to water deficiency (Campbell et al., 2012). A. Chen et al. (2006) showed for *A. thaliana* that transgenic plants with over-expressed *RGS1* gene have an increased drought tolerance.

The *NHL3* gene appeared to be in the eighth place; this gene encodes NDR1/HIN1-like protein 3, which is involved in response to pathogens (Chong et al., 2008). The *A. thaliana* transgenic line, in which increased expression of the *NHL3* gene was observed, showed the increased resistance to the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Varet et al., 2003).

The tenth place in Table 2 was taken by the *CYP21-4* gene, encoding cyclophilin, localized in the Golgi apparatus, which is involved in tolerance to oxidative stress (Park et al., 2017). The authors consider the over-expression of the *CYP21-4* gene in crops as a new promising way to increase the productivity of plants. For potatoes and rice, it has been shown that transgenic plants in which the *CYP21-4* gene is over-expressed have increased yield, the stems and roots of the plants are longer, and the leaves are thicker. Also, such potatoes produced a bigger number of tubers of a larger size, and the microtubers were formed faster than in wild-type plants (Park et al., 2017).

Conclusion

In the current work, a search for new potential participants of molecular genetic mechanisms of resistance to adverse factors in plants was carried out. Prioritization of candidate genes has shown that the *PHYA*, *GF14*, *CNIH1*, *RCI1A*, *ABI5*, *CPK1*, *RGS1*, *NHL3*, *GRF8* and *CYP21-4* genes are the most promising for the further study of their relationship with resistance to adverse factors. The performed analysis reviled that the molecular-genetic relationships responsible for the formation of significant agricultural traits are complex and include many direct and indirect interactions. The representation of these interactions in the form of associative gene networks and their analysis using the SOLANUM TUBEROSUM knowledge base can be the basis for the search for target genes important for targeted mutagenesis and marker-oriented selection of potato varieties resistant to adverse environmental factors.

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ORCID ID

A.V. Kochetov orcid.org/0000-0003-3151-5181

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Оптимизация параметров качества зерна для селекции озимой ржи

М.Л. Пономарева[1, 2] ✉, С.Н. Пономарев[1]

[1] Татарский научно-исследовательский институт сельского хозяйства – обособленное структурное подразделение Федерального исследовательского центра «Казанский научный центр Российской академии наук», Казань, Россия
[2] Казанский (Приволжский) федеральный университет, Казань, Россия
✉ e-mail: smponomarev@yandex.ru

Цель настоящей работы – оценить фенотипическую изменчивость показателей качества зерна и выявить комплекс наиболее информативных признаков для селекции разнообразных по направлению использования сортов озимой ржи. Исследования выполнены в Татарском научно-исследовательском институте сельского хозяйства – обособленном структурном подразделении Федерального исследовательского центра «Казанский научный центр Российской академии наук» в 2001–2015 гг. на 15 сортах озимой ржи. Оценены 20 параметров, определяющих качество зерна и сырьевую ценность: технологические показатели (масса 1000 зерен, натурная масса и выравненность зерна), параметры устойчивости к прорастанию зерна (число падения, высота амилограммы, температура пика клейстеризации крахмала), кинематическая вязкость водного экстракта (ВВЭ) зернового шрота, хлебопекарные свойства (органолептическая оценка пробной выпечки хлебцев). Наибольшее влияние генотипа выявлено по ВВЭ (34.8 %) и содержанию белка (27.8 %). Эти признаки должны быть в первую очередь предметом фенотипической оценки в селекционном процессе. Фенотипическая изменчивость критериев углеводно-амилазного комплекса и активности α-амилаз и технологических параметров определялась преимущественно (68.6 … 82.5 %) средовыми факторами. Между числом падения (ЧП) и содержанием белка отсутствовала значимая связь. ЧП существенно коррелировало с высотой амилограммы и температурой пика клейстеризации. Выявлена положительная сопряженность средней силы ВВЭ с высотой амилограммы и ЧП. На основании многолетней фенотипической оценки с помощью метода главных компонент проведена оптимизация анализируемых показателей качества зерна озимой ржи. Для селекционной оценки озимой ржи предлагается использовать четыре интегральных показателя, имеющих наибольшую весовую нагрузку: содержание белка, число падения, вязкость водного экстракта и масса 1000 зерен. Этот комплекс признаков будет обеспечивать объективность и полноту оценки создаваемого селекционного материала. Показано, что кинематическая вязкость водного экстракта должна стать важным селекционным показателем как для определения хлебопекарных качеств ржи, так и для выявления кормовых достоинств зерна.
Ключевые слова: озимая рожь; качество зерна; число падения; вязкость водного экстракта; белок; амилограмма; метод главных компонент.

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Optimization of grain quality parameters for winter rye breeding

M.L. Ponomareva[1, 2] ✉, S.N. Ponomarev[1]

[1] Tatar Scientific Research Institute of Agriculture – Subdivision of the "Kazan Scientific Center of Russian Academy of Sciences", Kazan, Russia
[2] Kazan (Volga region) Federal University, Kazan, Russia
✉ e-mail: smponomarev@yandex.ru

The purpose of this work was to evaluate the phenotypic variability of grain quality indicators and to identify the set of the most informative indicators for the selection of different use varieties of winter rye. The research was carried out in Tatar Scientific Research Institute of Agriculture – Subdivision of the "Kazan Scientific Center of Russian Academy of Sciences" in 2001–2015 on 15 varieties of winter rye. Twenty parameters defining quality of grain and raw value were estimated: technological indicators (thousand grain mass, full-scale weight and grain uniformity), parameters preharvest sprouting (falling number, rate of amylogram, temperature of peak of starch gelatinization), kinematic water extract viscosity (WEV) of grain meal, baking properties (organoleptic assessment of trial baking bread). The greatest influence of genotype was found on WEV (34.8 %) and protein content (27.8 %). These features should be primarily the subject of phenotypic evaluation in the breeding process. Phenotypic variability of criteria of carbohydrate-amylase complex, α-amylases activity and technological parameters was determined predominantly (68.6 … 82.5 %) by environmental factors. There was no significant relationship between falling number and protein content. The falling number correlated with the rate of amylogram and the temperature of gelatinization. Positive conjugation of the average power of WEV with the rate of amylogram and falling number was found. On the basis of a prolonged phenotypic evaluation using principal component analysis we have optimized the analyzed indicators of quality properties of winter rye grain. For breeding evaluation of winter rye it is proposed to use four integral indi-

cators having the greatest weight load: protein content, falling number, water extract viscosity and thousand grain mass. This complex of features will provide objectivity and completeness of the evaluation of the breeding material. It was shown that the kinematic viscosity of the water extract should become an important selection index, both for determining the baking qualities of rye, and for revealing the fodder grain advantages.
Key words: winter rye; grain quality; falling number; water extract viscosity; protein; amylogram; principal component analysis.

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Введение

Мировое производство ржи в 2016 г. составило 13 млн тонн с площади посева 5.4 млн га. Основными районами возделывания этой культуры остаются умеренно холодные и малоплодородные зоны Центральной и Восточной Европы. Р. Шлегель утверждает, что рожь является преимущественно культурой европейского континента, поскольку на его долю приходится 85 % производимого зерна (Schlegel, 2013).

Россия – одно из крупнейших государств мира, занимающее второе место по производству зерна ржи после Германии. В нашей стране рожь занимает пятое место по объему производства зерна (2.0 %). За последние четыре десятилетия посевные площади озимой ржи в Российской Федерации сократились в шесть раз (с 7.75 млн га в 1970 г. до 1.25 млн га в 2016 г.). Негативный процесс привел к тому, что стал наблюдаться спад товарного предложения зерна ржи на рынке в связи со снижением объемов его производства как в Российской Федерации в целом, так и в отдельных регионах страны. Регрессионный анализ показал, что средняя ежегодная потеря валового сбора зерна этой культуры составляла 342 тыс. тонн. Главной причиной подобной ситуации был низкий спрос на зерно со стороны потребителей в связи с узкой сферой использования ржи (Пономарева и др., 2014).

Современное состояние производства ржи характеризуется сложностью и противоречивостью. Несмотря на нежелательные тенденции, происходящие в ржесеянии, Россия продолжает занимать лидирующие позиции как по площади посева, так и по валовому сбору зерна.

В связи с этим важной задачей селекции озимой ржи является комплексное изучение качественных свойств зерна для создания сортов хлебопекарного направления, производства кормов для животных, спирта и сырья для глубокой переработки (Гончаренко, 2014).

По мнению А.И. Алтухова (2012), рыночный спрос формируется не на продовольственное зерно в целом, а на партии разного качества и целевого использования. Согласно критериям ЕС, ржаное зерно обладает хлебопекарными качествами, если число падения более 120 с, максимальная вязкость, оцениваемая по высоте амилограммы больше 200 е. а. и максимальная температура пика клейстеризации больше 63 °С (Muenzing et al., 2014). Доля ржи, соответствующая указанным выше нормативам, варьировала от 25 до 100 % в течение 1992–2014 гг., составила в среднем 80 %. Это обусловлено тем, что качество ржи очень отличается по годам (Bruemmer, 2005; Kucerova, 2009).

В отличие от пшеницы, где ключевую роль в определении качества играют концентрация белка, количество и свойства клейковины, для ржи наиболее важные характеристики – содержание пентозанов и крахмала, а также активность фермента альфа-амилазы.

За последние 20 лет все больше исследователей стали уделять особое внимание наряду с традиционным изучением процесса деградации крахмала под действием эндогенных ферментов зерна количественной оценке содержания пентозанов (высокомолекулярных арабиноксиланов), их состоянию (водопоглощение, вязкость и растворимость), а также их деятельности и последующих модификациях (Bengtsson et al., 1992; Nowotna et al., 2007).

Качество ржаной муки, используемой для хлебопечения, зависит от трех главных факторов: 1) количества и функциональных свойств арабиноксиланов; 2) особенностей крахмала; 3) присутствия и концентрации ферментов, используемых для гидролиза.

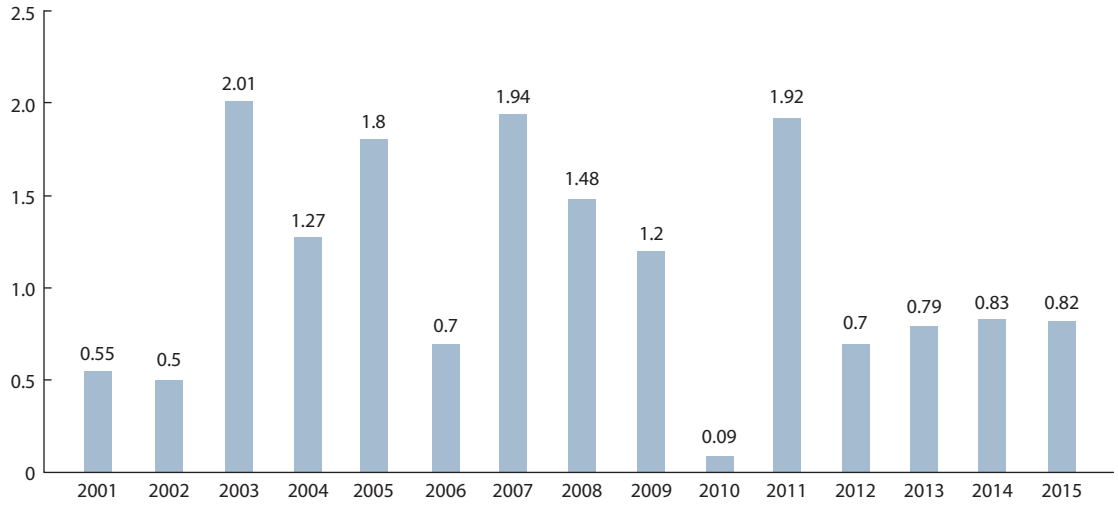
Многочисленными исследованиями подтверждено, что положительный эффект от общего содержания пентозанов и их водорастворимой фракции выражается в улучшении реологических показателей ржаного теста в процессе выпечки хлеба (D'Appolonia, 1973; Delcour,1995; Weipert, 1995).

Несмотря на то, что биохимические исследования зерна ржи довольно широко ведутся в нашей стране и за рубежом, многие вопросы селекционной направленности разработаны слабо. При этом возникает необходимость развития комплексных исследований селекционного плана, в частности методических подходов к созданию сортов с высоким качеством зерна целевого назначения.

Цель настоящей работы – оценить фенотипическую изменчивость показателей качества зерна и выявить комплекс наиболее информативных показателей для селекции разнообразных по направлению использования сортов озимой ржи.

Материалы и методы

Работа выполнена в Татарском научно-исследовательском институте сельского хозяйства – обособленном структурном подразделении Федерального исследовательского центра «Казанский научный центр Российской академии наук» (ТатНИИСХ ФИЦ КазНЦ РАН) в 2001–2015 гг. Опытные поля находятся в Лаишевском районе (55°38′60″ с. ш., 49°18′25″ в. д.) Республики Татарстан, находящейся на стыке Центральной России и Поволжья по среднему течению р. Волги и нижнему течению р. Камы. Агрохимические показатели селекционного севооборота являются типичными для среднесуглинистой серой лесной почвы. Сумма активных температур в зоне исследования колеблется в пределах 1700–2000°. Период активной вегетации растений равен 130–



Hydrothermal coefficient during the development and ripening of winter rye grain (June-July), 2001–2015.

135 дням. Среднемноголетнее количество осадков за год составляет 430–500 мм, за период май-сентябрь – 290–300 мм, но оно крайне неравномерно. Средняя высота снежного покрова за зиму составляет 35–45 см. Глубина промерзания почвы колеблется от 38 до 180 см.

Гидротермический коэффициент, характеризующий степень увлажнения территории в период с момента образования зерновки до полной спелости за 2001–2015 гг., показан на рисунке.

Из приведенных данных видно, что в наиболее ответственный период формирования качественных характеристик зерна складывались разнообразные условия: крайне засушливые (2002, 2010), сильно засушливые (2001, 2006, 2012), умеренно засушливые (2013, 2014, 2015), слабо засушливые (2009), влажные (2004, 2008) и избыточно влажные (2003, 2005, 2008, 2011) годы.

Для анализа было использовано 15 сортов озимой ржи конкурсного сортоиспытания, среди которых 9 – собственной селекции (Татарская 1, Эстафета Татарстана, Радонь, Огонек, Тантана, Спутник, перспективные популяции 8, 9, 10) и сорта других селекционных учреждений России (Антарес, Безенчукская 87, Саратовская 7, Марусенька, Татьяна, Роксана). Полевые эксперименты проведены на делянках 20 м^2, в четырех повторениях.

Всего оценивалось 20 параметров, определяющих качество зерна и сырьевую ценность. Технологические (масса 1000 зерен (МТЗ), натурная масса зерна (НЗ) и выравненность зерна (ВЗ)) и хлебопекарные свойства (органолептическая оценка пробной выпечки хлебцев) изучены по соответствующим методикам, принятым для государственного сортоиспытания сельскохозяйственных культур (Методика…, 1988). У всех сортов исследовались параметры устойчивости к прорастанию зерна (число падения (ЧП), высота амилограммы (ВА), температура пика клейстеризации крахмала (ТПК)).

Размол зерна осуществляли на лабораторной мельнице Perten Instruments Laboratory Mill 3100. Для помола использована средняя проба зерна массой 300 г. Число падения определяли на приборе Falling Number 1500 (Hagberg-Perten) в соответствии с требованиями международного стандарта ISO 3093 (2009), максимальную вязкость суспензии и температуру клейстеризации – на амилографе Brabender (ГОСТ ISO 7973-2013). Определение массовой доли белка (Б) проводили по методу Кьельдаля (ICC 167).

Вязкость водного экстракта (ВВЭ) ржаного шрота (кинематическая вязкость) оценивалась вискозиметрическим методом на приборе ВПЖ 1, согласно методике, опубликованной нами ранее (Пономарева и др., 2017), соотношение шрота и воды было 1:5.

Корреляционный и дисперсионный анализы проведены с использованием пакета Excel 7.0. Критическое значение коэффициента корреляции при 5 % уровне значимости равно 0.444. В качестве математической модели для многомерного анализа применяли метод главных компонент (пакет программ AGROS 2.13), который позволил выделить коррелирующие и взаимозависимые показатели качества зерна и выбрать из них самые информативные, а также снизить их размерность. Для построения биплот графика использована программа XLSTAT 2018.6.54644.

Результаты

Показатели качества зерна обусловлены большой группой сцепленных генов, экспрессия которых подвержена значительному влиянию генетических и средовых факторов, а также их взаимодействию. Согласно полученным данным, доля дисперсии, характеризующая влияние условий года на изменчивость обсуждаемых признаков, очень высока и ранжируется в порядке убывания следующим образом: высота амилограммы – 82.5 %, температура пика клейстеризации – 80.3 %, число падения – 73.7 %, выравненность зерна – 77.7 %, масса 1000 зерен – 77.2 %, натурная масса зерна – 68.6 %, вязкость водного экстракта – 49.0 %, содержание белка – 35.2 % (табл. 1).

Наибольшее влияние генотипа выявлено по ВВЭ и Б – соответственно 34.8 и 27.8 %. Суммарный эффект генотипа и генотип-средовых взаимодействий объяснял 64.8 % общего варьирования содержания белка и 51 % фенотипической изменчивости ВВЭ зернового шрота сортов озимой ржи. Это означает, что среди исследуемых в течение 15 лет

Table 1. Contributions of the genotype, environment and genotype × environment interaction to the variation of quality indices in winter rye grain, 2001-2015

Traits	Factor share, %		
	Genotype	Year	Genotype–year interaction
1000-grain weight	20.2	77.2	2.6
Hectoliter weight	21.8	68.6	9.6
Grain uniformity	15.5	77.7	6.8
Protein content	27.8	35.2	37.0
Amylograph peak viscosity	12.0	82.5	5.5
Temperature at peak viscosity	6.9	80.3	12.8
Falling number	11.2	73.7	15.1
Viscosity water extract	34.8	49.0	16.2

Table 2. Correlation coefficients of grain quality indices in winter rye

Index	TGW	HW	GU	P	APV	TPV	FN	VWE	H/D*	BV**
TGW	1.000	0.757	0.929	–0.480	–0.107	–0.079	–0.315	–0.522	–0.118	–0.210
HW		1.000	0.753	–0.575	0.294	0.416	0.133	–0.141	0.346	–0.336
GU			1.000	–0.504	–0.267	–0.151	–0.404	–0.624	–0.261	–0.116
P				1.000	–0.189	–0.208	0.072	–0.200	–0.098	0.242
APV					1.000	0.835	0.774	0.621	0.856	–0.563
TPV						1.000	0.821	0.386	0.750	–0.397
FN							1.000	0.471	0.774	–0.162
VWE								1.000	–0.211	0.450
H/D*									1.000	–0.449
BV**										1.000

* H/D, ratio of bread height to diameter; ** BV, bread volume.

генотипов имеется значительное сортовое разнообразие как по средней величине названных признаков, так и по конкретным значениям в отдельные годы испытаний.

Для селекционных целей важно знать не только генотипическую и средовую изменчивость, но и взаимосвязь между качественными показателями зерна ржи, выраженную коэффициентами парной корреляции (табл. 2).

Как и следовало ожидать, сопряженная изменчивость признаков МТЗ, НЗ и ВЗ имеет сильную корреляционную зависимость между собой (r = 0.753–0.929). Между ЧП и содержанием белка отсутствовала значимая связь (r = 0.072). ЧП коррелировало с ВА (r = 0.774) и ТПК (r = 0.821). Эти три признака находились в тесной связи с показателем отношения высоты хлеба к его диаметру: коэффициент парной корреляции ЧП с показателем отношения высоты хлеба к его диаметру составил 0.774, у признака «высота амилограммы» – r = 0.856, у признака «температура пика клейстеризации» – r = 0.750.

По годам мы наблюдали значительную вариацию тесноты корреляционной связи между анализируемыми признаками, поскольку их величина в сильной степени зависит от условий среды. Кроме того, при определении числа падения и показаний амилографа имеются существенные методические различия, связанные с продолжительностью анализа и температурой определения вязкости. ВВЭ была взаимосвязана умеренной отрицательной связью с МТЗ (r = –0.522) и ВЗ (r = –0.624).

В селекции сортов целевого использования очень большое значение имеют знания корреляционных отношений между числом падения (основной показатель качества, нормируемый ГОСТом РФ) и такими дополняющими параметрами, как вязкость водно-мучной суспензии, регистрируемая на амилографе, и вязкость водного экстракта зернового шрота, определяемая на вискозиметрах.

Выявлена положительная сопряженность средней силы ВВЭ с высотой амилограммы (r = 0.621) и ЧП (r = 0.471) (см. табл. 2). Однако достоверная связь между ЧП, ВА и ВВЭ зернового шрота прослеживалась только в отдельные годы, либо отсутствовала совсем.

Эффективность селекции озимой ржи на качественные характеристики зерна определяется тем, на каком этапе селекционного процесса и по каким признакам идет отбор в питомниках, поскольку начальным этапом формирования технологических свойств зерна озимой ржи является селекция. Пороговые значения тестируемых показателей и результаты селекции будут на многие годы предопреде-

Table 3. Factor loading values of quality indices in winter rye grain, 2001–2010

Index	Principal component				
	1	2	3	4	5
1000-grain weight, g	0.171	–0.459	0.331	0.287	–0.753
Hectoliter weight, g/l	–0.498	–0.376	0.617	–0.035	–0.478
Grain uniformity, %	–0.010	0.120	0.847	–0.341	–0.389
Protein content, %	0.644	0.442	–0.372	0.383	0.324
Amylograph peak viscosity, e.a.	–0.421	–0.129	0.890	–0.095	–0.073
Temperature at peak viscosity, °C	–0.723	0.197	0.637	–0.097	–0.151
Falling number, s	–0.871	0.101	0.468	–0.108	0.032
Viscosity water extract , cSt	0.176	–0.669	0.679	0.196	–0.147
Bread surface, score	0.122	–0.096	–0.918	–0.362	–0.050
Bread symmetry, score	–0.780	–0.608	–0.018	–0.012	–0.145
Bread appearance, score	0.160	–0.939	–0.008	0.306	0.001
Bread crust color, score	–0.321	–0.850	–0.399	–0.039	–0.120
Crumb color, score	0.436	–0.794	–0.303	0.241	–0.170
Bread porosity, score	–0.214	–0.452	–0.856	–0.027	–0.124
Elasticity, score	0.616	0.025	–0.646	–0.120	–0.434
Bread taste, score	–0.018	0.106	0.049	–0.992	–0.039
Ratio of bread height to diameter, H/D	–0.212	–0.454	0.767	–0.289	–0.277
Bread volume, mm	–0.082	0.958	–0.097	–0.054	0.252
Bread volume, score	0.309	0.922	–0.170	0.087	–0.135
General baking score	0.200	–0.545	–0.407	0.673	–0.208
Variance	3.738	6.175	6.263	2.246	1.577
Variance, %	18.7	30.9	31.3	11.2	7.9
Cumulative variance, %	18.7	49.6	80.9	92.1	100.0

лять качество зерна в соответствии с направлением его использования.

В селекционной работе мы анализировали восемь технологических параметров (включая ВВЭ) и двенадцать хлебопекарных показателей, получаемых при пробной выпечке хлебцев. Последние характеристики отличаются трудоемкостью, требуют значительного количества зерна и имеют некоторую оценочную субъективность, вследствие чего число анализируемых сортообразцов обычно ограниченно. Если хотя бы часть перекрывающихся (избыточных) признаков будет отсеяна, это, несомненно, приведет к оптимизации селекционного процесса на качество зерна. В условиях возрастающего объема селекционного материала и скудности материальных ресурсов выражение множества показателей качества через меньшее их число чрезвычайно важно. Фенотипическая оценка изучаемых показателей качества за годы исследований (средние значения для 15 сортов озимой ржи) приведена в Приложении 1[1].

На основе анализа вычисленной корреляционной матрицы получены вклады, с которыми признаки включены в главные компоненты, т. е. новые характеристики качества. Главные компоненты отражают несколько причин изменчивости качественных характеристик, а их значимость оценивается по доле дисперсии в общей дисперсии признака.

Значения факторных нагрузок на пять главных компонент показаны в табл. 3. Исходя из представленных данных, первая компонента описывала 18.7 % общей дисперсии. Мы назвали ее «белково-амилазной характеристикой зерна». Две следующие главные компоненты переменных имели примерно равные дисперсии: 30.9 и 31.3 %. Для визуализации группы тесно коррелирующих признаков метод главных компонент дополнен биплот анализом взаимосвязей признаков качества в системе двух основных компонент с наибольшей долей в дисперсии (Приложение 2). Для оптимизации оценки качественных характеристик желательно выбрать показатели из разных четвертей графика.

Вторая компонента охватывала признаки, обуславливающие «органолептические свойства хлеба»: внешний вид, симметричность, цвет корки и мякиша, объемные характеристики.

Третья компонента интегрировала в себе мукомольные качества (натурная масса, выравненность) и формоустойчивость хлеба, основанная на вязких свойствах водного

[1] Приложения 1 и 2 см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx6.pdf

экстракта и клейстеризованной суспензии (высота амилограммы и температура максимальной вязкости). Это доказывает, что перечисленные признаки изменяются согласованно. Независимо от генотипа, сорта сходным образом реагируют на изменение внешних факторов, но знаки их факторных нагрузок не одинаковы. Структура первых трех главных компонент оставалась неизменной при варимакс-вращении, что свидетельствует об их взаимной нескоррелированности.

Остальные главные компоненты ранжировались по мере уменьшения описываемых ими дисперсий. На четвертую компоненту приходилось 11.2 % общей дисперсии, где превалировали вкус и общая хлебопекарная оценка. Последнюю компоненту определяла масса 1000 зерен.

Необходимо отметить важность признака «вязкость водного экстракта», который присутствовал в двух компонентах, имеющих значительную долю изменчивости. Отсюда следует, что в селекционных исследованиях показатель ВВЭ может стать базовым, так как является обобщающей характеристикой хлебопекарных свойств зерна, в которой учтены многие слагающие их параметры.

Следующий шаг исследований – выявление наиболее информативных показателей, которые можно применять на самых ранних этапах селекции. По нашему убеждению, ими должны стать: белок, число падения, вязкость водного экстракта, масса 1000 зерен. Эти показатели приняты за основу при оценке и браковке селекционного материала по качеству зерна. Показатели, выявляемые в ходе традиционного хлебопекарного анализа, вполне заменимы легко определяемой вязкостью.

Обсуждение

В результате проведенных исследований сортов озимой ржи различного эколого-географического происхождения на протяжении 15 различающихся лет испытания установлено, что наибольшую генотипическую изменчивость имеют признаки Б и ВВЭ. Варьирование содержания белка на 27.8 % от общей дисперсии признака в исследуемом комплексе определялось генотипом, на 37 % взаимодействием генотип–среда и на 35.2 % условиями года. По вязкости водного экстракта одноименные источники варьирования составили 34.8, 16.2 и 49 % соответственно. Следовательно, названные признаки (Б и ВВЭ) должны быть, в первую очередь, предметом фенотипической оценки в селекционном процессе. Фенотипическая изменчивость критериев углеводно-амилазного комплекса и активности α-амилаз (ЧП, ВА, ТПК) и технологических параметров (МТЗ, ВЗ, НЗ) определялась преимущественно (68.6…82.5 %) средовыми факторами, что значительно превышает влияние генотипа (6.9…21.8 %). В исследовании был взят пятнадцатилетний временной отрезок, в который попали годы, значительно различающиеся по гидротермическому режиму в период созревания зерна (молочная и восковая спелость). Поэтому погодные условия сыграли первостепенную роль в вариабельности качества зерна, а различия между сортами на фоне влияния средовых факторов были относительно невелики.

При исследовании 19 различных гибридных и популяционных сортов, выращенных в течение трех лет, H.B. Hansen с коллегами (2004) обнаружили, что вариация концентрации белка зависела в основном от генотипа, а масса 1000 зерен и содержание пентозанов в большей степени – от года испытания. Авторы выявили также, что свойства крахмала, измеряемые ЧП и ТПК, были сильнее подвержены влиянию условий года сбора урожая. В.М. Бебякин (2008) предлагает пересмотреть подходы к тестированию селекционного материала, поскольку роль углеводно-амилазного комплекса в определении хлебопекарных качеств ржи преувеличена. Согласно проведенным нами исследованиям, высокая средовая вариансa показателей ЧП, ВА и ТПК затрудняет выявление перспективных форм.

Встает резонный вопрос, на какие признаки следует ориентироваться в селекции на целевое использование – хлебопечение или кормовые цели? Обобщение ряда публикаций показывает, что имеются умеренно сильные положительные взаимосвязи между ЧП и ТПК и между ЧП и вязкостью. В то же время не выявляются или существуют слабые корреляции между концентрацией белка и числом падения, а также между максимальной амилографической вязкостью и температурой клейстеризации (Rattunde et al., 1994; Gomez et al., 2009; Laidig et al., 2017). A. Repeckiene с коллегами (2001) обнаружили, что высокие значения ЧП соответствовали высоким значениям вязкости клейстеризованной суспензии ($r = 0.87$). В исследованиях А.А. Гончаренко с коллегами (2002), напротив, показано, что, несмотря на положительную корреляцию ЧП с ВА ($r = 0.56$), содержанием водорастворимых пентозанов ($r = 0.46$) и качеством мякиша формового хлеба ($r = 0.53$), оценивать качество ржаного хлеба только по ЧП можно лишь косвенно. Это обусловлено тем, что хлебопекарные качества зерна ржи и качество конечного продукта (ржаной хлеб) определяются большой группой свойств: вязкость по амилографу и уровень активности амилолитических ферментов отражают особенности ретроградации крахмала, а содержание водорастворимых пентозанов, регистрируемое по ВВЭ, – водопоглотительную способность различных компонентов набухания ржаной муки. При выпечке ржаного теста пористая и эластичная структура мякиша поддерживается благодаря высокой вязкости слизистых веществ, закрепляемых клейстеризованным крахмалом. Изменение крахмала при клейстеризации имеет решающее значение для образования пористого мякиша хлеба. Структура теста, создаваемая главным образом пентозанами, формируется при температуре 30 °С, тогда как роль крахмала превалирует при температурах выше 45 °С, поскольку крахмал ржи образует желеобразную структуру при более низкой температуре, чем крахмал пшеницы (Gudmundsson, Eliasson, 1991). Поэтому особый интерес представляет создание методами селекции сортов ржи с контрастной ВВЭ. Разнонаправленный отбор позволяет изменить биохимическую структуру некрахмальных полисахаридов в соответствии с задачами селекции: 1) увеличить содержание трудногидролизуемых арабиноксиланов, улучшив хлебопекарные свойства, или 2) снизить их долевое участие, улучшив кормовую ценность зерна (Гончаренко, 2014).

ВВЭ не связана с крахмалом ржи, клейстеризационные свойства которого проявляются под влиянием температурного фактора, а также с активностью амилолитических

ферментов, гидролизующих его, поэтому не прослеживается достоверная взаимосвязь между ВВЭ и показателями, определяющими вязкость водно-мучной суспензии (r = 0.03...–0.29) (Гончаренко и др., 2005).

В наших исследованиях выявлена положительная сопряженность средней силы вязкости водного экстракта с высотой амилограммы (r = 0.621) и числом падения (r = 0.471).

Объяснением неодинаковых результатов, полученных разными авторами в отношении ВВЭ, служат несколько причин. Во-первых, взаимосвязи между параметрами технологической и хлебопекарной оценки зерна, муки и факторами среды произрастания неоднозначны. Это связано с тем, что каждый признак формируется под действием множества внешних факторов и сам по себе является интегральной характеристикой группы свойств и признаков, изменение которых может иметь как однонаправленные, так и противоположные векторы, причем значимость их далеко не одинакова. Во-вторых, разные авторы при анализе ВВЭ используют неодинаковые методики и аппаратуру для ее определения, что также отражается на получаемых результатах.

Заключение

С помощью метода главных компонент проведена редукция анализируемых показателей качества зерна. Поиск структуры в матрице нагрузок и использование варимакс-вращения в пространстве главных компонент позволили дать их интерпретацию: белково-амилазная характеристика, органолептические свойства хлеба, мукомольные качества и формоустойчивость, вкус и общая хлебопекарная оценка, масса 1000 зерен. Для селекционной оценки озимой ржи предлагается использовать четыре интегральных показателя, имеющих наибольшую весовую нагрузку: содержание белка, число падения, вязкость водного экстракта и масса 1000 зерен.

Результаты наших исследований показали необходимость обязательной оценки селекционного материала по содержанию белка и вязкости водного экстракта. Чем раньше начинается селекция на эти качественные характеристики, а не только на урожайность, тем результативнее будет работа по созданию высококачественных сортов. Кинематическая вязкость вытяжек из ржаного шрота должна стать важным селекционным показателем как для определения хлебопекарных качеств ржи, так и для выявления кормовых достоинств зерна.

Среди множества направлений в работах по селекции ржи на первый план выдвигается следующее требование – соответствие качества зерна требованиям рынка. Каждому сорту должны быть присущи свои генетически детерминированные технологические свойства, позволяющие выявить его пригодность как сырья для конкретной отрасли. Только селекционная работа позволяет направленно создавать сорта для целевого использования и расширения технологий, связанных с переработкой ржи. Именно в этом нам видится реновация ржи – переоткрытие ржи с новых позиций, что должно привести к изменениям в отношении к культуре в целом и ее производству.

На основании многолетней фенотипической оценки проведена оптимизация показателей качества зерна озимой ржи и обоснован полноценный комплекс признаков (белок, число падения, вязкость водного экстракта, масса 1000 зерен), который будет обеспечивать объективность и полноту оценки создаваемого селекционного материала.

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ORCID ID

M.L. Ponomareva orcid.org/0000-0002-1648-3938
S.N. Ponomarev orcid.org/0000-0001-8898-4435

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Направленная селекция психрофильного штамма *Trichoderma asperellum* Г-034 ВИЗР для ускоренной утилизации полимеров растительных остатков и оздоровления почвы

И.И. Новикова ✉, Ю.А. Титова ✉, И.В. Бойкова, И.Л. Краснобаева

Всероссийский научно-исследовательский институт защиты растений, Пушкин, Санкт-Петербург, Россия
✉ e-mail: irina_novikova@inbox.ru; juli1958@yandex.ru

Штаммы рода *Trichoderma* – природные биодеструкторы растительных остатков, высокоактивные антагонисты почвенных фитопатогенов и фиторегуляторы с широчайшим диапазоном оптимальных для своего развития условий, масштабно используются в создании биопрепаратов. Огромное значение в северных регионах России, особенно при выращивании озимых культур, имеет способность штамма микроорганизма, используемого в агротехнологиях, сохранять жизнеспособность и целевую биологическую активность при низких температурах. В связи с этим цель данной работы – отбор психротолерантного штамма *T. asperellum* для ускоренной утилизации основных полимеров растительных остатков и оздоровления почвы при низкой температуре, а также оценка его активности в лабораторных и полевых условиях. В процессе работы решали задачи по отбору психротолерантных штаммов *T. asperellum* с высокой целлюлозолитической активностью; дальнейшей направленной селекции психрофильных штаммов, способных к быстрому росту, активной колонизации растительного субстрата и высокой споропродуктивности при 4–8 °С; оценке целевой активности отселектированного психрофильного штамма в качестве целлюлозолитика и антагонистической активности в отношении фитопатогенов зерновых культур; получению лабораторных образцов препаративных форм путем глубинно-поверхностного культивирования на нестерильном торфе и мультиконверсионных отходах производства съедобных грибов при их последовательном культивировании на одном и том же субстрате и оценке их эффективности в полевых мелкоделяночных опытах. Использовали методы культивирования и создания инокулюма, определения споропродуктивности, модифицированной влажной камеры, оценки антагонистической активности и качества биопрепаратов, организации полевых испытаний, количественных оценок потерь биомассы и содержания целлюлозы и лигнина. Селекцию активного психрофильного штамма для ускоренной утилизации полимеров растительных остатков и оздоровления почвы осуществили в процессе четырехступенчатого скрининга 29 штаммов *T. asperellum* из Государственной коллекции микроорганизмов ФГБНУ Всероссийский НИИ защиты растений (ВИЗР) с высокими целлюлозолитической и антагонистической активностями. По показателям линейной скорости роста, антагонистической и гиперпаразитической активности при 4–8 °С, высокой скорости колонизации пожнивных остатков пшеницы и кукурузы отобран перспективный психрофильный штамм *T. asperellum* Г-034 для наработки на его основе лабораторных образцов биопрепаратов и проведения полевых опытов. В полевых испытаниях выявили активное разложение пожнивных остатков кукурузы под воздействием *T. asperellum* Г-034, приводящее к биодеструкции более 80 % целлюлозы и более 20 % лигнина, а за 12 месяцев – к полной потере растительными остатками интактного состояния. Максимальные потери биомассы пожнивными остатками кукурузы за 12 месяцев составили более 70 %. Штамм-продуцент *T. asperellum* Г-034 находился в активном состоянии после перезимовки в полевых условиях в количестве $\times 10^4$ КОЕ/г, приводящем к нарастанию титра с сезонными возрастанием температуры и расширением доступности трофической базы.

Ключевые слова: *Trichoderma asperellum* Г-034 ВИЗР; направленная селекция; психротолерантный штамм-продуцент; психрофильные штаммы-продуценты; мультиконверсионные биопрепараты; биопрепараты для оздоровления почвы; биодеструкторы пожнивных остатков; целевая активность штаммов-продуцентов; эффективность биопрепаратов; разложение растительных остатков.

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Controlled breeding of the psychrophilic strain G-034 VIZR of *Trichoderma asperellum* for fast crop residues' polymers utilization and soil enhancement

I.I. Novikova ✉, J.A. Titova ✉, I.V. Boykova, I.L. Krasnobaeva

All-Russian Research Institute of Plant Protection, Pushkin, St. Petersburg, Russia
✉ e-mail: irina_novikova@inbox.ru; juli1958@yandex.ru

Genus *Trichoderma* strains as the natural plant residues' biodestructors, highly active antagonists of soil phytopathogens and phytoregulators with the widest range of optimum conditions for their development, are widely used in biologics development. Of particular importance in Russia's northern regions, especially in winter crop cultivation, is the ability of a

microorganism's strain used in agro-technologies to maintain viability and target biological activity at low temperatures. In this connection, this work purpose is to select a psychrotolerant strain of *T. asperellum* for the rapid crop residues' polymer utilization and soil enhancement at low temperature, as well as to evaluate its activity under laboratory and field conditions. In the work process, the following tasks were addressed: selecting psychrotolerant strains of *T. asperellum* with high cellulolytic activity; further controlled breeding of psychrophilic strains capable of rapid growth, active colonization of plant substrates and high sporulation at 4–8 °C; evaluating the target activity of the selected psychrophilic strain as a cellulolytic as well as antagonistic activity against cereal pathogens; obtaining laboratory samples of bioformulations by deep-surface cultivation on non-sterile peat and multirecycled wastes from the edible mushrooms production and assessing their efficacy in field small-plot trials. The methods for inoculum cultivation, sporulation capacity determination, modified wet chamber, estimating antagonistic activity and biologics' quality, field small-plot trials management, quantitative estimates of biomass losses, cellulose and lignin content were used in the work. The active psychrophilic strain for the rapid crop residues' polymer utilization and soil enhancement controlled breeding was selected during a four-step screening of 29 *T. asperellum* strains from All-Russian Research Institute of Plant Protection (VIZR) State Microorganisms' Collection with high cellulolytic and antagonistic activities. In terms of linear growth rate, antagonistic and hyperparasitic activities at 4–8 °C, a high rate of wheat and maize stubble residues' colonization, a perspective psychrophilic strain G-034 of *T. asperellum* was selected for developing the laboratory samples of biologics and for running field trials. In small-plot trials, the active maize crop residues' decomposition under the *T. asperellum* G-034 influence was revealed, resulting in the complete loss of plant intact state in 12 months due to more than 80 % cellulose and 20 % lignin biodestruction. The maximum loss of maize crop residues biomass for 12 months was more than 70 %. The *T. asperellum* strain G-034 was active after field hibernation in an amount of $\times 10^4$ cfu/g, resulting in a titer increase with seasonal temperature rising and the trophic base bioavailability growth.

Key words: *Trichoderma asperellum* G-034 VIZR; controlled breeding; psychrotolerant strain-producer; psychrophilic strain-producers; multirecycled biologics; biologics for soil inhancement; stubble residues' biodestructors; producer strains' target activity; biologics' efficacy; plant residues' destruction.

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Введение

Улучшению структуры почвы и накоплению гумуса способствует запахивание пожнивных остатков или внесение соломы на поля. При этом снижается эрозия почвы, улучшаются водный и воздушный режимы, ее впитывающая способность, а также стимулируются процессы азотфиксации. При разложении пожнивных остатков в почву поступает на 1 га около 12–15 кг азота, до 7 кг фосфора, 30 кг калия, 4 кг натрия (Лабынцев, Целуйко, 2013). Пожнивные остатки – источник питания почвенных микроорганизмов, которые в значительной степени обеспечивают доступность отдельных нутриентов для сельскохозяйственных растений (Зиганшин и др., 2016). Вместе с тем растительные остатки на полях являются резерватором и источником фитопатогенов – возбудителей болезней сельскохозяйственных культур. Без создания необходимого пула антагонистов, способствующих восстановлению и усилению супрессивности почвы, невозможно стабилизировать фитосанитарную ситуацию в агроценозах, сохранить плодородие и получить высокие урожаи (Новикова, 2007, 2016).

Решению этой проблемы способствует все возрастающее использование микробиологического метода в защите растений. Современные биопрепараты обладают ростостимулирующей активностью, их продуценты выделяют множество биологически активных веществ, подавляющих развитие популяций фитопатогенов, повышающих болезнеустойчивость и урожайность сельскохозяйственных растений (Whipps, Lumsden, 2001; Садыкова и др., 2010). Успешность применения микробиологического метода зависит от эффективности агентов биоконтроля. Для создания биопрепаратов наибольший интерес представляют виды и штаммы микроорганизмов, обладающие разнообразием метаболических процессов, неприхотливостью к условиям культивирования, высокой технологичностью и экологической пластичностью (Haran et al., 1996; Новикова, 2007, 2016; Зиганшин, Сироткин, 2017). Всеми этими качествами обладают штаммы рода *Trichoderma* – природные биодеструкторы растительных остатков и других целлюлозосодержащих материалов, занимающие особое положение как продуценты полифункциональных биопрепаратов (Коломбет и др., 2001; Boureghda, Renane, 2011). Они способны быстро осваивать органический субстрат путем активного разложения простых и сложных соединений, в десятки раз ускоряя процесс их минерализации и улучшая физико-химические свойства почвы (Алимова, 2005; Bheemaraya et al., 2011; Рязанова и др., 2014). Благодаря высокой антагонистической и гиперпаразитической активности в отношении почвообитающих возбудителей болезней, штаммы *Trichoderma* в 2.5–3 раза снижают заболеваемость растений, повышают их болезнеустойчивость, проявляют фиторегуляторную активность за счет стимуляции развития азотфиксирующих бактерий, способствуя обогащению почвы аминным азотом, связыванию солей минеральных удобрений, усилению мобилизации фосфора и калия (Садыкова и др., 2009; Alamri et al., 2012; Devi et al., 2012; Heidi, Abo-Elnaga, 2012; Parra, Maniscalco, 2012). Особое значение в условиях северных регионов России имеет способность штамма микроорганизма, используемого в агротехнологиях, сохранять жизнеспособность и целевую биологическую активность при низких температурах, особенно при выращивании озимых культур.

Целью работы были отбор психрофильного штамма *Trichoderma asperellum* для ускоренной утилизации основных полимеров растительных остатков, оздоровления почвы при низкой температуре и оценка его активности в лабораторных и полевых условиях. Для этого решались

следующие задачи: отбор из «Государственной коллекции микроорганизмов, патогенных для растений и их вредителей» (ГКМ) Центра коллективного пользования научным оборудованием «Инновационные технологии защиты растений» ФГБНУ Всероссийский НИИ защиты растений (ВИЗР) ФАНО, сайт http://www.vizrspb.chat.ru (Постановление Правительства РФ № 725-47 от 24 июня 1996 г., приказ по Министерству сельского хозяйства и Правительству РФ от 15 августа 1996 г., зарегистрирована в WFCC WDCM 760 (Япония) 28.01.98) психротолерантных штаммов *T. asperellum* с высокой целлюлозолитической активностью; дальнейшая направленная селекция психрофильных штаммов, способных к быстрому росту, активной колонизации растительного субстрата и высокой споропродуктивности при 4–8 °С; оценка целевой активности отселектированного психрофильного штамма в качестве целлюлозолитика при колонизации пожнивных остатков пшеницы и кукурузы, а также антагонистической активности в отношении фитопатогенов зерновых культур; получение лабораторных образцов препаративных форм (ЛО) путем глубинно-поверхностного культивирования на различных органических субстратах, в том числе отходах производства съедобных грибов, и оценка их эффективности в полевых мелкоделяночных опытах.

Материалы и методы

Работу проводили на базе лаборатории микробиологической защиты растений с использованием чистых культур из ГКМ ФГБНУ ВИЗР. Объектами исследований были штаммы *T. asperellum* с высокой целлюлозолитической и антагонистической активностью, тест-объектами служили штаммы фитопатогенов зерновых культур – возбудители наиболее вредоносных болезней, и сухие пожнивные остатки пшеницы и кукурузы (табл. 1). Для получения и хранения чистых культур микромицетов для лабораторных опытов *in vitro* использовали питательные среды: синтетическую среду Чапека (ООО «Биокомпас-С», Углич, Россия); полусинтетические (селективные) агаризованные среды на основе растительных экстрактов (картофеля, зерна злаков и т. п.). Растительный субстрат предварительно измельчали и кипятили в течение часа в объеме 200 г/л, фильтровали, восстанавливали до исходного объема с добавлением агар-агара (20 г/л), в некоторых случаях – с добавлением сахарозы (20 г/л). Режим стерилизации для всех сред, содержащих сахара в низкомолекулярной форме, – 30 мин при 0.5–0.8 атм (Методы…, 1982).

Материалами исследований были субстраты для опытно-промышленного культивирования съедобного макромицета *Lentinula edodes* (шии-таке) на основе отходов техногенной сферы, блоки с развивающимся мицелием шии-таке, а также отработанные в процессе жизнедеятельности последнего целлюлозо-лигнинсодержащие отходы. Кроме того, материалами служили мультиконверсионные субстраты, полученные после последовательного культивирования на отработанном отходе производства плодовых тел шии-таке другого вида съедобного макромицета – *Pleurotus ostreatus* НК-35 (вешенка). Используемые для мультибиоконверсии субстраты в интактном и отработанном состояниях имеют оформленный состав (табл. 2). В отработанных (конверсионных) субстратах все ингредиенты (см. табл. 2) находятся в переработанном предыдущим участником биоконверсии состоянии, происходит накопление водорастворимых легко усваиваемых веществ – аминокислот, витаминов, моносахаридов и др.

При этом наблюдается сужение соотношения азота к углероду, что делает отработанные субстраты наиболее доступными для последующей утилизации как макро-, так и микромицетами и бактериями (Бисько и др., 1986; Бисько, Дудка, 1987; Титова, 2013; Титова и др., 2014, 2017а–в). Превалировал в конверсионных субстратах грибной белок в виде мицелия предыдущего участника биоконверсии, пронизывающего практически всю толщу используемого субстрата (как компонент в табл. 2).

В работе использовали следующие методы исследований: культивирования и создания инокулюма, определения споропродуктивности (титра), модифицированной влажной камеры, оценки антагонистической активности, оценки качества лабораторных образцов биопрепаратов, организации полевых испытаний, количественных оценок потерь биомассы и содержания целлюлозы и лигнина.

Культуры микромицетов поддерживали на агаре Чапека с обеспечением необходимой чистоты материала. Чистые культуры вводили в работу по созданию инокулюмов путем жидкофазной и твердофазной ферментаций при температуре 24–28 °С. Для определения линейного роста изучаемый штамм высевали в центр поверхности агаризованной питательной среды одинакового слоя немногочисленным инокулюмом практически однородной плотности. Культивирование вели в условиях холодильной камеры при 4–8 °С. Через определенные промежутки времени (двое суток) измеряли диаметр колоний в двух взаимоперпендикулярных направлениях от точки инокулирования до конца зоны роста мицелия. Повторность пятикратная. Расчетные параметры линейного роста мицелия и споропродуктивности: дифференциальная скорость роста мицелия, представляемая как производная функции приращения диаметра в единицу времени (мм/сут); время наступления спороношения; споропродуктивность (Методы…, 1982).

Определение споропродуктивности штаммов проводили с помощью создания маточных суспензий колониеобразующих единиц (КОЕ) в единице объема, массы и т. п., в которых число КОЕ подсчитывали прямым способом в камере Горяева или путем последовательных десятичных разведений маточной суспензии. Наиболее оптимальное для количественного и качественного учета споропродуктивности серийное разведение маточной суспензии КОЕ исследуемого штамма высевали на агаризованные среды (Лилли, Барнетт, 1953; Методы…, 1982). Для оценки целевой активности штаммов *T. asperellum* при колонизации пожнивных остатков пшеницы и кукурузы использовали метод модифицированной влажной камеры в чашках Петри. Кусок фильтровальной бумаги обрезали по размеру камеры и помещали в ее основание. Чашки Петри с фильтровальной бумагой стерилизовали автоклавированием при 132 ± 2 °С в течение 1 ч. Далее образцы (по 10 г сухих пожнивных растительных остатков пшеницы и кукурузы) помещали поверх фильтровальной бумаги так, чтобы отрезки стеблей закрыли основание, но

Table 1. Micromycete strains studied

Species	Characterization	Origin
Trichoderma asperellum Samuels, Lieckf. et Nirenberg (29 strains)	Deposited and categorized in the State Microorganism Collection of the All-Russia Institute of Plant Protection (SCM FSBSI VIZR)	Leningrad region (LR). Natural conditions (NC)
Fusarium oxysporum Schlecht. (strains 173 and 11D)	Deposited and categorized in SCM FSBSI VIZR (*Fusarium* fungi)	LR, NC (wheat and barley root rot)
F. sambucinum Fuckel	»	»
F. graminearum Schwabe	»	LR, NC (in the *Fusarium* head blight complex)
F. sporotrichioides Sherb., mycotoxins' producer	»	»
Alternaria alternata (Fr.) Keissl., mycotoxins' producer	Deposited and categorized in SCM FSBSI VIZR	LR, NC (in leaf spot complex)
Rhizoctonia solani J.G. Kuhn.	»	LR, NC (wheat and barley root rot)
Cochliobolus sativus (S. Ito et Kurib.) Drechsler ex Dastur	»	LR, NC (in leaf spot complex and cereal root rot)

Table 2. Composition of intact (for inoculation with edible basidiomycetes) and multirecycled substrates

Edible macromycete species	Substrate*	
	for inoculation	wasted (recycled)
L. edodes	Sawdust of hardwood trees, wheat bran (10 % of the substrate weight), $CaCO_3$ (whitening) 0.1 %, $CaSO_4 \times 7H_2O$ (gypsum) 1 %	Sawdust of hardwood trees, wheat bran (10 % of the substrate weight), $CaCO_3$ (whitening) 0.1 %, $CaSO_4 \times 7H_2O$ (gypsum) 1%, shiitake mycelium deep in the substrate
L. edodes + *P. ostreatus* НК-35	Sawdust hardwood trees, wheat bran (10 % of the substrate weight), $CaCO_3$ (whitening) 0.1 %, $CaSO_4 \times 7H_2O$ (gypsum) 1 %, shiitake mycelium deep in the substrate	Sawdust hardwood trees, wheat bran (10 % of the substrate weight), $CaCO_3$ (whitening) 0.1 %, $CaSO_4 \times 7H_2O$ (gypsum) 1 %, shiitake and oyster mushrooms mycelium deep in the substrate

* Percentages are indicated with respect to substrate weight at 70 % humidity.

не накладывались друг на друга. Добавляли достаточное количество стерильной дистиллированной воды, чтобы образец был погружен в воду. Затем чашки закрывали и инкубировали 1 сут при комнатной температуре для впитывания образцами максимального количества воды. На следующий день остатки воды сливали, образцы инокулировали 2 мл суспензии исследуемого штамма с титром $\times 10^8$ КОЕ/мл. Образцы инкубировали в течение 14 сут в термостатированных условиях как при 24–28 °С, так и при 4–8 °С в темноте. В последующие сутки воды не добавляли. Влажные камеры ежесуточно просматривали в течение периода инкубации, на ранних сроках развития с использованием лупы с увеличением ×16–54 или бинокулярной лупы (Методы…, 1982).

Для оценки антагонистической активности отобранных штаммов *T. asperellum* на тест-объектах в лабораторных опытах *in vitro* использовали метод встречных культур на агаризованных питательных средах (Рудаков, 1981, 1986; Методы…, 1982; Егоров, 2004; Алимова, 2005; Богданов, Титова, 2014). Исследовали взаимодействие четырех отобранных психрофильных штаммов *T. asperellum* Г-004, Г-007, Г-025 и Г-034 с восемью видами фитопатогенных микромицетов – возбудителей заболеваний сельскохозяйственных культур: *F. oxysporum* 173, 11Д, *F. sambucinum*, *F. graminearum*, *F. sporotrichioides*, *A. alternata*, *R. solani*, *C. sativus* (см. табл. 1). Для встречных культур использовали агар Чапека. Культивирование проводили в термостатах при 24–28 и 4–8 °С в темноте. Вносили взаимодействующие культуры немногочисленным инокулюмом практически однородной плотности (уколом) в центр половины чашки Петри. Повторность опытов пятикратная. Регистрацию производили ежесуточно в течение 30 сут до и после образования зоны контакта культур.

Количественной характеристикой антагонистической активности штаммов *T. asperellum* служили изменения скоростей роста мицелия участников взаимодействия. Качественную характеристику типов взаимоотношений изучаемых микромицетов определяли в соответствии с классификацией взаимодействий мицелиев, разработанной на основе качественных параметров взаимодействий мицелиев: наличие границы между колониями; переплетение гиф с образованием мицелиального валика различной структуры, текстуры, размера и плотности и без него; пигментация зоны контакта; наличие зоны отталкивания или барража; ускорение, замедление или остановка роста колонии (Богданов, Титова, 2014). Показатели гиперпаразитической активности штаммов по отношению к тест-культурам рассчитывали, исходя из зависимости соотношения площадей, занимаемых культурами штаммов исследуемых объектов взаимодействия, от роста культур: исследуемый штамм *T. asperellum* обладает гиперпаразитической активностью, если соотношение площадей, занимаемых культурами, будет не менее 1.9 (ТУ 9291-005-59147141-2006).

Полевые испытания проводили на участке со взаимно ортогональной организацией опыта со сплошным размещением организованных повторений стандартного размещения вариантов общей площадью 133.0 м^2 (66.5 × 2 м): три повторности на вариант опыта (19 вариантов), учетная делянка – 2.0 м^2 (1 × 2 м); защитная полоса – 1.0 м^2 (0.5 × 2 м), с убранной основной культурой (кукуруза) и максимально измельченными, запаханными в почву пожнивными остатками. Повторность опыта трехкратная (Доспехов, 1979).

Для полевых испытаний эффективности были наработаны ЛО гранулированных мультиконверсионных и торфяных биопрепаратов на основе *T. asperellum* Г-034 с титрами: торфяной – ЛО Г-034, П (2.1×10^8 КОЕ/г); мультиконверсионные – ЛО Г-034, ШГ (шиитачный) (0.9×10^8 КОЕ/г) и ЛО Г-034, ШВГ (шиитачно-вешеночный) (1.1×10^8 КОЕ/г). В полевых мелкоделяночных опытах для оценки эффективности использовали следующие нормы применения ЛО: 2.5, 5 и 10 г/м^2 при норме расхода рабочего раствора 1.5 л/м^2. Для проведения опыта готовили водные суспензии образцов биопрепарата и вносили их в почву путем полива. В контроле почву поливали водой.

Качество ЛО оценивали по титрам и степени контаминации: по культуральным признакам и с помощью последовательных серийных разведений. Учет результатов полевых испытаний проводили на 10-е, 20-е, 30-е, 180-е и 360-е сутки, дважды через 6 мес после применения биопрепаратов (6 и 12 мес). Анализировали смешанные усредненные почвенные пробы с пожнивными остатками по показателям числа КОЕ/г штамма-продуцента *T. asperellum* Г-034 в почве учетных делянок, его жизнеспособности и целевой активности, колонизации пожнивных остатков кукурузы и их габитусу, потерям биомассы растительными остатками и изменению содержания мажорных компонентов (целлюлозы и лигнина). В процессе лабораторного контроля полевого опыта 20 смешанных усредненных почвенных проб из вариантов полевого опыта (примерно по 300 г каждая) помещали в керамические вазоны объемом 1 л и выдерживали в условиях 22–25 °С и перманентного увлажнения в течение 6 мес для обеспечения разложения остатков кукурузы комплексом почвенных микроорганизмов на естественном фоне, а также с внесением психрофильного штамма *T. asperellum* Г-034 в разных нормах применения ЛО. Выявление штамма-продуцента и его жизнеспособности в образцах почвы проводили ежемесячно.

Для оценки степени разложения пожнивных остатков применяли визуальные и органолептические характеристики состояния (габитуса) ткани стеблей и корневой шейки кукурузы. Перед проведением химических анализов образцы пожнивных остатков кукурузы высушивали до воздушно-сухого состояния, измельчали на электрической мельнице и просеивали через сито с диаметром пор 1 мм. Содержание целлюлозы определяли по ГОСТ 31675-2012, лигнина – по ГОСТ 26177-84, влажность остатков – по ГОСТ 13525.19-91. Также определяли процентное содержание целлюлозы и/или лигнина от веса исходной пробы растительных остатков по формуле $C = 100 \times m_i/m_0 - m_0 \times \rho$, где ρ – влажность образца растительных остатков; m_i – масса искомого биополимера (целлюлоза, лигнин); m_0 – общая масса образца растительных остатков (Оболенская и др., 1955; Schwanninger, Hinterstoisser, 2002). Статистическую оценку результатов и визуализацию материала производили с помощью программных пакетов Excel 2010 и STATISTICA 6.

Результаты

Селекцию активного психрофильного штамма рода *Trichoderma* для ускоренной утилизации полимеров растительных остатков и оздоровления почвы осуществили в процессе четырехступенчатого скрининга 65 штаммов *T. asperellum* из ГКМ ФГБНУ ВИЗР с высокими целлюлозолитической и антагонистической активностями. На первом этапе было отобрано 29 психрофильных штаммов *T. asperellum*, недостоверно различающихся по скорости роста при 4–8 и 26–28 °С (табл. 3).

Table 3. *T. asperellum* growth dynamics at 4–8° C

Strain accession no.	Mean growth rates at 4–8 °C, mm/per day		
	5 days	7 days	10 days
G-001	6.4	11.8	8.5
G-002	4.1	4.3	6.8
G-003	4.3	6.2	7.5
G-004	7.0	12.0	8.5
G-005	4.1	3.2	5.6
G-006	3.0	2.1	3.4
G-007	6.5	11.5	8.5
G-011	4.2	5.9	7.3
G-012	3.5	3.6	3.5
G-016	4.4	8.8	8.5
G-018	3.6	10.7	8.5
G-020	2.2	4.4	3.5
G-021	3.7	6.5	7.7
G-022	2.8	5.7	7.3
G-024	5.6	9.9	8.5
G-025	6.4	10.7	8.5
G-026	2.6	5.6	7.6
G-027	2.0	3.6	3.5
G-029	4.5	5.7	7.0
G-030	2.7	5.0	8.0
G-033	1.0	4.9	7.8
G-034	6.8	11.9	8.5
G-035	3.4	5.5	7.7
G-039	3.4	9.4	8.5
G-040	4.6	9.1	8.5
G-045	5.3	10.2	8.5
G-047	0.6	4.3	7.4
G-049	5.2	6.2	8.5
G-051	3.4	6.0	7.9
$HCP_{0.5}$	0.2	1.2	0.8

На втором этапе из этих 29 штаммов были отобраны 5 психрофильных штаммов (Г-001, Г-004, Г-007, Г-025 и Г-034), отличающихся высокими скоростями роста и споропродуктивностью при низких температурах (4–8 °С). При исследовании антагонистической активности отселектированных штаммов на второй ступени скрининга были отобраны 4 штамма (Г-004, Г-007, Г-025 и Г-034), показавшие высокие начальные скорости линейного роста при взаимодействии с тест-культурами фитопатогенных грибов. Максимальную начальную скорость роста при взаимодействии с тест-культурами выявили у штамма Г-034 *T. asperellum*:

Штамм	Средняя скорость роста, мм/сут
Г-004	1.52
Г-007	1.94
Г-025	1.51
Г-034	2.11
$НСР_{0.5}$	0.2

Анализ антагонистической активности отобранных штаммов Г-004, Г-007, Г-025 и Г-034 выявил как неполный, так и полный паразитизм *T. asperellum* на большинстве тест-культур и при высоких, и при низких температурах встречного культивирования (Богданов, Титова, 2014). В табл. 4 приведены коэффициенты гиперпаразитической активности отобранных штаммов, соответствующие неполному и полному паразитизму в отношении *F. oxysporum* 173, *F. oxysporum* 11Д, *F. sambucinum*, *F. graminearum*, *F. sporotrichioides*, *A. alternata*, *R. solani*, *C. sativus*.

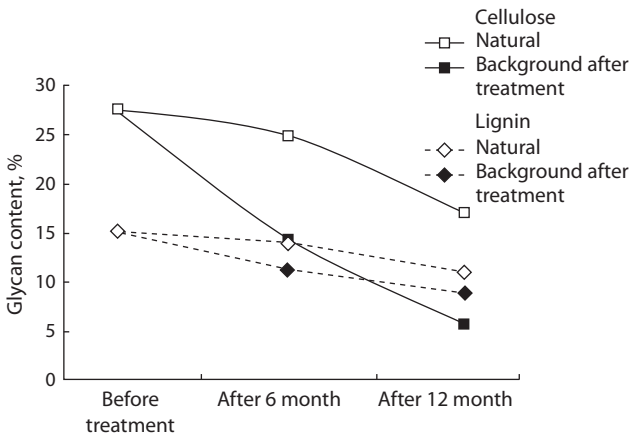
По совокупности психрофильных показателей – высокой скорости линейного роста и высоким коэффициентам гиперпаразитической активности при 4–8 °С – для оценки эффективности колонизации пожнивных остатков был отобран штамм *T. asperellum* Г-034. Было показано, что в лабораторных условиях колонизация пожнивных остатков пшеницы и кукурузы при 26–28 °С произошла в течение 2 сут с формированием обильного спороношения штамма *T. asperellum* Г-034 на растительных остатках. Таким образом, отобранный психрофильный штамм *T. asperellum* Г-034 обладает высокой антагонистической активностью в отношении фитопатогенных микромицетов – возбудителей фузариозов и пятнистостей зерновых культур, и способностью эффективно колонизировать растительные остатки в модельных лабораторных опытах.

На следующем этапе исследований необходимо было наработать ЛО препаративных форм и оценить способность штамма-продуцента утилизировать полимеры растительных остатков в условиях полевых испытаний. ЛО на основе штамма-продуцента *T. asperellum* Г-034 получены путем иммобилизации нестерильного торфа, а также дву- и трехступенчатой биоконверсии отходов сельского хозяйства и деревоперерабатывающей промышленности, первично конвертированных *L. edodes*, далее *P. ostreatus* НК-35 с титрами 2.1×10^8, 1.0×10^8, 1.1×10^8 КОЕ/г соответственно. В контроле полевого опыта аборигенных штаммов *Trichoderma* не было выявлено.

После двукратного применения с 6-месячным перерывом ЛО гранулированных биопрепаратов штамм-продуцент *T. asperellum* Г-034 при всех испытанных нормах применения находился в активном и жизнеспособном состоянии в количестве $\times 10^4$ КОЕ/г. Потери биомассы растительных остатков кукурузы перманентно за 10, 20, 30, 180 и 360 сут в два раза превышали этот показатель в контроле с увлажненными пожнивными остатками без обработки *T. asperellum* Г-034. Максимальное снижение биомассы по отношению к воздушно-сухим пожнивным остаткам растений без обработки за 10, 20, 30 и 180 сут составило 5.4, 10.5, 13.8 и 74.7 % соответственно, а по отношению к увлажненным пожнивным остаткам без обработки биопрепаратами – 1.8, 6.1, 11.4 и 72.7 % соответственно. Наблюдали сходное во всех вариантах опыта общее состояние растительных остатков (габитус): изменение цвета до темно-бурого и серого; легкость скручивания и разрыва тканей как мелких, так и крупных фрагментов; оголение лигнинового скелета во фрагментах остатков стеблей; мацерацию кутикулярного слоя и лигнинового остова мелких фрагментов стеблей; полную мацерацию фрагментов листовых пластинок. Габитус разлагаемых остатков кукурузы после двукратного применения штамма (12 мес. с перезимовкой в поле) соответствовал практически полному оголению и значительной мацерации лигнинового скелета стеблей кукурузы. Наряду с исследованием габитусов растительных остатков кукурузы, про-

Table 4. Hyperparasitic activity coefficients of selected *T. asperellum* strains in the interaction with test objects

Test object	G-004		G-007		G-025		G-034	
	Temperature, °C							
	26–28	4–8	26–28	4–8	26–28	4–8	26–28	4–8
F. oxysporum 173	1.79	2.55	1.92	3.21	1.92	2.37	2.21	2.06
F. oxysporum 11D	1.21	1.14	1.57	1.57	1.57	1.29	1.57	1.68
F. sambucinum	2.75	1.68	1.92	3.47	2.21	1.57	2.21	2.37
F. graminearum	1.79	1.92	1.79	1.79	1.79	2.21	1.11	1.92
F. sporotrichioides	2.21	1.47	2.37	2.37	2.55	2.55	2.21	1.79
A. alternata	1.38	2.55	1.29	2.96	1.57	2.96	1.07	2.96
R. solani	1.57	1.47	1.68	1.47	1.68	1.29	1.68	1.11
C. sativus	1.57	6.02	2.21	6.02	2.21	6.02	1.68	6.02



Variation of cellulose and lignin contents in corn crop residues after treatments with a granulated lab specimen (LS, G) based on *T. asperellum* G-034 strain (substrate multirecycled with Shiitake-Oyster mushrooms) at the application rate 10 g/m^2).

вели реизоляцию штамма-продуцента *T. asperellum* Г-034, осуществляющего их разложение в комплексе почвенных микроорганизмов естественного фона, присутствующего во всех вариантах опыта. Содержание целлюлозы в растительных остатках после обработки ЛО биопрепаратов на основе *T. asperellum* Г-034 при нормах применения 5 и 10 г/м^2 уменьшилось в 2 раза за 6 мес. и в 5 раз за 12 мес., а содержание лигнина – в 1.5 раза за 6 мес. и в 2 раза за 12 мес. В контроле содержание целлюлозы и лигнина в растительных остатках на естественном фоне за 6 и 12 мес. снизилось незначительно (см. рисунок).

Обсуждение

Таким образом, по показателям линейной скорости роста, антагонистической и гиперпаразитической активности при 4–8 °С, высокой скорости колонизации пожнивных остатков пшеницы и кукурузы отобран перспективный психрофильный штамм *T. asperellum* Г-034 для наработки на его основе лабораторных образцов биопрепаратов и проведения полевых опытов. Мелкоделяночные полевые испытания показали высокую эффективность штамма *T. asperellum* Г-034 для ускоренного разложения растительных остатков кукурузы и оздоровления почвы. При проведении исследований по лабораторному контролю полевого опыта выявили активное разложение пожнивных остатков кукурузы под воздействием почвенных микроорганизмов-антагонистов, а главное, под воздействием высокоактивного психрофильного штамма-продуцента *T. asperellum* Г-034, приводящее к полной потере за 12 мес. интактного состояния растительными остатками за счет биодеструкции более 80 % содержащейся в них целлюлозы и более 20 % лигнина, обеспечивающего механическую прочность. Максимальные потери биомассы пожнивными остатками кукурузы за 12 мес составили более 70 %. Штамм-продуцент *T. asperellum* Г-034 после перезимовки в полевых условиях находился в активном состоянии в количестве ×10^4 КОЕ/г, приводящем к нарастанию титра с сезонными возрастанием температуры, увеличением и расширением биодоступности трофической базы.

Микромицеты *Trichoderma* spp. занимают особое положение как продуценты полифункциональных биофунгицидов, синтезирующих богатые комплексы гидролаз. Для реализации антагонистической активности у этой группы микромицетов синтез гидролитических ферментов, таких как хитиназы, глюканазы, протеазы и липазы, лизирующих клеточные стенки фитопатогенных грибов и разрушающих ряд эффекторных молекул фитопатогенов, имеет определяющее значение (Benítez et al., 2004; Аринбасарова и др., 2017). В ряде работ приведены данные о способности штаммов *Trichoderma* существенно обогащать почву подвижными и доступными для растения формами питательных веществ, участвуя в разложении органических соединений (Kubicek et al., 2001; Алимова и др., 2006). Очевидно, именно этим обусловлено повышение эффективности усвоения азота вследствие активизации развития популяций азотфиксаторов и, в целом, биологической активности почв.

Заключение

Микромицеты рода *Trichoderma*, обладая высокой гиперпаразитарной и антагонистической активностью в отношении почвообитающих возбудителей болезней, синтезируют широкий спектр биологически активных веществ, повышают болезнеустойчивость и продуктивность растений (Коломбет и др., 2001; Алимова и др., 2006; Садыкова и др., 2009). Полученные нами результаты согласуются с данными других авторов, а также позволяют существенно расширить возможности применения штаммов *Trichoderma* для разложения растительных остатков и биоконтроля почвообитающих фитопатогенных видов при низких температурах, характерных для северных регионов России.

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ORCID ID

I.I. Novikova orcid.org/0000-0003-2816-2151
J.A. Titova orcid.org/0000-0002-8188-1852

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An optimized method for counting viral particles using electron microscopy

B.N. Zaitsev ✉, O.S. Taranov, N.B. Rudometova, N.S. Shcherbakova, A.A. Ilyichev, L.I. Karpenko

State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk Region, Russia
✉ e-mail: zaitsev@vector.nsc.ru

Viruses can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea. When studying samples containing viruses, one confronts an unavoidable question of the quantitative determination of viral particles in the sample. One of the simplest and efficient approaches to quantitative determination of viral particles in preparation includes the use of electron microscopy; however, a high detection threshold is a significant limitation of this method (10^7 particles per ml). Usually, such sensitivity is insufficient and can result in error diagnosis. This study aims to develop a method making it possible to detect the number of viral particles more precisely and work with samples in which the concentration of particles is lower than 10^7/ml. The method includes a concentration of viral particles on the polyethersulfone membrane applied in centrifugal concentrators and subsequent calculation using an electron microscope. We selected env-pseudoviruses using a lentiviral system making it possible to obtain standardized samples of virus-like particles that are safer than a live virus. Suspension of viral particles (a volume of 20 ml) was placed into the centrifugal concentrator and centrifuged. After that, we took a membrane out of the centrifugal concentrator and evaluated the number of particles on the ultrathin section using an electron microscope. The number of viral particles on the whole surface of the filter (a square of 4 cm^2) was 4×10^7 virions, the initial concentration of pseudoviruses in the sample was 2×10^6 per 1 ml (4×10^7 particles per 20 ml). As a result, the developed method enables one to evade the major disadvantage of quantitative determination of viruses using electron microscopy regarding a high detection threshold (concentration of particles 10^7/ml). Furthermore, the centrifugal concentrator makes it possible to sequentially drift a considerable volume of the suspension through the filter resulting in enhancement of test sensitivity. The developed approach results in increased sensitivity, accuracy, and reproducibility of quantitative analysis of various samples containing animal, plant or human viruses using electron microscopy.
Key words: electron microscopy; pseudoviruses; concentrating; number of viral particles.

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Оптимизированный метод подсчета количества вирусных частиц с помощью электронной микроскопии

Б.Н. Зайцев ✉, О.С. Таранов, Н.Б. Рудометова, Н.С. Щербакова, А.А. Ильичев, Л.И. Карпенко

Государственный научный центр вирусологии и биотехнологии «Вектор» Роспотребнадзора, р.п. Кольцово, Новосибирская область, Россия
✉ e-mail: zaitsev@vector.nsc.ru

Вирусы поражают все типы организмов, от растений и животных до бактерий и архей. При исследовании образцов, содержащих вирусы, неизбежно встает вопрос количественного определения вирусных частиц в пробе. Одна из наиболее простых и эффективных методик количественного определения вирусных частиц в препарате – использование электронной микроскопии, однако основным ограничением метода является относительно высокий предел обнаружения (10^7 частиц/мл). Часто такая чувствительность недостаточна и может приводить к ошибочной диагностике. Цель данной работы заключалась в разработке методики, позволяющей более точно оценивать количество вирусных частиц и работать с образцами, в которых концентрация ниже, чем 10^7 частиц/мл. Метод заключается в концентрировании вирусных частиц на мембране из полиэфирсульфона, применяемой в центрифужных концентраторах, с последующим подсчетом с помощью электронного микроскопа. В качестве модельного объекта были выбраны env-псевдовирусы, созданные с использованием лентивирусной системы, которая позволяет получать стандартизованные образцы вирусоподобных частиц. Суспензию вирусных частиц (объемом 20 мл) помещали в центрифужный концентратор и центрифугировали. Затем извлекали мембрану из концентратора и оценивали количество осажденных на мембране частиц с помощью электронного микроскопа, используя метод ультратонких срезов. Количество вирусных частиц на всей поверхности фильтра (площадь 4 $см^2$) составляло 4×10^7 вирионов, исходная концентрация псевдовирусов в образце – 2×10^6 на 1 мл (4×10^7 частиц/20 мл). Таким образом, предложенная

методика позволяет преодолеть основной недостаток количественного определения вирусов с помощью электронной микроскопии, связанный с относительно высоким пределом обнаружения (10^7 частиц/мл). Кроме того, центрифужный концентратор дает возможность последовательно прогнать через один и тот же фильтр значительные объемы суспензии, содержащей вирусы, что также может привести к повышению чувствительности метода. Предложенный подход позволяет повысить чувствительность, точность и воспроизводимость количественного анализа различных образцов, содержащих вирусы животных, растений и человека, с использованием электронной микроскопии.
Ключевые слова: электронная микроскопия; псевдовирусы; концентрирование; количество вирусных частиц.

Introduction

At current, the interest in viruses has increased tremendously. Viruses affect all types of organisms, from plants and animals to bacteria and archaea. According to recent release of the International Committee on Taxonomy of Viruses, nearly 5000 species of viruses are presently known, and new viruses are discovered every year.

Modern diagnostic is an important element in the system of protection against infectious diseases; it determines adequate preventative measures and efficacy of further therapy. Visual detection and identification of an infectious agent with microscopy is an explicitly positive outcome of diagnostics. Electron microscopy is used widely to study viruses (Goldsmith, Miller, 2009). Apart from diagnostic purposes, electron microscopy is also employed for structural studies of nanoparticles, such as artificial VLP (virus-like particles), created to construct vaccine and for genome studies. Along with identification of particular nano-dimensional objects, electron microscopy gives important information about their morphology, survival rate under an impact of various physical and chemical (including pharmacological) factors, and enables to determine their content in biological fluids.

The main obstacle for use of electron microscopy in studies of viral objects is insufficient concentration of particles in fluids for the purposes of detection. To apply the simplest and the most popular method – negative staining, the concentration of virions (or other nano-scale objects) should be no less than 10^7 particles/ml (Reid et al., 2002; Malenovska, 2013). Such level of sensitivity, however, is often insufficient and can result in error diagnosis. To increase sensitivity, concentrating nanoparticles by means of ultracentrifugation is broadly used. It helps increase concentration of the analyzed particles per unit volume approximately thousandfold. At the same time, such work would require expensive equipment and a lengthy period of sample preparation.

Our study presents an original method for more accurate counting of viral particles. We illustrate the method proposed using env-presudoviruses as an example.

Materials and methods

Production of pseudoviruses. Pseudoviruses were obtained and characterized according to an earlier described method (Ryzhikov at al., 2012) with some modifications. HEK293T/17 cells were seeded in the 5×10^5 cells/hole concentration on a 6-hole cultural plate and incubated in an CO_2-incubator at +37 °C in DMEM, containing 10 % fetal bovine serum, 600 mg/ml L-glutamine and 50 μg/ml gentamicin. To obtain pseudoviruses, HEK293T cells were transfected simultaneously with two plasmids: pcDNA3.1env (SF162.LS) (NIH) and backbone pSG3Δenv (with the defective *env* gene) using MATra (PromoKine) according to the protocol recommended by the manufacturer. The plates were put into the CO_2-incubator and incubated at +37 °C. In 4–6 hours the medium in the plates was replaced. The cultural medium was collected after 48 hours of incubation. Pseudoviruses were separated from cells by low-frequency centrifugation followed by filtration through a nitrocellulose filter with 0.45 μm pore diameter. The obtained pseudoviral samples were stored at –80 °C in the DMEM medium containing 20 % fetal bovine serum.

Determining functional activity. Functional activity of the obtained pseudoviruses was determined using TZM-bl cells. 5×10^4 TZM-bl cells were put in each hole of a 96-hole cultural plate, then 50 μl of suspension of the analyzed env-pseudoviruses were added to each hole, four hopes per pseudovirus. The plates were incubated at 37 °C in 5 % CO_2. After 48 hours of incubation, luciferase activity was determined in cells with a LAR (Promega) set in accord with the manufacturer's recommendations. Pseudovirus-free TZM-bl cells were used as the control. A luminescent signal was measured on a STATFAX® 4400 (Lumate) luminometer and registered in RLU (relative light units), the intensity of luminescence correlates with the amount of a pseudovirus in a cell (Ryzhikov at al., 2012). Env-pseudovirus was used in further work if RLU exceeded the twofold value of spontaneous luminescence of TZM-bl cells by 50 times or more.

Electron microscopy. Cell-purified supernatant, containing pseudo-viral particles, was fixed by adding the equal volume of 8 % paraformaldehyde solution. The fixation time was 48 hours at +4 °C.

A standard negative staining was used for control counting, studying the forms and dimensions of virus-like particles (Harris, Horne, 1994). The supernatant was put on copper grids for electron microscopy, covered with carbon-stabilized formvar film. The samples were stained with 2 % aqueous solution of uranyl acetate.

The cell culture was separated from the surface with a rubber spatula and fixed in 4 % paraformaldehyde solution at +4 °C for 24 hours. Then it was rinsed in a buffer, addi-

tionally fixed with 1 % solution of osmic acid, dehydrated according to the standard procedure in increasing-concentration solutions of ethanol and acetone, and embedded in epon-araldite mixture. The procedure was described earlier in the literature (Sergeev at al., 2016). The semithin (1 μm) and ultrathin (50–60 nm) sections were prepared on a Reichert-Jung microtome (Austria). Semithin sections were stained with azure-II solution and studied in an AxioImagerZ1 light microscope (Zeiss, Germany). The ultrathin sections were stained with uranyl acetate and lead citrate and studied in a JEM 1400 electron microscope (Jeol, Japan) at accelerated voltage 80 kV. Image acquisition, image analysis and processing were performed using a Veleta digital camera (SIS, Germany) and iTEM software suit (SIS, Germany).

Pseudoviruses concentration. 20 ml of cell-purified supernatant were successively run through a Vivaspin 6 (300 000 MWCO) centrifugal concentrator (Sartorius, UK) in 5 ml doses (5 min per dose at the speed of 3000 rotations/min). We used the concentrator with a membrane filter with a 300 000 kDa molecular weight cutoff threshold, holding objects with a molecular weight higher than 300 000 kDa and letting pass macromolecules with a smaller molecular weight. Then the filter was taken out and some pieces, sized approximately 1 × 1 mm, were cut off from its different parts. Further sample preparation followed the technique described above for the cell culture. Embedding into resin, membrane pieces were oriented in such a way as to obtain a section through the entire thickness of the filter.

To determine a physical titre of pseudoviral particles in suspension, the average amount of such particles per length unit of the upper (from the side of the filtrated fluid) edge of the filter on the cutoff was counted. Based on the assumption about homogeneity of the fluid flow in the course of filtering on the filter area and the isotropy of the filter itself, it was believed that the density of particles on the filter is uniform in all directions, and, therefore, is the square of the linear density. The value was calculated for the full filter area – 4 cm^2.

Results

To count the amount of viral particles several methods can be used, including plaque-forming cells; quantitative RT-PCR; immunofluorescence microscopy; analytical flow cytometry; electron microscopy, etc. (Ferris et al., 2002; Reid et al., 2003; Malenovska, 2013; Heider, Metzner, 2014; Rossi et al., 2015).

Electron microscopy has some advantages over the above-listed methods since it gives information not only about the amount of all viral particles (regardless of whether they are infectious) but also about the morphology of an analyzed virus (Malenovska, 2013). Briefly, the method can be described as follows. An analyzed sample containing viral material is added a certain amount of suspension with a known concentration of latex beads. The mixture is put on copper grids covered with supporting film or adsorption for a particular period of time, or spraying of the mixture over the grids with an ultrasound probe. Then the electron-microscopic grids are analyzed in an electron microscope. Latex particles and viral particles are counted simultaneously in several grid elements. Knowing the latex concentration and comparing it with the amount of the detected viral particles, it is possible to estimate concentration in the initial sample. Naturally, this method is not very accurate, but convenient, and it is widely used for quantitative assessment of some viruses.

With such counting, errors are due to heterogeneous properties of the surface of the supporting film, aggregations of both latex and viral material, deviations from the experimental design, such as insufficient mixing or contamination during the spraying. The main limitation of negative staining method for counting the amount of viral particles by means of electron microscopy is insufficient sensitivity: the level of concentration required to obtain reliable results should be at least 10^7 particles/ml (Reid et al., 2003).

The study presents an original method (on the case of env-pseudoviruses) enabling to evaluate the amount of VLP in the samples, where their concentration is lower than 10^7 particles/ml.

HIV-1 virus-like particles (env-pseudoviruses) were chosen as a model. Env-pseudoviruses can penetrate into the cell similarly to live human immunodeficiency virus. Due to the deficient genome, though, they cannot form adequate virus progeny so they are safe to work with. Two types of plasmids are used to obtain env-pseudoviruses: envelope and packaging. Packaging, or core plasmid (pSG3Δenv) codes all HIV-1 proteins except the envelope ones. The second plasmid (pEnv), on the contrary, codes only HIV-1 envelope proteins. To verify infectivity of env-pseudoviruses, TZM-bl genetically engineered cells are used; their genome contains a luciferase gene that becomes active when getting to an HIV-1 or pseudovirus cell (Montefiori, 2009).

Co-transfection of 293T cells with two plasmids forms pseudoviral progeny capable of only single cells infecting, without further replication. Electronic microscopy of ultrathin sections was used to confirm pseudoviral particles assembly and yield (Fig. 1).

Pseudovirus was also characterized by defining functional activity with a single infection cycle in TZM-bl cell-target culture. The results are shown on Fig. 2. Signal intensity is given in luminescence standard units – RLU/ml. The findings are indicative of the functional activity of the virus.

However, using negative staining we did not reveal virus-like particles (VLP) with positive functional activity in the supernatant fluid. The most probable reason was a low particle concentration in the original fluid. To solve the task, we proposed a method of concentrating viral particles on polyethersulfone membranes used in centrifugal concentrators. Viral particles are deposited from suspension

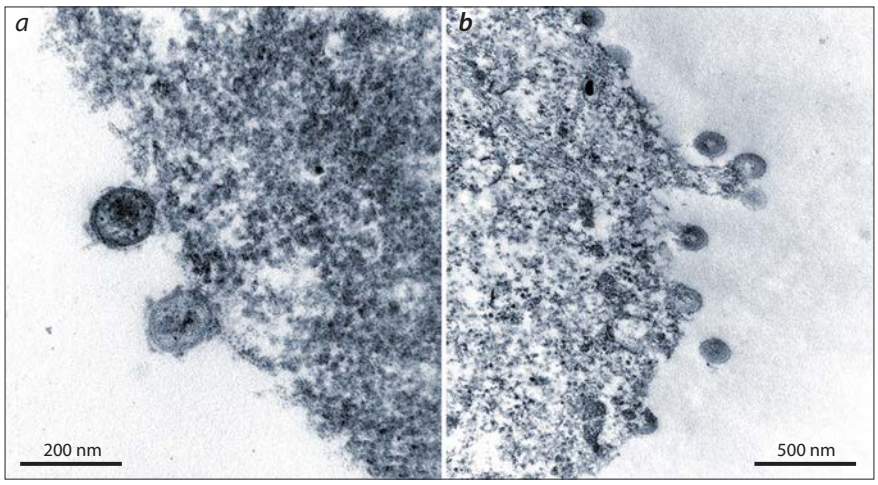

Fig. 1. *a*, Electron microscopic image of 293T cells with SF162 budded pseudovirus. *b*, Several pseudovirus particles of different maturity level.

Transmission electron microscopy, an ultrathin section.

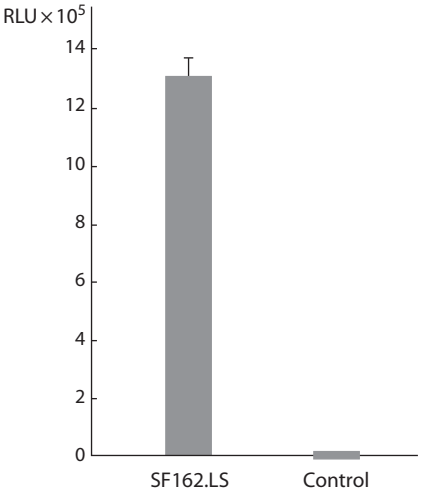

Fig. 2. Functional activity of SF162.LS pseudoviruses.

on a concentrator membrane by low-speed centrifugation. Henceforth, a part of the membrane is embedded into epon-araldite and used to make ultrathin sections for analysis with electron microscope. We used membranes with the 300 000 kDA cutoff threshold to entrap pseudoviral particles of around 100 nm diameter.

Figure 3 demonstrates VLP captured in a nanofilter during centrifugation. The particles are clearly discernable in the body and on the surface of the nanofilter, which allows to identify them morphologically and count (determine the average number per length unit of a filter section).

Assuming homogeneity of the fluid flow in the course of filtering on the filter area and the isotropy of the filter itself, we believed that the density of particles on the filter is uniform in all directions, and, therefore, is the square of the linear density. Particles were counted for the full filter area – 4 cm^2. On average, 3 virions per run-

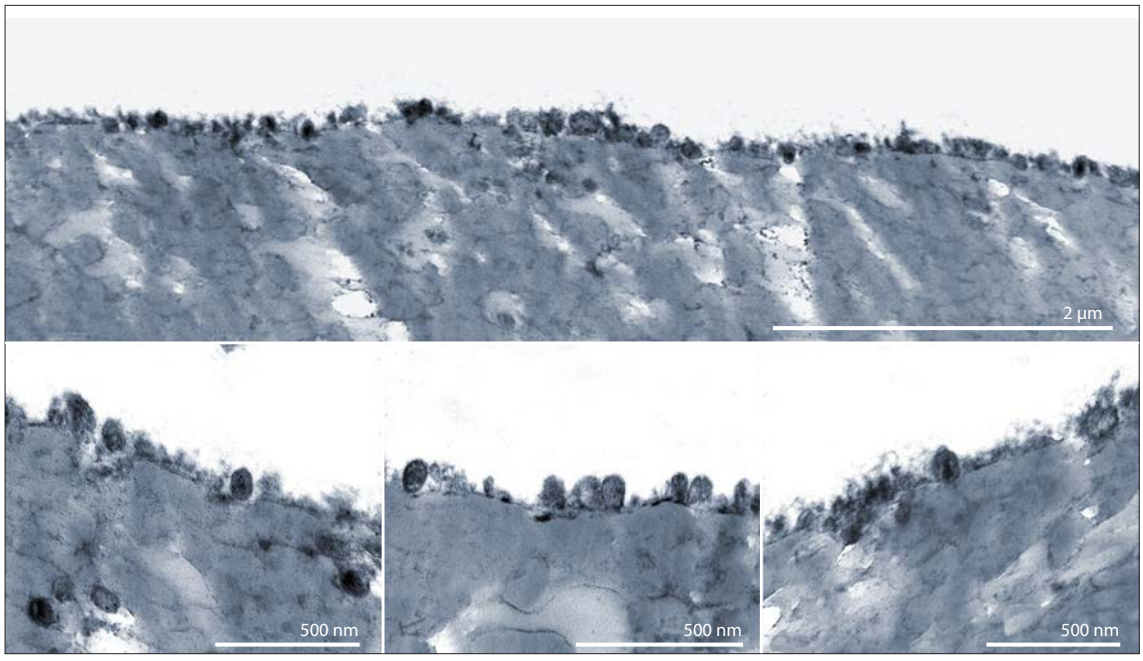

Fig. 3. Pseudovirus particles captured by a Vivaspin 6 filter (300 000 MWCO).

Transmission electron microscopy, ultrathin sections.

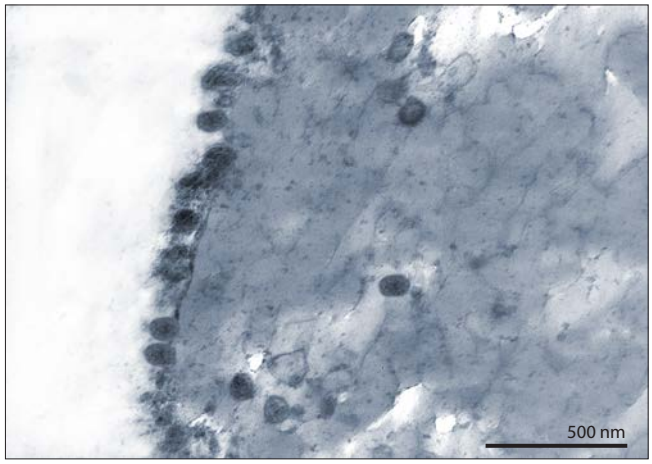

Fig. 4. Pseudovirus particles captured by a Vivaspin 6 (300 000 MWCO) filter. Particles are located both on the surface and inside the filter.
Transmission electron microscopy, ultrathin section.

ning 10 μm are observed on a section. Considering that the perpendicular density is the same, there are $3 \times 3 = 9$ virions per 100 μm^2. Thus, there are around 4×10^7 virions on the entire filter surface (the area of 4 cm^2). Understanding that the volume of supernatant containing pseudoviruses was 20 ml, the input concentration of pseudoviruses in a sample (4×10^7 particles/20 ml = 2×10^6 particles per 1 ml) can be determined. This value is by order lower than the value that can be registered when negative staining is used, which confirms our assumption (negative staining did not detect pseudoviral particles).

Conclusion

Thus, the proposed method overcomes the main disadvantage of virus quantitative estimation by means of electron microscopy, associated with a relatively high detection threshold (particles concentration – higher than 10^7/ml). As demonstrated on Fig. 4, the density of pseudoviruses on the section is quite high. It permits to expect that decreasing virion concentration in suspension by two more orders will also allow identifying the captured virions. In addition, centrifugal concentrator successively runs considerable suspension volumes through the same filter, which can also increase sensitivity of the method.

Comparing the proposed method with a widely used plaque-technique, we can emphasize the advantages related to the speed of work execution and possibility to determine the total titer of viral particles, including "noninfectious" particles. The plaque-technique detects only the particles that possess infectivity under the given conditions. In some cases, for instance, when characterizing vaccine specimens, it is necessary to know the amount of all particles, including noninfectious ones because they can also influence the host immune response (Blancett et al., 2017).

The developed method can be used for quantitative analysis of various samples containing viruses of animals, plants and humans, as well as noninfectious nanoparticles and virus-like particles. The method is especially valuable for analyzing specimens with a low content of viral particles. This approach increases sensitivity, accuracy and reproducibility of quantitative analysis made by means of electron spectroscopy.

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ORCID ID

B.N. Zaitsev orcid.org/0000-0001-6359-465X
O.S. Taranov orcid.org/0000-0002-6746-8092
N.B. Rudometova orcid.org/0000-0002-1684-9071
N.S. Shcherbakova orcid.org/0000-0002-0953-6333
A.A. Ilyichev orcid.org/0000-0001-5356-084
L.I. Karpenko orcid.org/0000-0003-4365-8809

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Molecular-genetic bases of plumage coloring in chicken

A.V. Makarova, O.V. Mitrofanova, A.B. Vakhrameev, N.V. Dementeva

Russian Research Institute of Farm Animal Genetics and Breeding – Branch of the L.K. Ernst Federal Science Center for Animal Husbandry, Pushkin, St. Petersburg, Russia
e-mail: dementevan@mail.ru

The color of plumage in birds is an important feature, often determining descent to a particular species or breed. It serves as a key factor in the interaction of birds with each other due to their well-developed visual perception of the surrounding world. In poultry including chickens, the color of the plumage can be treated as a genetic marker, useful for identifying breeds, populations and breeding groups with their specific traits. The origin of diverse color plumage is the result of two interrelated physical processes, chemical and optical, due to which pigment and structural colors in the color are formed. The pigment melanin, which is presented in two forms, eumelanin and pheomelanin, is widely spread in birds. The basis for the formation of melanin is the aromatic amino acid tyrosine. The process of melano-genesis involves many loci, part of the complex expression of plumage color genes. In birds, the solid black color locus encodes the melanocortin 1 receptor (*MC1R*), mutations in which lead to a change in receptor activation and form different variants of the E locus. Using the GWAS analysis, possible genes affecting the formation of color in chickens were detected. The biosynthesis and types of melanin are affected by the activity of the enzyme tyrosine, and mutations in the tyrosinase gene (*TYR*) cause albinism in different species. The formation mechanism of brown, silver, gold, lavender and a number of other shades is determined by the influence on the work of the *MC1R* genes and *TYR* specific modifier genes. Thus, locus I currently associated with the *PMEL17* gene inhibits the expression of eumelanin, and the *MLPH* gene affects tyrosinase function. Research on the mechanisms of formation of the secondary coloring of plumage in chickens is being actively conducted nowadays. The formation of a marble feather pattern is associated with the mutation of the endothelin B2 receptor (*EDNRB2*), in the coding part of the gene of which a polymorphism is found associated with the mo locus. The molecular base that causes the feather banding (locus B and autosomal recessive banding) is identified. Today, only some genes that determine the color of the plumage of chickens are studied and described. Different genes can produce similar plumage patterns, and different phenotypes can be determined by the polymorphism of a single gene. Using molecular methods, you can more accurately identify these differences. This overview shows the nature of melanin coloration in birds using the example of chickens of various breeds and also attempts to systematize knowledge about the molecular-genetic mechanisms of the appearance of various types of coloration.
Key words: chickens; coloring plumage; genes; locus; expression; markers.

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Молекулярно-генетические основы формирования окраски оперения у кур

А.В. Макарова, О.В. Митрофанова, А.Б. Вахрамеев, Н.В. Дементьева

Всероссийский научно-исследовательский институт генетики и разведения сельскохозяйственных животных – филиал Федерального научного центра животноводства – ВИЖ им. академика Л.К. Эрнста, Пушкин, Санкт-Петербург, Россия
e-mail: dementevan@mail.ru

Окраска оперения – важный признак у птиц, нередко определяющий принадлежность к тому или иному виду или породе. Окраска является результатом действия веществ, которые поглощают определенную длину волны и формируют так называемые пигментные цвета, и оптическим эффектом, обусловленным интерференцией света, отраженного биологическими микроструктурами пера. Основой для формирования окраски служит синтез меланина. Эумеланин ответственен за черные и коричневые оттенки, а феомеланин отвечает за красновато-коричневые оттенки. Молекулярно-генетический механизм появления того или иного типа окраски еще до конца не изучен, поскольку на один и тот же признак могут влиять несколько генов. Первичная пигментация оперения определяется взаимодействием полиморфных вариантов гена *MC1R* и генов, участвующих в регуляции меланогенеза. Гены-модификаторы вызывают изменение окраски любого генотипа по локусу E и могут как уменьшать или увеличивать экспрес-

сию эумеланина, так и разрушать меланоциты. Вторичная пигментация оперения определяется белыми пятнами или специфическим распределением эумеланина на отдельных перьях. Современные методы анализа ДНК, такие как секвенирование, полногеномный анализ с использованием чипов различной плотности, анализ экспрессии генов, позволяют получать новые данные о генах, определяющих окраску оперения.
Ключевые слова: *Gallus domesticus*; куры; окраска оперения; гены; локус; экспрессия; маркеры.

Introduction

Plumage color is an important feature in birds, often determining assignment to a particular species or breed. It was the color of the plumage that formed the basis for the development of such paradigms in biology as the theory of speciation. The color of the plumage largely determines how animals communicate with each other and plays an important role in adapting to environmental conditions (Cott, 1940). Birds have a variety of feather color patterns, which gave rise under the pressure of natural selection (Roulin, 2004; Roulin, Ducrest, 2013).

Plumage coloration is the result of two different but interrelated physical processes: (a) the chemical mechanism creates coloration as a result of substances that absorb a certain wavelength and form so-called pigment colors; and (b) the optical mechanism due to interference of light reflected by the biological microstructures of the feathers, which creates structural colors. The latter mechanism allows the creation of colors that cannot be generated only by pigments, but specialized microstructures often require the presence of pigments that absorb certain wavelengths to produce structural colors (D'Alba et al., 2012). Consequently, pigment and structural colors are not the result of two independent processes, but rather are the basis responsible for all the variety of color.

Birds are characterized by a wide variety of color plumage. This is due to the fact that they, unlike mammals and humans, in birds take place visual perception of relatives, interaction with them plays a leading role (Negro et al., 2016). The pigments responsible for this diversity are deposited not only in the feathers but also in the not feathered parts of the body such as the beak and legs. In birds, three groups of pigments that give variations in the color of the plumage are described: melanin, carotenoids and unusual colors (for example, porphyrin). Most of these pigments are present only in certain groups of birds птиц (Lopes et al., 2016; Brelsford et al., 2017; Cooke et al., 2017). The most widespread in birds are melanins and carotenoids. Melanins are usually more common, and in some species (e. g. swallows) melanin levels are an order of magnitude higher than carotenoid levels (McGraw et al., 2004).

In poultry, including chickens, plumage color can serve as a genetic marker, useful for the identification of breeds, populations and breeding groups with their characteristic features (Moiseyeva et al., 2012; Mitrofanova et al., 2017). The molecular genetic mechanism of the appearance of a particular type of color is not yet fully understood, since several genes can affect the same trait. Some genes cause primary effects of color, others play the role of modifiers and regulators that affect the zonal and regional distribution of the pigment, its distribution within individual feathers (banding, spotting, edging and other patterns) (Yurchenko et al., 2015). This division is conditional and the manifestation of the pigment may differ in the color of down, juvenile and adult plumage of chickens (Serebrovsky, 1926; Crawford, 1991; Yang et al., 2017).

In this review we consider the nature of melanin coloration in birds on the example of chickens of different breeds, as well as molecular genetic mechanisms of the appearance of different types of this color. As an example of different colors gene pool breeds from the Bioresource Collection "Genetic collection of rare and endangered breeds of chickens" (http://vniigen.ru/ckp-geneticheskaya-kollekciya-redkix-i-ischezayushhix-porod-kur/) is presented.

Biochemistry of melanin synthesis

The most common pigment in birds is melanin, which describes 2 types – eumelanin and pheomelanin. Eumelanin – a larger form responsible for black and brown shades, pheomelanin – is responsible for reddish-brown shades. These pigments are produced endogenously in peripheral tissues such as skin, in specialized melanocyte cells.

Melanocytes are most common in skin, hair, follicles of the feathers and in the eyes (Dupin, Le Douarin, 2003). They are also found in the inner ear, esophagus, thyroid gland, bones, heart and even brain, for example, neuromelanin (Zucca et al., 2014).

In mammals and poultry, melanin is produced in small organelles called melanosomes, which contain all the enzymes necessary for the pigmentation process. Depending on the structure and location of the melanosomes, the colour of the birds plumage may change (Maia et al., 2013; Nordén et al., 2018). Figure 1 shows the structure of the feather follicle during the rest and growth phases. Resting melanocyte progenitor cells are present at the base of the feather. If the feather is broken or lost as a result of molting, melanocyte progenitor cells activate and migrate up the growing stem of the feather, divide and differentiate into melanocytes producing the pigment.

Avian melanins are formed from the aromatic amino acid tyrosine (Lerner, Fitzpatrick, 1950). The enzyme tyrosinase catalyzes the initial oxidation of tyrosine to dopaquinone, which is an intermediate for the synthesis of both types of melanin. If additional enzymes TRP1 and TRP2/DCT are activated, the synthesis of black eumelanin will occur. In addition to genetic control, melanogenesis can be affected by environmental or physiological conditions, and the color will depend on the season, sex and shape of the cover. Melanin is influenced by 4 classes of hormones: androgens, estrogens, pituitary hormones (luteinizing hormone) and thyroid hormones. Melanin can interact with other pigments, giving a complex manifestation of the color of feathers. In addition, the colouring due to the feather structure is also applied (Rzepka et al., 2016).

Yellow pigment requires additional amino acid – cysteine. For example, high levels of cysteine in the environment

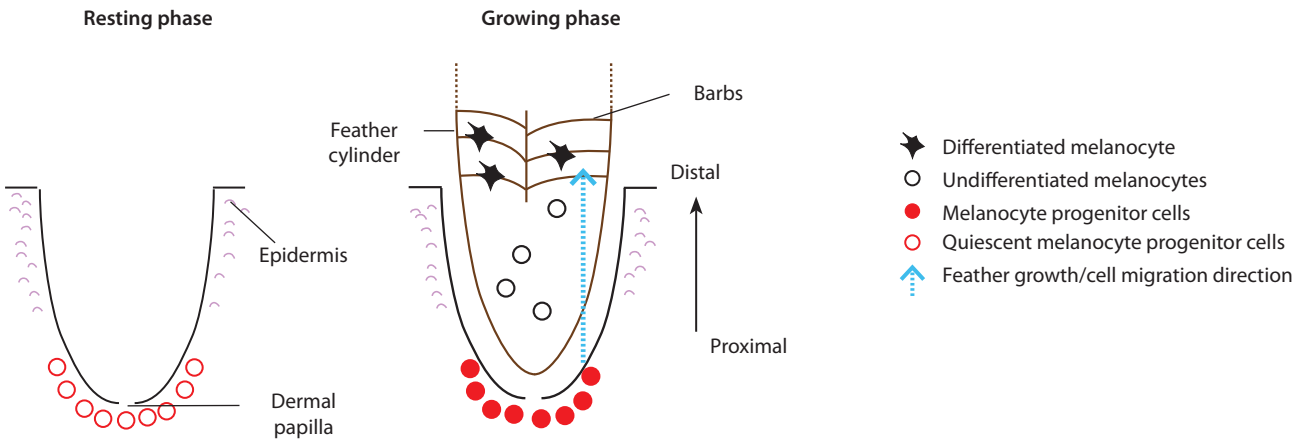

Fig. 1. Anatomy of the feather follicle during resting and growing phase (Schwochow-Thalmann, 2018).

may lead to increased synthesis of pheomelanin (Smit et al., 1997; Land, Riley, 2000). This situation will also be influenced by other factors (Ancans et al., 2001), or, for example, when *TYR* concentration or activity is low (Ozeki et al., 1997; Ito et al., 2000), when pathways that suppress eumelanin production are activated, for example, the agouti signal pathway (Takeuchi et al., 2000; Wolff, 2003). Higher expression of *TRP1* and *TRP2*/DCT correlates with dark pigmentation in several birds such as chickens, ducks, Chinese painted quails, pigeons, and geese (Galvan, Solano, 2016; Galvan et al., 2017). In addition, some hormones such as α-melanocytic stimulating hormone (αMSH) as well as steroid hormones (e. g. testosterone) affect melanogenesis, usually by increasing the production of eumelanin (Strasser, Schwabl, 2004; Eising et al., 2006) (Fig. 2).

Given the diversity of bird pigments and their functions, understanding the molecular basis of these processes remains poorly understood. Most of all, to date, knowledge about the synthesis of melanin has been accumulated.

The process of melanogenesis includes phases with multiple loci involved in the complex expression of the plumage color genes (Doucet et al., 2004; Baiao et al., 2007; Uy et al., 2009; Johnson et al., 2012). Molecular studies in mammals and birds have shown that the solid black locus encodes the melanocortin receptor 1 (*MC1R*) (Takeuchi et al., 1996; Mundy, 2005). This receptor is embedded in the melanocyte membrane and encoded by a small gene (less than 1000 bp). When

Fig. 2. The effect of testosterone level on sexual dimorphism of the color of Faverolles (*a*, *b*) and Leghorn Light Brown (Italian Partridge) (*c*, *d*).

In the photographs, gene pool breeds from the Bioresource Collection "Genetic collection of rare and endangered breeds of chickens". Authors of all the photos A. Sanganaeva, A.B. Vakhrameev.

Fig. 3. Reduction *MC1R* activity from eumelanin of completely black color (Pantsirevskay Black breed) – allele E (*a*), through birch color E^R (Uzbek Game) (*b*) to strengthening of feomelanin color at e^{Wh} (New Hampshire) (*c*) and e^y – buff (Buff Leghorn) (*d*).

linking with his antagonist αMSH there is a change in conformation of the receptor, activation of adenylylcyclase that causes the transition of ATP to form cyclic AMP. Increasing the level of cyclic AMP leads to activation of the *CREB* (cAMP response element-binding protein) and *MITF* (microphthalmia-associated transcription factor) trascription factors (Schiaffino, 2010). Higher *MC1R* activity usually results in darker pigmentation, while lower activity contributes to the production of pheomelanin (Garcia-Borron et al., 2005).

Primary pigmentation of plumage

The basic or zonal distribution of black eumelanin throughout the body of chickens is determined by mutations in the *MC1R* gene, leading to a change in receptor activation, which explains the color options for the locus E in chickens (Smyth, 1990; Sazanov et al., 1998; Kerje et al., 2003; Ling et al., 2003; Hoque et al., 2013). The locus E alleles includes: E – all black paint (Minorca, Black Australorp, Pantsirevskaya); E^R – birch colour (Yurlov crower breed); e^{Wh} – dominant wheaten (New Hampshire); e^+ – wild type colour (Italian Partridge, see Fig. 2, *c*, *d*); e^b – brown (Zagorsk salmon, Faverol; see Fig. 2, *a*, *b*); e^{bc} – Buttercup (Sicilian Buttercup); and e^y – recessive wheat (Rhode island) (Fig. 3). These alleles influence the distribution of melanin pigments (eumelanin and pheomelanin) in feathers (Serebrovsky, 1926; Somes et al., 1988; Davila et al., 2014).

Studies of Davila et al. (2014) showed that haplotypes of the gene *MC1R* explain the color changes of the *CSD* locus E of different breeds. Eleven haplotypes for 7 significant SNPs have been discovered. The association for the distribution of these haplotypes for alleles of locus E has been found. The greatest number of haplotypes known to breeds with a black, birch and blue colors of the plumage, whereas partridge and red breed was monomorphic. Davila et al. (2014) suggested that the Glu92Lys mutation may be responsible for activating the receptor for producing eumelanin, being a necessary but not always sufficient condition for maximum expression of the black phenotype. Another mutation Arg213Cys may be the cause of the loss or reduction of function of the receptor for the production eumelanin, and mutation Ala137Thr might be a candidate to mitigate Glu92Lys. The observed joint segregation of alleles and polymorphisms in E and *MC1R* confirms that E locus is equivalent to *MC1R*.

Recently, attempts have been made to conduct a genome-wide association search (GWAS) of black plumage with individual SNPs on chips of different densities. Park's general study with co-authors (2013) using the Illumina 60K chip revealed 12 significant color-associated SNPs. In the intron region of *AKT3*, SNP (rs14339964) was found, located on chromosome 3, and which is known to be one of the key genes in the formation of melanoma cells (Tsao et al., 2012). Thus, the authors conclude that *AKT3* mutations may be associated with pigmentation of the plumage. The other two SNPs (GGaluGA344987 and rs14641648 on chromosome 3 and 8, respectively) are located in the intergene region near the genes *KRT7* and *PAP2*, which are associated with pigmentation. *PAP2* (*LPPR5*) increases pigmentation (Shan et al., 2009), and *KRT7* is a member of the keratin gene family and is associated with melanocytic tumors (Blum et al., 2010). The detected polymorphism in the intron of the *DDX6* gene may also be associated with coloration, as it is an established gene causing vitiligo skin disease (Tang et al., 2012).

Yang and co-authors (2017) identified 13 significant SNPs in 10 genes using Affymetrix 600K HD chip. They found most likely affecting the synthesis eumelanin, candidate genes *SHH* and *NUAK*. Based on previous studies of model species, Yang and co-authors (2017) suggested that *NUAK 1* kinase genes and *SHH* signaling gene may

play a role in the development of melanoblast cells during the embryonic period, which also affects feathers pigmentation.

On the biosynthesis and types of melanin influences the activity of tyrosinase (Chang et al., 2006). It was found that the lack of its function leads to a complete loss of melanin in the skin, feather, retina and causes albinism in different species. Tyrosinase is an important enzyme in the biogenesis of melanin in pigment cells (Niwa et al., 2002). In studies of Liu et al. (2010), the tyrosinase (*TYR*) and melanocortin 1 (*MC1R*) genes were recognized as the main genes involved in pigmentation of chick plumage. Profiles change the color of the plumage and the gene expression levels of *TYR* and *MC1R* were observed from birth until the age of 112 days. The level of expression of *TYR* was maximum in 1-day age and then sharply decreased during the studied ages; the expression level of *MC1R* was higher on day 28 in comparison with other ages. *TYR* expression in chickens carrying E/E and E/e alleles at the *MC1R* locus was higher from birth to 28 days than in those carrying e/e alleles. These studies have shown that the mechanisms that affect the color of down in the 1-day age and those that regulate the color of the plumage at a later age are different. In addition, although the *TYR* gene in interaction with the *MC1R* gene are the determining factors for plumage coloration, different phenotypes did not correspond to different genotypic classes for both the *TYR* and *MC1R* genes, and the recessive white variation of the *TYR* gene could not completely block melanin synthesis until 28 days. Therefore, day-old chickens were colored according to the allele of locus E (Liu et al., 2010).

A study by Chang et al. (2006) showed the insertion of a full-size retrovirus inside the intron 4 of the *TYR* gene in recessive white chickens (Chang et al., 2006; Kuliawat, Santambrogio, 2009) resulting in impaired tyrosinase expression. Such recessive epistasis is typical for some white-colored breeds, for example, Silkie (Fig. 4). Deletion in the *TYR6* gene of nucleotides (-GACTGG) led to autosomal albinism (Tobita-Teramoto et al., 2000).

Genes *MC1R* and *TYR* are the molecular genetic basis for the formation of color plumage in chickens. Other genes are modifiers of their expression.

Genes-modifiers

A change in the color of any genotype at locus E can be induced by the dominant allele I, which inhibits the expression of eumelanin, destroying melanocytes. Locus I is associated with *PMEL17* gene, located on chromosome 33 in chickens, encodes protein specific to melanocytes which are important for the normal development of eumelanosomes (Keeling et al., 2004; Kerje et al., 2004; Natt et al., 2007). Locus I has 4 alleles: dominant white (I), Smoky (I^S), partially restores pigmentation and gives grayish phenotype, it is recessive for dominant white, but partially dominant for wild type allele (i), Dun (I^D) inhibits only eumelanin expression and gives brown color (Galeotti et al., 2003; Karlsson et al., 2010; Gaudet et al., 2011). Dominant white was found in White Leghorn and was associated with the insertion of 9 bp in exon 10 *PMEL17*, which led to the introduction of three amino acids into the transmembrane region. Similarly, there was a deletion of five amino acids in the transmembrane region in a protein encoded by Dun. Allele Smoky appeared already in White Leghorn and includes both 9-bp-insertion in exon 10 and deletion of 12 nucleotides in exon 6, excluding four amino acids from the protein.

Fig. 4. Silkie White breed of chickens. Recessive epistasis of the *TYR* gene.

Dark brown (Db) mutation in chickens reduces the expression of black eumelanin and increases the expression of red pheomelanin, but only in certain parts of the plumage. Gunnarsson with co-authors (2011) suggested association of the Db phenotype with 8.3 kb deletion located 14 kb above the *SOX10* gene on chromosome 1, which is an important transcription factor in melanocytes and some other cell types. The mechanism of action of this mutation suggests that deletion leads to a decrease in the expression of the *SOX10* gene, which in turn reduces the expression of key enzymes in the synthesis of pigments, such as tyrosinase. Further, tyrosinase leads to a shift towards more pheomelanin (reddish) colors of the plumage, which is characteristic of genotype *Db*. The dark brown allele is particularly interesting because it affects the nature of pigmentation rather than the presence or absence of pigmentation. A simple diagnostic test to determine the *Db* genotype will facilitate the study of other loci associated with feather color.

One more gene influencing the expression of the tyrosinase gene is *MLPH* (Vaez et al., 2008; Bed'hom et al., 2012; Xu et al., 2016). Vaez and co-authors (2008) studied the blue (LAV) coloration of chicken plumage based on orthology with the gene found in mice. They found a single-nucleotide polymorphism that weakens the color of gene E. Later, mutations in the *MLPH* gene leading to the formation of lavender color of the plumage were found in quails (Bed'hom et al., 2012). Xu and co-authors on the example of Ani chickens confirmed the connection of LAV color with mutations in this gene (Xu et al., 2016).

On chromosome Z in *Gallus gallus* there is a gene which determines golden and silver feathers (Gunnarsson et al., 2007). It forms a series of alleles S*S (silver), S*N (wild type/Golden) and S*AL (sex-related imperfect albinism) (Fig. 5).

In the orthologous locus AL, sex-related albinism (AL*A) was also found in the Japanese quail (*Coturnix japonica*). The determining color factor is the protein SLC45A2, which plays an important role in the sorting of vesicles in melanocytes. Mutation 106delT in allele S*AL chickens leads to a shift of the reading frame, formation of stop codons and degradation of the corresponding mRNA.

Fig. 5. Allele S – silver plumage color in Pervomayskaya breed (*a*, *b*) and allele s – Golden color in Poltavskaya clay breed (*c*, *d*).

Mutation in Japanese quail A-allele causes slippage of exon 4. Two independent missens mutations Tyr277Cys and Leu347Met were associated with silver allele in chickens. A special feature of the SLC45A2 variants is the specific inhibition of red pheomelanin in silver chickens (Gunnarsson et al., 2007). It remains unknown why mutations at this locus cause an almost complete absence of both eumelanin and pheomelanin, while some missens mutations are dominant and cause specific inhibition of pheomelanin production.

Oribe and co-authors (2012) studied the signal protein aguchi (ASIP), a paracrine factor that stimulates the synthesis of pheomelanin and inhibits the synthesis of eumelanin in follicular melanocytes. In mammals, the distal promoter of the *ASIP* gene acts exclusively on the ventral side of the body, creating a protective spotty color of pigmentation, stimulating the synthesis of pheomelanin on the abdominal side. ASIP produces spotting in chickens and adult females, similar to mammals. In addition, the promoter of class 1 of this gene plays an important role in creating estrogen-controlled sex differences.

Secondary color of plumage

Secondary pigmentation of the plumage is determined by white spots or specific distribution of eumelanin on individual feathers (Smyth, 1990). Molecular genetic studies have significantly expanded the field of knowledge of the genetic mechanisms of formation of such a color. In some breeds of chickens around the world there is a motley color of the plumage, where the tip of the plow is painted white. The formation of such a marble feather pattern is associated with a mutation of the endothelin receptor B2 *(EDNRB2*) located on chromosome 4 (Kinoshita et al., 2014). These studies found a polymorphism in the coding region of *EDNRB2*, leading to the replacement of Arg332His, which is associated with the locus *mo*.

Another G1008T mutation causes replacement of the amino acid Cys244Phe in exon 5 and provokes defective protein binding to endothelins. As a result of such replacement, the differentiation, proliferation and migration of melanocytes is changed. The plumage of mo^w/mo^w chickens is lightened to almost white color with several partially pigmented feathers. It is proved that such a phenotype is not associated with the tyrosinase gene and showed an autosomal recessive type of inheritance against the pigmented phenotype. Unlike albinos, mutant chickens mo^w/mo^w had painted the iris of the eyes and some pigmented spots on a whitish-yellow fluff. This mutation was also present in individuals of four Japanese breeds with white plumage. The results indicate that EDN3 (endothelin 3) – *EDNRB2* signaling is necessary for normal pigmentation in birds (Kinoshita et al., 2014).

Somes (1980) speaks of six phenotypes produced in different combinations of the gene *mo* with other color genes. In the Bioresource Collection "Genetic collection of rare and endangered breeds of chickens" there are several breeds containing allele *mo* in its genotype and having three different phenotypes (Fig. 6): black-and-white Australorp, mille fleur breed chickens, and Pushkin breed.

In modern breeds of chickens there is often a striped color of the plumage, coupled with the sex, which is characterized by a completely white stripe on the main background of the plumage and caused by the so-called barring effect. Weakening of the color is observed both in the plumage of an adult bird and in the fluff of day-old chickens (Campo, 1991; Alekseevich et al., 2000; Dorshorst, Ashwell, 2009).

Sex-linked banding is determined by the B-locus associated with the *CDKN2A* gene (Hellström et al., 2010, 2011). Locus B lightens the dermal pigment in

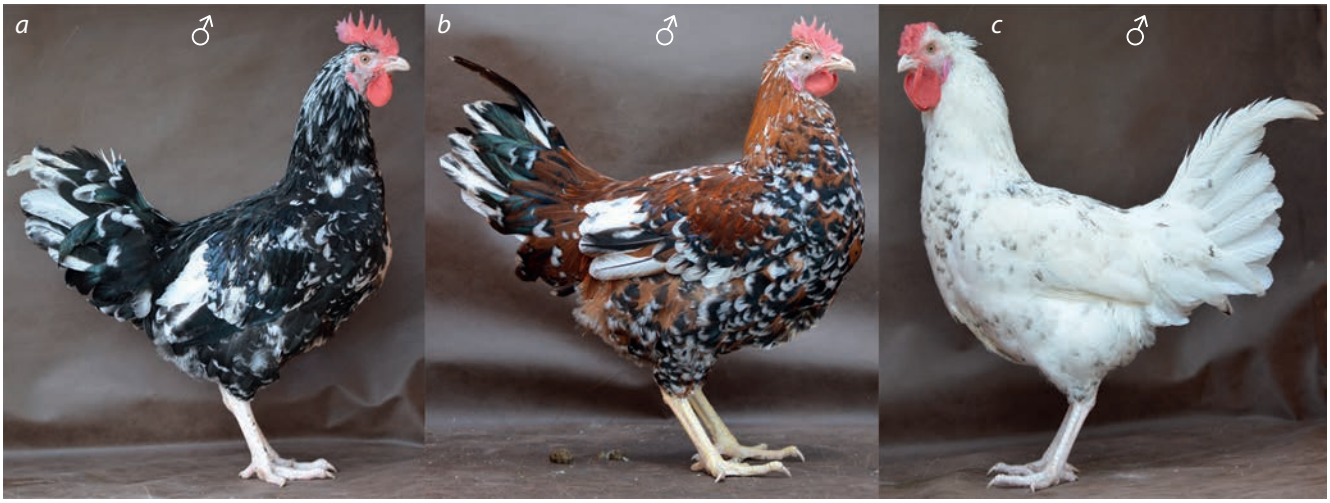

Fig. 6. Three different phenotypes in different combinations of the mo gene with other color genes: *a*, black-and-white color of plumage (Australorp black-and-white); *b*, mille fleur color of plumage (Leningradskaya Mille Fleur); *c*, white slightly-motley color of plumage (Pushkinskaya breed).

the shanks, beak and limits the spread of black pigment, creating a striped feather pattern (Jerome, 1939). Since the B gene is located in the Z chromosome, it can only be homozygous in roosters, and hemizygous in hens. The degree of pigmentation weakening depends on the homo- or heterozygous state of the allele (Kogan, 1979) (Fig. 7).

In studies by Schwochow Thalmann with co-authors (2017) it was found that the sex-linked striped pattern of plumage in chickens is associated with two non-coding and two coding mutations affecting the transcription of ARF in the locus of the *CDKN2A* tumor suppressor. These mutations form four functionally different alleles – BN, B1, B2 and B0. The last allelic variant is characterized by extreme dilution of melanin (Schwochow Thalmann et al., 2017). These allele variants were formed from four SNPs located in the 12 kb region, including exon 1 *CDKN2A*. Two of the SNPs were in non-coding regions, SNP1 in the promoter and SNP2 in intron 1. The other two SNPs are missense mutations. SNP3 causes valine to be replaced by aspartic acid (V9D), while SNP4 causes arginine to be replaced by cysteine (R10C). Haplotype B1 forms SNP1, SNP2, SNP3. Haplotype B2 includes SNP1, SNP2, SNP4 and B0 – SNP1, SNP2.

In addition to the striped color of the plumage linked to the sex, there is an autosomal striped pattern of the plumage in chickens. Black stripes on a white or red background, in this case,

Fig. 7. Plymouth Rock Barred. Lighter color of rooster plumage (B1/B1) compared to hen (B1/–).

are induced, perhaps, not by blocking, but by increasing melanogenesis against the background of recessive variants E. The molecular basis of such expression has not yet been sufficiently studied.

Change of the color type largely depends on changes in the number eumelanin and pheomelanin feather pigment (Guernsey et al., 2013), which creates a lot of different variations in the basic plumage. For example, the brown color in different breeds of chickens varies from dark brown (Rhode Island Red) to Golden or pale yellow (Brama pale yellow, experimental Tsarskoselskaya population).

Genes that regulate the variability in color, may have a pleiotropic effect and influence other economically useful traits of chickens. It is possible to use it as a marker of the intensity of growth and identification of certain diseases of the bird. For example, the endothelin receptor gene *EDNRB2* is associated with the ability of Tibetan chickens to hypoxic adaptation in mountain conditions (Zhang et al., 2017). Polymorphism in *TYR* tyrosinase gene promoter determines the black color of skin and bones in chickens, which is important in the selection of birds for breeding on these traits (Yu et al., 2017). Interactions between pigmentation genes and

Loci forming the basic types of plumage in chickens

Locus	Chromosome	Genotype	Color	Breed	Mutation type	References
MC1R	11	E	Black	Minorca, Australorp Black	Haplotype H1 (G274A)	Dávila et al., 2014
		E^R	Birchen	Yurlov Crower	Haplotypes H1, H4, H5, H6	
		e^{Wh}	Dominant wheaten	New Hampshire	Haplotype H7 (A427G)	
		e^+	Wild type	Leghorn Light Brown (Italian Partridge)	Haplotype H0 (reference sequence)	
		e^b	Brown	Zagorsk Salmon, Faverolles	Haplotype H9 (4SNP)	
		e^{bc}	Buttercup	Buttercup	Haplotypes H10, H7, H1	
		e^y	Recessive wheaten	Some lines of Rhode Island	Haplotype H11 (C637T)	
EDN3	20	FM	Intense black pigmentation of internal connective tissue and the exterior skin	Silkie White	Duplication and inversion of *EDN3*	Dorshorst et al., 2011
SOX10	1	Db	Dark brown	Friesian Fowl	8.3-kb deletion upstream of the SOX10 transcription start site	Gunnarsson et al., 2011
CDKN2A	Z	B0	White	Intercross between the red Junglefowl and the White Leghorn	A combination of three SNPs: two in a gene promoter, an SNP in an intron, a combination of two SNPs	Schwochow Thalmann et al., 2017
		B1	Sharp white and pigmented stripes	Plymouth Rock	A combination of three SNPs: one in a gene promoter, an SNP in an intron, a combination of two SNPs	
		B2	Light male chicken, Striped female chicken	Intercross between the red Junglefowl and the White Leghorn	A combination of three SNPs: one in a gene promoter, an SNP in an intron, a combination of two SNPs	
PMEL17	33	I (dominant white)	White with red/brown	White Leghorn	9-bp insertion in exon 10	Kerje et al., 2004
		D	Lighter than wild type	Dun	15-bp deletion	
		S	Smoky	Smoky	12-bp deletion in exon 6	
SLC45A2	Z	Al	White	Intercross between the red Junglefowl and the White Leghorn	A 1-bp deletion (106delT)	Gunnarsson et al., 2007
		S	Silver	Yurlov Crower	Two independent missense mutations (Tyr277Cys and Leu347Met)	
MLPH	7	LAV*L	Lavender	Orpington	C103T transition	Vaez et al., 2008
TYR	1	C*C	White	Silkie White	Insertion of a complete avian retroviral sequence of 7.7 kb in intron 4	Chang et al., 2006
		c^a	White	White Leghorn	Deletion of six nucleotides (–ΔGACTGG)	Tobita-Teramoto et al., 2000
EDNRB2	4	mo^w	Recessive White	Minohiki	G1008T substitution in exon 5	Kinoshita et al., 2014
		mo	Mottled	Cochin Dwarf, Australorp Black Speckled	C300T, A320G and G1272A	

the environment can contribute to the formation of melanoma and tumors (Gudbjartsson et al., 2008; Ibarrola-Villava et al., 2012). In quails, several mutations in the *MLPH* gene linked to the lavender color of the plumage lead to a decrease in live weight (Bed'hom et al., 2012).

The Table shows the characteristics of loci mapped on the chromosomes of chickens and determine the basic variants of plumage color, and also lists the main genes involved in the processes of pigmentation of plumage in chickens

The evolution of the *MC1R* gene

Melanocortin 1 receptor, which plays an important role in the formation of plumage color in chickens, is a representative of the whole family of G-protein-binding receptors, which are involved in a number of important functions of the body, including the regulation of energy balance.

Endogenous ligands-agonists in melanocortin system are α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH). It is now known that five MCR subtypes mediate the action of these ligands (Schiöth, 2001). Apparently, all of them are found in most mammals, as well as in chickens (Takeuchi, Takahashi, 1998).

The melanocortin 1 receptor (*MC1R*) is primarily expressed in the skin and plays a role in skin, hair or fur pigmentation in most mammals, as shown in study of several mutations in this gene (Rees et al., 1999). In chickens, mutations in the gene *MC1R* are correlated with pigmentation of feathers (Takeuchi et al., 1996). This receptor also mediates the anti-inflammatory action of MSH peptides.

Little is known about the evolutionary origin of the melanocortin receptor gene family. Hen MCR is found in a much wider range of tissues compared to mammals, but their physiological effects are still unclear.

Schiöth and co-authors (2003) used available comparative cartographic information to determine the likely chromosome associated with the *MC1R* gene. *MC1R* in *Gallus domesticus* is located on *GGA11*, confirmation of this was obtained in two-color FISH experiments, which clearly showed consistent hybridization labeled with Biotin *MC1R* on the same chromosome, as labeled with digoxygenin ADL02232 and MCW0097. The latter are known to be present on *GGA11* (Schiöth et al., 2003).

The latest work also carried out phylogenetic analysis of the MCR family based on the method of maximum economy (MEGA2) using full-size amino acid sequences of each receptor. It was shown that the genes responsible for *MCR1* receptors form a separate cluster of genes, which probably arose during duplication.

Conclusion

The color of the plumage in birds is a trait that used as a key factor in the interaction of birds with each other due to their well-developed visual perception of the world. In poultry, including chickens, plumage color determines decorative qualities and is a marker for the identification of breeds, populations and breeding groups. The variety of plumage color is formed as a result of two interrelated physical processes – chemical and optical, through which pigment and structural colors are formed. The most common pigment in birds is melanin, for which 2 types are described – eumelanin and pheomelanin. Pigmentation of the plumage is caused by the distribution of black eumelanin throughout the body of chickens and is determined by mutations of the *MC1R* gene, which describes several haplotypes that explain changes in the color of chickens at the E locus in different breeds.

Melanogenesis can be influenced by hormones and enzymes. Genes *DN3E*, *SOX10*, *PMEL17*, *SLC45A2*, *MLPH* and *TYR* are molecular genetic modifiers in the formation of plumage color in chickens. Mutations in these genes alter the level of expression, which determines the biosynthesis and types of melanin. Some of them inhibit or reduce the formation of black eumelanin, others increase the amount of red pheomelanin. Variants of the specific distribution of pigments on individual feathers, forming a marble and striped pattern, which is associated with a mutation of the endothelin receptor B2 *(EDNRB2*) and mutations in the *CDKN2A* gene, are described. This review examines the nature of melanin coloration in birds on the example of chickens of different breeds, and also attempts to systematize knowledge about the molecular genetic mechanisms of the appearance of different types of coloration.

Despite the fact that the genome of chickens is well studied, not all genes affecting the color are described. Additional difficulties are associated with the fact that different genes sometimes produce the same pattern of plumage, and polymorphism of one gene can determine different phenotypes. The use of new modern methods of DNA analysis, such as sequencing, genomic analysis using chips of different densities, expression analysis on poultry from gene pool populations will provide new data on genes that determine the color of the plumage.

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ORCID ID

A.V. Makarova orcid.org/0000-0002-3281-4581
O.V. Mitrofanova orcid.org/0000-0003-4702-2736
A.B. Vakhrameev orcid.org/0000-0001-5166-979X
N.V. Dementeva orcid.org/0000-0003-0210-9344

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Chromosome synapsis, recombination and epigenetic modification in rams heterozygous for metacentric chromosome 3 of the domestic sheep *Ovis aries* and acrocentric homologs of the argali *Ovis ammon*

T.I. Bikchurina[1, 2, 3], E.K. Tomgorova[3], A.A. Torgasheva[1, 2, 3], V.A. Bagirov[3], N.A. Volkova[3], P.M. Borodin[1, 2, 3] ✉

[1] Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
[3] Federal Scientific Center for Animal Husbandry – VIZH named after academician L.K. Ernst, Dubrovitsy, Russia
✉ e-mail: borodin@bionet.nsc.ru

Hybridization of domestic animal breeds with their wild relatives is a promising method for increasing the genetic diversity of farm animals. Resource populations derived from the hybridization of various breeds of domestic sheep with mouflon and argali are an important source of breeding material. The karyotypes of argali and domestic sheep differ for a Robertsonian translocation, which occurred in the common ancestor of mouflon and domestic sheep (*Ovis aries*) due to the centric fusion of chromosomes 5 and 11 of the argali (*O. ammon*) into chromosome 3 of sheep. It is known that heterozygosity for translocation can lead to synapsis, recombination and chromosome segregation abnormalities in meiosis. Meiosis in the heterozygotes for translocation that distinguishes the karyotypes of sheep and argali has not yet been studied. We examined synapsis, recombination, and epigenetic modification of chromosomes involved in this rearrangement in heterozygous rams using immunolocalization of key proteins of meiosis. In the majority of cells, we observed complete synapsis between the sheep metacentric chromosome and two argali acrocentric chromosomes with the formation of a trivalent. In a small proportion of cells at the early pachytene stage we observed delayed synapsis in pericentromeric regions of the trivalent. Unpaired sites were subjected to epigenetic modification, namely histone H2A.X phosphorylation. However, by the end of the pachytene, these abnormalities had been completely eliminated. Asynapsis was replaced by a nonhomologous synapsis between the centromeric regions of the acrocentric chromosomes. By the end of the pachytene, the γH2A.X signal had been preserved only at the XY bivalent and was absent from the trivalent. The translocation trivalent did not differ from the normal bivalents of metacentric chromosomes for the number and distribution of recombination sites as well as for the degree of centromeric and crossover interference. Thus, we found that heterozygosity for the domestic sheep chromosome 3 and argali chromosomes 5 and 11 does not cause significant alterations in key processes of prophase I meiosis and, therefore, should not lead to a decrease in fertility of the offspring from interspecific sheep hybridization.
Key words: *Ovis aries*; immunostaining; meiosis; synaptonemal complex; recombination; Robertsonian translocation.

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Синапсис, рекомбинация и эпигенетическая модификация хромосом у баранов, гетерозиготных по метацентрической хромосоме 3 домашней овцы *Ovis aries* и акроцентрическим гомологам архара *Ovis ammon*

Т.И. Бикчурина[1, 2, 3], Е.К. Томгорова[3], А.А. Торгашева[1, 2, 3], В.А. Багиров[3], Н.А. Волкова[3], П.М. Бородин[1, 2, 3] ✉

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
[3] Федеральный научный центр животноводства – ВИЖ им. академика Л.К. Эрнста, Дубровицы, Россия
✉ e-mail: borodin@bionet.nsc.ru

Гибридизация пород домашних животных с их дикими сородичами может служить перспективным методом повышения генетического разнообразия сельскохозяйственных животных. Ресурсные популяции, полученные на основе гибридизации различных пород домашних овец с муфлоном и архаром, являются важным источником селекционного материала. Кариотипы архара и домашней овцы различаются по робертсоновской транслокации, возникшей у общего предка муфлона и овец (*Ovis aries*) за счет центрического слияния хромосом 5 и 11 архара (*O. ammon*) с образованием хромосомы 3 овцы. Известно, что гетерозиготность по транслокациям может приводить к нарушениям синапсиса, рекомбинации и сегрегации хромосом в мейозе. Осо-

бенности протекания мейоза у баранов, гетерозиготных по транслокации, различающей кариотипы овец и архаров, до сих пор не исследованы. Мы изучали синапсис, рекомбинацию и эпигенетическую модификацию хромосом, вовлеченных в данную перестройку у гетерозигот, с использованием иммунолокализации ключевых белков мейоза. В большинстве клеток наблюдался полный синапсис между метацентрической хромосомой овцы и двумя акроцентрическими хромосомами архара с образованием тривалента. В небольшой доле клеток на стадии ранней пахитены наблюдалась задержка синапсиса в перицентромерных районах тривалента. Неспаренные участки подвергались эпигенетической модификации: фосфорилированию гистона H2A.X. Однако к концу пахитены эти нарушения полностью устранялись. Асинапсис замещался негомологичным синапсисом между перицентромерными районами акроцентрических хромосом. К концу пахитены сигнал γH2A.X сохранялся только на половом биваленте и отсутствовал на триваленте. По числу и распределению рекомбинационных сайтов, степени центромерной и кроссоверной интерференции транслокационный тривалент не отличался от нормальных бивалентов метацентрических хромосом. Таким образом, установлено, что гетерозиготность по хромосоме 3 домашней овцы и хромосомам 5 и 11 архара не вызывает существенных изменений в ключевых процессах профазы I мейоза и, следовательно, не должна приводить к снижению плодовитости у потомков от межвидовой гибридизации овец.
Ключевые слова: *Ovis aries*; иммуноокрашивание; мейоз; синаптонемные комплексы; рекомбинация; робертсоновские транслокации.

Introduction

Hybridization of domestic animals with their wild relatives is a promising method for increasing the genetic diversity of farm breeds and introducing factors of resistance to diseases and extreme environmental factors into their genomes (Serebrovsky, 1935). This approach has been used in sheep breeding. Resource populations obtained from hybrids of various breeds of domestic sheep (*Ovis aries*: OAR) with mouflon (*O. orientalis*) and argali (*O. ammon*: OAM) serve as a source of valuable alleles and allelic combinations for subsequent selection (Deniskova et al., 2016).

However, karyotypic differences between domestic and wild species might affect the fertility of the hybrids. The argali karyotype contains two pairs of metacentric chromosomes ($2n = 56$), and the karyotypes of the mouflon and domestic sheep contain three pairs of metacentrics ($2n = 54$). Differences in the diploid number of chromosomes occurred due to the Robertsonian fusion between the chromosomes 5 and 11 argali: rob (OAM5;11) that originated in the common ancestor of mouflon and sheep and resulted in the sheep chromosome 3 (OAR3) (Bunch et al., 1998). Hereinafter, for the argali chromosomes we use the standard nomenclature of the Bovidae chromosomes (Popescu et al., 1996). Theoretically, heterozygosity for Robertsonian translocations should lead to significant disruptions of meiosis and a decrease in fertility. Disturbances of meiosis can occur due to the spatial complexity of the presynaptic alignment and subsequent synapsis between metacentric and acrocentric homologues (Borodin et al., 1998). Delayed synapsis may lead to transcriptional inactivation of unpaired chromatin, apoptosis and death of generative cells (Burgoyne, Mahadevaiah, 1993; Burgoyne et al., 2009). Even in the case of successful synapsis and normal recombination, nondisjunction of chromosomes involved in the trivalent may lead to the formation of unbalanced gametes and a decrease in fertility of heterozygotes (Garagna et al., 2014).

Despite this, Robertsonian translocations are the most common variant of evolutionary chromosomal rearrangements in mammals and the common cause of karyotypic difference between closely related species (Ferguson-Smith, Trifonov, 2007). Polymorphism for Robertsonian translocations is widespread in populations of many species (Dobigny et al., 2017) including breeds of domestic sheep (Broad et al., 1997) because they rarely cause phenotypic effects. In most cases, fertility does not decrease in simple heterozygotes carrying the metacentric chromosome and two homologous acrocentric chromosomes. Moreover, fertility remains normal even in the case of simple heterozygosity for several Robertsonian translocations (Bruère, Ellis, 1979; Dobigny et al., 2017). Reduced fertility is usually found in complex heterozygotes for several Robertsonian translocations involving the same shoulders of chromosomes: hybrids with monobrachial homology (Medarde et al., 2015).

Domestic sheep shows intraspecific polymorphism for five Robertsonian translocations: rob(6;24), rob(9;10), rob(7;25), rob(5;8), and rob(8;22) (Broad et al., 1997). Electron microscopic analysis of synaptonemal complexes (SC) in male heterozygotes for the translocations rob(6;24), rob(9;10) and rob(7;25) revealed a relatively high frequency of cells with delayed synapsis in trivalents formed by metacentric chromosome and two acrocentric homologues. Another abnormality detected was associations between the unpaired pericentromeric areas of the acrocentric elements of trivalent and the XY bivalent (Dai et al., 1994a, b). At the same time, it was shown that simple heterozygotes for one or several of these translocations retain normal fertility, although they have a slightly increased level of chromosome nondisjunction (Bruère, Ellis, 1979).

Meiosis in rams heterozygous for the translocation rob(OAM5;11), have not been studied yet. Analysis of meiosis is important in the light of experiments for creating resource populations based on hybrids of various breeds of domestic sheep with mouflon and argali (Deniskova et al., 2016).

In this study, we investigated synapsis, recombination, and epigenetic modification of chromosomes involved in rob(OAM5;11), using immunolocalization of key meiotic proteins. The lateral elements of the SC were visualized using antibodies to the SYCP3 protein. Epigenetic modifications of the regions containing unrepaired double-strand DNA breaks were detected by antibodies to the phosphorylated form of histone H2A.X (γH2A.X) (Rogakou et al., 1998). We evaluated the number and distribution of recombination nodules using antibodies to MLH1, the mismatch repair protein (Anderson et al., 1999). It is known that the global distribution of recombination events by chromosomes is mainly determined by the

size of the chromosome and the centromeric-telomeric gradient (Kleckner et al., 2003). The genetic content determines the positions of recombination hot spots within 1–2 thousand base pairs and does not make a significant contribution to the chromosome-wide recombination pattern (Lichten, Goldman, 1995). Chromosomes of most of the studied species of vertebrates show the distal peaks of recombination (Ruiz-Herrera et al., 2017). The distribution of crossover sites is also affected by centromeric interference (suppression of recombination near the centromere) and crossover interference (reduction of the probability of a new crossing over to occur near to one that has already arisen) (Zickler, Kleckner, 2015). The greater the distance between adjacent recombination points, the greater the interference. Little is known about the number and distribution of MLH1 sites in domestic and wild sheep species (Muñoz-Fuentes et al., 2015; Ruiz-Herrera et al., 2017). The study of these characteristics in heterozygotes for species-specific evolutionary translocation rob(OAM5;11) is of particular interest.

Materials and methods

Meiotic chromosome spreads were prepared from the testes of mature 6–9 months old rams breed in the experimental farm of the Federal Scientific Center for Animal Husbandry. Rams heterozygous for rob(OAM5;11) were obtained in the crosses shown in Fig. 1. Rams of the Romanov breed were used as representatives of the standard karyotype.

Testes were isolated during castration. The maintenance, anesthesia and castration were carried out in accordance with the international and national rules of humane treatment of animals according to the protocol approved by the Commission on Bioethics of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (protocol No. 35 of October 16, 2016).

SC spreads were prepared by the method of Peters et al. (1997) with modifications. The testes were removed from *tunica albuginea* and a small fragment of the testis about 0.5 cm^3 in size was placed in a hypotonic extraction buffer (30 mM Tris, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, pH 8.2) for 90–110 minutes. Then the seminiferous tubules were macerated in 40 µl of a 0.1 M sucrose solution at pH 8.2. The debris was removed and a suspension of testicular cells was re-suspended several times. One drop (20 µl) of the resulted suspension was placed onto slides moistened with 1 % paraformaldehyde solution and slowly distributed over the surface by tilting the slide. The slides were left to dry in a humid chamber for 2 hours. The preparations were washed in 0.4 % Kodak PhotoFlo, dried and stored until staining in sealed containers at –20 °C.

Immunostaining of the SC spread was performed according to the method of Anderson et al. (1999) with modifications. A solution of 400 µl of 10 % PBT (PBS (phosphate-buffered saline), 0.05 % Tween-20, 3 % BSA (Sigma-Aldrich, USA)) was applied to the slides at room temperature for 45 minutes to block non-specific antibody binding. Then the preparations were incubated for 12 hours at 37 °C with primary antibodies. Depending on the task, the following antibodies were used: rabbit polyclonal antibodies to human SYCP3 protein (Abcam, UK) at a dilution of 1:500; mouse monoclonal antibodies to human MLH1 protein (Pharmingen, USA) at a dilution of 1 : 30; rabbit polyclonal antibodies to human γH2A.X (Abcam) at a dilution of 1:150; human antibodies to human centromere proteins (Sigma-Aldrich) at a dilution of 1:70 in PBT. The preparations were washed three times for 15 minutes in PBS with 0.1 % Tween-20 and incubated for 60 minutes at 37 °C with the following secondary antibodies, which were used in various combinations: donkey antibodies to rabbit immunoglobulins conjugated with a Cy3 fluorescent label (Jackson Laboratories, USA) at a dilution of 1:500; goat antibodies to mouse immunoglobulins conjugated with a fluorescent FITC label (Jackson Laboratories) at a dilution of 1:30; goat antibodies to rabbit immunoglobulins conjugated with a fluorescent FITC label (Jackson Laboratories) at a dilution of 1:150 and goat antibodies against human immunoglobulins conjugated with a fluorescent label AMCA (Vector Laboratories) at a 1:50 dilution. The preparations were washed in PBS, dried, and 15 µl of antifade solution was applied (Vectashield; Vector Laboratories) to prevent fluorescence quenching and covered with a cover glass.

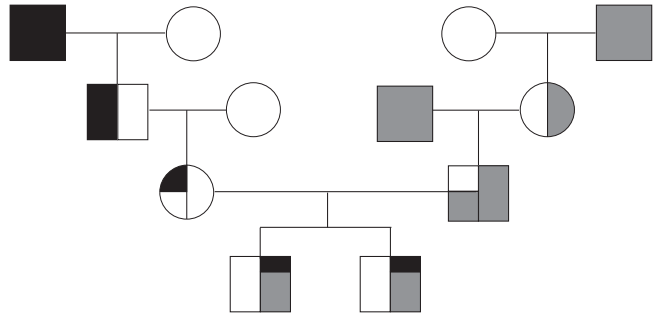

Fig. 1. Pedigree of the heterozygotes for translocation rob(OAM5;11).

The squares show males, the circles indicate females, the black fill shows argali genome, the gray shows mouflon genome, the white shows the Romanov sheep genome.

Microscopic analysis was performed at the Microscopy Center of the Siberian Branch of the Russian Academy of Sciences. The preparations were analyzed under Axioplan 2 microscope (ZEISS, Germany) equipped with a CCD video camera (CV M300, JAI Corporation, Japan), a set of CHROMA filter kits and ISIS4 image processing software (MetaSystems GmbH, Germany). Image brightness and contrast were edited using Corel PaintShop Photo Pro X3.

We analyzed 93 spermatocytes of the Romanov rams and 101 spermatocytes of the translocation heterozygotes. For analysis, we selected the cells at the pachytene stage, in which all autosomal bivalents of the standard *O. aries* karyotype were completely synapsed. Chromosome lengths and the relative position of MLH1 foci were measured using MicroMeasure 3.3 (Reeves, 2001).

To analyze the distribution of MLH1 foci along the chromosomes, we calculated the absolute position of each MLH1 focus, multiplying the relative position of each focus by the absolute length of the corresponding chromosome arm, averaged for all metacentrics. To construct a recombination map, we divided the chromosomal arms into equal intervals corresponding to 1 µm. For each interval, we calculated the proportion of MLH1 foci located on it out of the total number of foci located on a given chromosome.

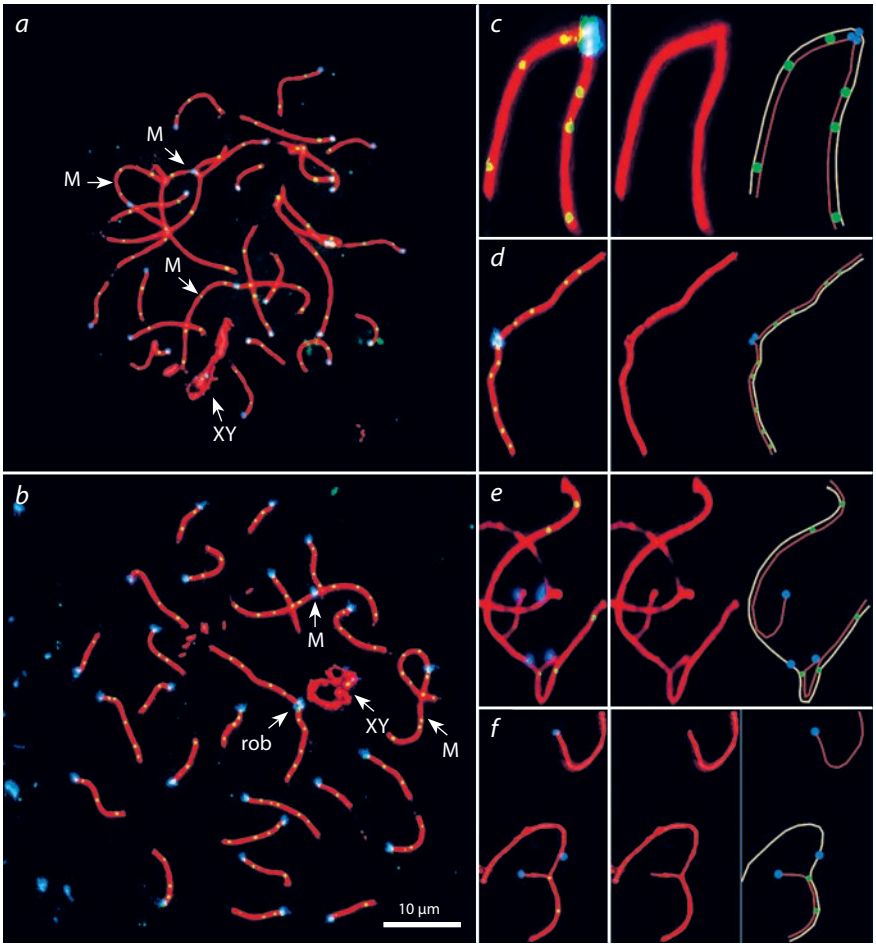

Fig. 2. Spermatocytes of Romanov rams (*a*), heterozygotes for rob(OAM5;11) (*b*) and magnified images of various synaptic configurations of the Robertsonian trivalent (*c–f*).

c – trivalent is completely synapsed; *d* – pericentromeric regions of acrocentrics are synapsed and form a side arm; *e* – the pericentromere region of trivalent is partially asynapsed; *f* – one of the acrocentrics in trivalent is completely asynapsed. First column shows merge of three color channels, second column shows the red channel only, third column shows a schematic image of the trivalent. The red signal represents SYCP3; green – MLH1; blue – centromere. M indicate metacentric bivalents, rob – Robertsonian trivalent, XY – sex bivalent.

We used ANOVA to test the effect of heterozygosity for the chromosomal rearrangement on the recombination characteristics of chromosomes involved. Statistical tests were performed using Statistica 6.0 (StatSoft). The average values of the SC lengths and the number of MLH1 foci are given with standard deviations (±SD).

Results

The karyotype of pachytene cells of Romanov rams contained three large metacentric bivalents, indistinguishable from each other, 23 acrocentric bivalents, forming a continuous series in descending length, and the sex bivalent (Fig. 2, *a*). Pachytene spermatocytes of the heterozygotes for the Robertsonian translocation contained two metacentric bivalents and trivalent formed by the sheep chromosome 3 and acrocentric argali homologs (Fig. 2, *b*). The trivalents did not differ in the average SC length from the bivalents of the two other metacentric chromosomes in heterozygotes ($t = 0.87$, $p = 0.38$), but they were shorter than the bivalents of all three metacentric chromosomes in the normal karyotype ($t = 5.36$, $p < 0.001$) (see the Table). Therefore, in the further analysis, we compared the recombination characteristics of the translocation trivalent with the combined data on the bivalents of two metacentric chromosomes in heterozygotes.

Figure 2, *c–f* present various variants of synaptic configurations found in the heterozygotes. The most frequent was the variant with almost complete pairing between metacentric chromosome 3 and its acrocentric homologues (see Fig. 2, *c*). Sometimes centromeres of acrocentrics superimposed on each other, forming a small lateral arm (*d*). Asynapsis of the pericentromeric regions of one or both acrocentrics was found in 5.0 ± 2.2 % of cells (*e*). The average size of the unpaired region was 24.2 ± 14.3 % of the trivalent length. In one case, we observed complete asynapsis of a longer acrocentric (*f*).

Immunolocalization of the phosphorylated form of histone H2A.X (γH2A.X) allowed us to visualize areas containing non-repaired DNA double-strand breaks (Fig. 3). At the leptotene stage, such areas were numerous and were present on all chromosomes (see Fig. 3, *a*). In the early pachytene, we observed γH2A.X signals on unpaired sites of autosomes, including the asynapsed areas of the acrocentric elements of the translocation trivalent (see Fig. 3, *b*). In rare cases, the entire arm of the trivalent, including its synaptic part, was subjected to epigenetic modification (see Fig. 3, *c*). By the end of the pachytene, the γH2A.X signal remained on the sex bivalent only and was absent on the trivalent (see Fig. 3, *d*).

Visualization of recombination nodules using antibodies to the MLH1 protein (see Fig. 2) allowed us to estimate the number of crossovers on the chromosomes of interest. We did not find differences in the average number of MLH1 foci at the Robertsonian trivalent with that at the bivalents of the two other metacentric chromosomes in the heterozygotes for rob(OAM5;11) ($t = 1.33$, $p = 0.18$) whereas the bivalents of the three metacentric chromosomes, including chromosome 3, in the normal homozygotes had fewer MLH1 foci ($t = 3.59$, $p < 0.001$).

The distribution of MLH1 foci along chromosome 3 of the translocation trivalent was similar to the distribution observed on the bivalents of metacentric chromosomes 1–2 in heterozygotes for rob(OAM5;11) and on the bivalents of chromosomes 1–3 in the standard karyotype (Fig. 4). It was relatively uniform. Unlike the chromosomes of many other mammals (Ruiz-Herrera et al., 2017), the metacentric chromosomes of the rams did not have pronounced peaks in the distal chromosome regions. The frequency of MLH1 foci in the

Recombination characteristics of the chromosomes 1–3 in the rams of the standard karyotype (+/+) and chromosomes 1–2 and 3 in heterozygotes for rob(OAM5;11) (+/rob)

Chromosomes	Number of chromosomes studied	Length of synaptonemal complex, μm	Number of MLH1 foci	Relative distance between adjacent MLH1 foci (chromosome length fraction)	Percent of MLH1 foci in pericentromeric regions*
1–3 (+/+)	279	35.8 ± 8.4	5.7 ± 1.2	0.18 ± 0.01	6.9 ± 0.6
1–2 (+/rob)	202	31.6 ± 8.3	6.0 ± 1.4	0.17 ± 0.01	5.8 ± 0.7
3 (+/rob)	101	30.7 ± 7.8	6.2 ± 1.4	0.16 ± 0.01	4.8 ± 0.9

* ± 2 μm from the centromere.

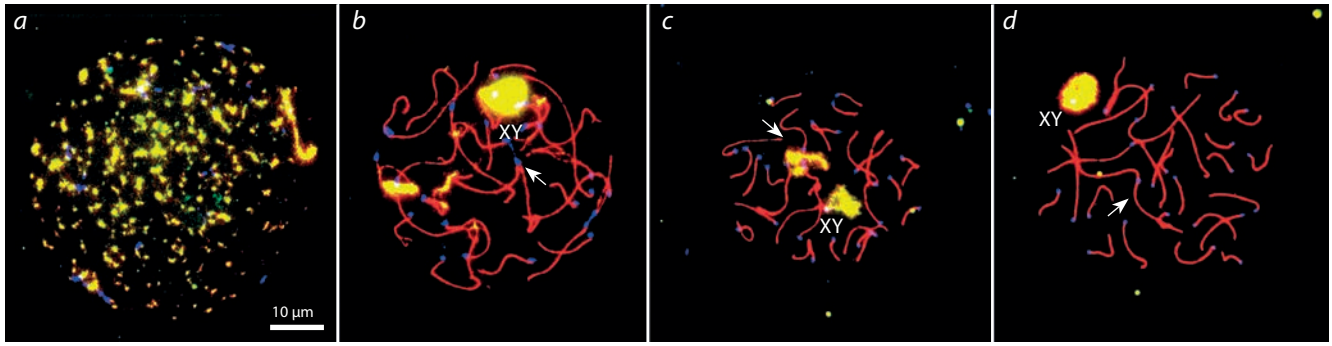

Fig. 3. Spermatocytes of rams heterozygous for rob(OAM5;11) at different stages of prophase I.

a, leptotene: γH2A.X signals are localized along the fragments of lateral SC elements; *b*, early pachytene: γH2A.X signals are localized at the asynapsed regions of autosomes and sex bivalent, whereas the trivalent does not show the signal; *c*, mid-pachytene: γH2A.X signals are localized at the asynapsed regions of the trivalent and sex bivalent; *d*, late pachytene: γH2A.X signal is localized at the sex bivalent only. Arrowhead indicate trivalent with translocation, XY – sex bivalent. Red signal represents SYCP3; yellow – γH2A.X; blue – centromere.

pericentromeric regions of trivalent and normal bivalents was reduced only in a short (1–2 μm) interval. However, we did not observe significant differences in the MLH1 foci number in pericentromeric regions ($p > 0.05$) between trivalent and two metacentric bivalents (see the Table). We also found no differences between trivalent and bivalents in the degree of crossover interference: the average distance between adjacent MLH1 foci was almost the same (see the Table).

Discussion

We found that rob(OAM5;11) heterozygotes showed a delayed synapsis of the Robertsonian trivalent in a small percentage of pachytene spermatocytes. This is in agreement with the results of electron microscopic studies of the sheep spermatocytes heterozygous for rob(6;24), rob(9;10) and rob(7;25) (Dai et al., 1994a, b). However, in the cited papers, the percentage of abnormal spermatocytes was higher than in our experiment. The carriers of these translocations showed rather high percentage of associations between the asynapsed regions of trivalent and the sex bivalent. We did not observe such associations. These differences in trivalent synapses in heterozygotes for translocations that are polymorphic within a species, and for translocations that distinguish different species, might occur due to either the methodological differences and/or the genetic properties of the chromosomes involved in these translocations.

Delayed synapsis in the pericentromeric region of trivalent lead to delays in repair of DNA double-strand breaks, which in turn caused epigenetic modification of this region: H2A.X histone phosphorylation at serine 139. Typically, such a modification of an unpaired chromatin results in transcriptional inactivation of genes localized in modified regions (Burgoyne et al., 2009; Turner, 2015). If such events occurred in pachytene spermatocytes of the rams heterozygous for the translocation, they affected only a small percentage of the cells. Even so, the pericentromeric chromosome regions are usually enriched in repeated sequences and contain few genes. Therefore, it is unlikely that inactivation of such areas may lead to germ cell death. In addition, in the spermatocytes at the late pachytene stage, we did not observe either asynapsed regions or signals of epigenetic modification. Most likely, asynapsis was replaced by a nonhomologous synapsis, and the modification of chromatin turned out to be reversible. We cannot exclude a possibility that the cells with the delayed synapsis were eliminated earlier and did not reach the pachytene stage. However, even if such elimination occurred, it affected only a small percentage of the germ cells and should not have affected the fertility of the heterozygotes.

The number and distribution of crossovers on the bivalents of normal metacentric chromosomes and on the translocation trivalent were similar. A rather common effect of heterozygosity for Robertsonian translocations is the distalization of the distribution of crossovers along the arms of the involved chromosomes (Dumas, Britton-Davidian, 2002; Borodin et al., 2008; Dumas et al., 2015). We did not detect such distalization in the rob(OAM5;11) trivalents. The degree of centromeric interference in the trivalents did not exceed that of homozygotes for the normal metacentric chromosomes. In a number of cells, we observed MLH1 foci in the close vicinity of the centromere (see Fig. 2, *c*, *e*, Fig. 4). In terms

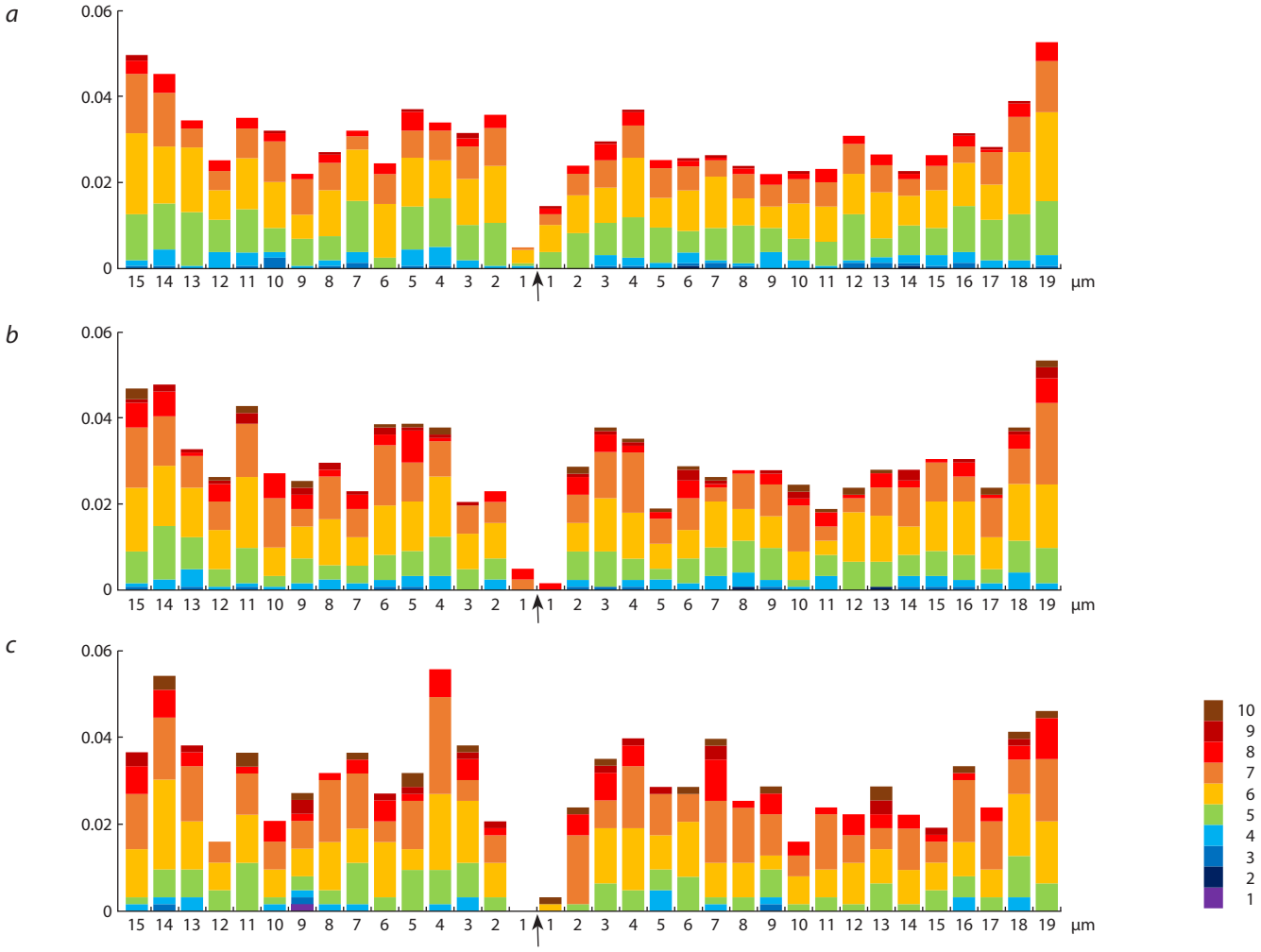

Fig. 4. Distribution of MLH1 foci along the bivalents of chromosomes 1–3 in normal homozygotes (*a*), along the bivalents of chromosomes 1–2 (*b*) and along trivalent chromosome 3 (*c*) in heterozygotes for translocation.

The X-axis shows the position of MLH1 foci at the bivalent relative to the centromere (indicated by arrow). The marks are equivalent to 1 μm. The Y-axis shows the proportion of MLH1 foci at each interval. The colors indicate the frequency of trivalents and bivalents containing different number of MLH1 foci within each interval, from 1 to 10.

of crossover interference, the translocation trivalent did not differ from normal bivalents either. Normal recombination in the Robertsonian trivalent ensures normal chromosome segregation and makes unlikely unbalanced gametes formation.

Conclusion

Thus, we found that heterozygosity for the evolutionary Robertsonian translocation involving chromosome 3 of domestic sheep and chromosomes 5 and 11 of argali does not cause significant changes in the key stages of meiosis and, therefore, should not lead to a decrease in fecundity in the offspring from interspecific hybridization of sheep.

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ORCID ID

T.I. Bikchurina orcid.org/0000-0003-0921-7970
E.K. Tomgorova orcid.org/0000-0001-5398-8815
A.A. Torgasheva orcid.org/0000-0002-8933-8336
V.A. Bagirov orcid.org/0000-0001-5398-8815
N.A. Volkova orcid.org/0000-0001-7191-3550
P.M. Borodin orcid.org/0000-0002-6717-844X

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Филогеографическая структура евразийской свиязи (*Mareca penelope*) в Голарктике

И.В. Куликова[1] ✉, Ю.Н. Журавлев[1], И.Г. Коробицын[2], Г.А. Немкова[1], К.Г. МакКрекен[3, 4, 5], Дж.Л. Петерс[6]

[1] Федеральный научный центр биоразнообразия наземной биоты Восточной Азии Дальневосточного отделения Российской академии наук, Владивосток, Россия
[2] Биологический институт Томского государственного университета, Томск, Россия
[3] Отделение биологии, Университет Майами, Корал Кэйблз, Флорида, США
[4] Отделение морской биологии и экологии, Институт морской и атмосферной науки им. Розенштейна, Университет Майами, Майми, Флорида, США
[5] Институт геномики человека им. Хассмана, Медицинский институт им. Миллера Университета Майами, Майми, Флорида, США
[6] Отделение биологических наук, Государственный университет Райта, Дэйтон, Огайо, США
✉ e-mail: Kulikova@biosoil.ru

Евразийская свиязь (*Mareca penelope*) – один из самых многочисленных перелетных видов уток в Палеарктике. Ежегодно значительная часть всего вида совершает сезонные перелеты на расстояния от десятков до тысяч и более километров. По данным кольцевания выделено пять географических популяций, однако четких границ между популяциями не обнаружено. В то же время филогеографическая структура евразийской свиязи на всем протяжении ее ареала до сих пор не изучена. Помимо фундаментального значения подобного исследования, знание генетической структуры популяций необходимо для разработки мер по сохранению и увеличению численности этого ценного охотничье-промыслового вида. В связи с этим целью нашей работы был филогеографический анализ свиязи на обширной территории ее ареала в Палеарктике с привлечением образцов тканей птиц, зимующих в Северной Америке. С помощью секвенирования 5′-фрагмента контрольного региона митохондриальной ДНК была изучена генетическая дифференциация популяций свиязи, выделенных по данным кольцевания, реконструированы филогенетические отношения гаплотипов мтДНК и демографическая история популяций и вида в целом. Всего было проанализировано 195 последовательностей длиной 661 п. н. Генетическое разнообразие было высоким во всех изученных популяциях. Филогенетические реконструкции показали отсутствие кластеризации по географическому признаку. Анализ молекулярной изменчивости (AMOVA) выявил две группы популяций: европейско-сибирскую и восточноазиатскую. В первую группу, помимо европейской и сибирской, также вошла выборка из Атлантического побережья Северной Америки, вторая включила особей из Дальнего Востока, Камчатки, Чукотки, Алеутских островов, Аляски и Тихоокеанского побережья Северной Америки.
Ключевые слова: филогеография; популяционно-генетическая структура; свиязь; *Mareca penelope*; контрольный регион; мтДНК.

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Holarctic phylogeographic structure of Eurasian wigeon (*Mareca penelope*)

I.V. Kulikova[1] ✉, Y.N. Zhuravlev[1], I.G. Korobitsyn[2], G.A. Nemkova[1], K.G. McCracken[3, 4, 5], J.L. Peters[6]

[1] Federal Scientific Center of the East Asia Terrestrial Biodiversity, FEB RAS, Vladivostok, Russia
[2] Biological Institute of Tomsk State University, Tomsk, Russia
[3] Department of Biology, University of Miami, Coral Gables, Florida, USA
[4] Department of Marine Biology and Ecology, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Miami, Florida, USA
[5] Human Genetics and Genomics, Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, Florida, USA
[6] Department of Biological Sciences, Wright State University, Dayton, Ohio, USA
✉ e-mail: Kulikova@biosoil.ru

The Eurasian wigeon (*Mareca penelope*) is one of the most numerous migrant species of waterfowl in the Palearctic. Annually, significant part of the world's wigeon population makes seasonal flights over distances from tens to thousands or more kilometers. According to different estimates based on banding data, five geographic populations of the species were described in the Palearctic. However, distinct borders between the populations have not been identified. At the same time, no phylogeographic studies have been carried out for the complete native range of wigeon so far. In addition to the fundamental importance of such a study, knowledge of the genetic structure of populations is necessary for the development of measures to increase the number of and preserve this valuable game species. The aim of our work was a phylogeographic analysis of the wigeon across its vast native range in the Palearctic including ducks wintering in North America. We examined genetic diversity and differentiation of wigeon

populations identified with banding data, phylogenetic relationships of mtDNA haplotypes and demographic history of populations and species as a whole by sequencing a 661 base-pair 5′-fragment of the mitochondrial control region from 195 individual ducks collected throughout the Palearctic and Nearctic. Genetic diversity was high in all studied populations. A reconstruction of haplotypes phylogeny revealed the absence of geographic structure in the data. Nonetheless, analysis of molecular variance (AMOVA) identified two groups of populations: European-Siberian and East Asian. The former included wigeons from Europe, Siberia and the Atlantic coast of North America, and the latter comprised ducks from Russian Far East, Kamchatka Peninsula, Chukotka Autonomous District, the Aleutian Islands, Alaska, and the Pacific coast of North America.
Key words: phylogeography; population genetic structure; wigeon; *Mareca penelope*; control region; mtDNA.

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Введение

Евразийская свиязь (*Mareca penelope*) – широко распространенный и многочисленный вид промысловых утиных птиц Палеарктики. Гнездится в северной части Палеарктики, от Британских островов и Исландии на западе до бассейна р. Анадырь и полуострова Камчатка на востоке, от высоких северных широт Евразии до приблизительно 50-й параллели северной широты (Дементьев, Гладков, 1952). Встречается евразийская свиязь также и в Неарктике, а именно на Алеутских островах, Аляске и вне гнездового периода на Атлантическом и Тихоокеанском побережьях Северной Америки. По данным кольцевания в Палеарктике выделено пять географических популяций вида: исландская, европейская, западносибирская, восточносибирская и дальневосточная (Павлов и др., 1997). Предполагается, что между популяциями нет четких границ. Так, данные кольцевания выявили высокий уровень обмена мигрантами между популяциями (от 5 до 30 %). Однако миграции происходят преимущественно на периферии популяций, в местах наложения популяционных ареалов и во время зимовки (Павлов и др., 1997).

В последние годы было проведено много филогеографических исследований утиных птиц. Распространенным результатом таких исследований стало обнаружение парафилии или полифилии митохондриальной и ядерной ДНК, особенно в случае анализа геномов близкородственных видов. В качестве наиболее вероятного объяснения были предложены незавершенная сортировка линий ядерной ДНК/гаплотипов мтДНК или межвидовая гибридизация (McCracken et al., 2001; Kulikova et al., 2004, 2005; Peters et al., 2005, 2007; Peters, Omland, 2007). Это неудивительно, учитывая относительно молодой с точки зрения эволюции возраст таксонов (Peters et al., 2007; Lavretsky et al., 2014) и широко известную способность уток к гибридизации в неволе и в дикой природе (Johnsgard, 1960; Greig, 1980). Было показано, что между географическими популяциями многих видов утиных существуют лишь незначительные генетические различия, что, по мнению ряда авторов, обусловлено генным потоком и расселением (Cronin et al., 1996; McCracken et al., 2001; Kulikova et al., 2005; Peters, Omland, 2007; Kraus et al., 2011).

В настоящей работе была впервые изучена филогеографическая структура широкоареального вида *Mareca penelope* в Голарктике. В качестве молекулярного маркера мы выбрали 5′-фрагмент контрольного региона мтДНК. Анализ вариабельности мтДНК является рутинным методом филогеографических исследований, что объясняется гаплоидностью, отсутствием рекомбинации, матриархальным наследованием и быстрой по сравнению с ядерной ДНК скоростью эволюции митохондриального генома. Контрольный регион, или D-петля – это протяженный некодирующий участок мтДНК, скорость эволюции которого в три-пять раз превышает таковую остальной мтДНК (Taanman, 1999). В области D-петли расположены промоторы транскрипции легкой и тяжелой цепей и точка репликации тяжелой цепи мтДНК. В составе контрольного региона выделяют три домена, которые отличаются по степени вариабельности: консервативный центральный домен II и фланкирующие домены I и III, характеризующиеся высокой скоростью нуклеотидных замен. Длина контрольного региона птиц варьирует от 1028 до 1581 п. н., составляя в среднем 1127 п. н. (Ruokonen, Kvist, 2002). У пекинской породы уток, например, длина контрольного региона составляет 1048 п. н., а длины доменов I, II и III равны 348, 478 и 222 п. н. соответственно (Ramirez et al., 1993). Благодаря высокой скорости эволюции контрольный регион весьма успешно применяется в изучении внутривидовой изменчивости утиных птиц (Cronin et al., 1996; McCracken et al., 2001; Kulikova et al., 2005; Peters, Omland, 2007; Kraus et al., 2011). С помощью анализа полиморфизма 5′-фрагмента контрольного региона мтДНК мы пытались выяснить, окажутся ли популяции свиязи, выделенные по данным кольцевания, генетически дифференцированными и существуют ли генетические различия между особями, зимующими в Северной Америке, и евразийскими популяциями.

Материалы и методы

В Евразии и Северной Америке были собраны 115 образцов тканей евразийских свиязей; использованы также 80 опубликованных ранее последовательностей (Peters et al., 2005, 2014; Куликова, Журавлев, 2010). Информация об образцах и местах их сбора представлена в табл. 1. Региональные выборки были выделены в соответствии с данными кольцевания (Павлов и др., 1997) и в настоящем исследовании приравнены к популяциям. (1) Европейская популяция состояла из образцов, собранных в Европе ($n = 8$). (2) Сибирская популяция ($n = 22$) включила главным образом образцы из западной и центральной частей Сибири, за исключением восьми свиязей из Томской области, собранных во время миграции, которые могли иметь как западносибирское, так и восточносибирское происхождение. В эту региональную выборку попали также девять особей из Турции, Египта и Бангладеш, поскольку

Table 1. Experimental material

Continent	Region	Code	*n*	Sampling locality	*n*	Reference
Eurasia *n* = 164	Europe	EUR	8	Germany	5	Peters et al., 2005
				Scotland	2	»
				Spain	1	»
	Siberia	SIB	22	Turkey	2	Unpubl.
				Egypt	6	»
				Yamalo-Nenets Autonomous Okrug	1	Peters et al., 2005
				Bangladesh	1	Unpubl.
				Kazakhstan	2	Peters et al., 2005
				Tomsk oblast	10	Unpubl.
	Far East	FE	106	Primorsky Krai	105	Kulikova, Zhuravlev, 2010; Peters et al., 2005; unpubl.
				Magadan oblast	1	Peters et al., 2005
	Western Beringia	WB	30	Chukotka Autonomous Okrug	8	Kulikova, Zhuravlev, 2010
				Kamchatka oblast	22	Unpubl.
n = 8	Aleutian Islands and Alaska	ALA	8	Aleutian Islands, United States	6	Peters et al., 2014
				Alaska, United States	2	Peters et al., 2005
North America *n* = 21	Pacific coast of North America	PAC	17	California, United States	5	Peters et al., 2005; неопубл.
				Oregon, United States	5	»
				Washington, United States	7	»
	Atlantic coast of North America	ATL	4	Delaware, United States	1	Unpubl.
				Massachusetts, United States	1	Peters et al., 2005
				Maryland, United States	1	Unpubl.
				Nova Scotia, Canada	1	»

в этих странах зимуют особи из западносибирской популяции, и две образца из Казахстана, собранные во время весенней миграции в районе одного из миграционных путей, характерных для западносибирской популяции (Павлов и др., 1997). (3) Дальневосточная популяция (*n* = 106) состояла из особей, собранных во время миграции в Приморском крае, и одного образца из Магадана. Птицы этой популяции зимуют главным образом в Корее, Китае и на севере Вьетнама, а гнездится на севере и северо-востоке Якутии (Павлов и др., 1997). (4) Популяция западной Берингии (*n* = 30) включила гнездящихся на Камчатке и в бассейне Анадыря птиц, которые зимуют на Курильских островах и в Японии. (5) В одну популяцию были объединены утки Алеутских островов и Аляски (*n* = 8). (6) Тихоокеанская (*n* = 17) и (7) атлантическая (*n* = 4) североамериканские популяции состояли из образцов, собранных на побережье Тихого и Атлантического океанов Северной Америки соответственно.

Тотальную ДНК выделяли из печени и мышц птиц с помощью ДНК DNeasy Tissue Kit (Qiagen, США). 5′-участок контрольного региона митохондриальной ДНК (78–774 п. н. митохондриального генома курицы) амплифицировали с помощью праймеров L78 и H774 (Sorenson, Fleischer, 1996). Амплификацию проводили на приборе UNOII Thermoblock (Biometra, Германия) в 25 мкл реакционной смеси, содержащей геномную ДНК, 1.25 мкл каждого праймера (10 мМ), 2.5 мкл каждого dNTP (10 мМ), 2.5 мкл $MgCl_2$ (25 мМ), 2.5 мкл десятикратного буфера для PCR и 0.2 мкл Taq-ДНК-полимеразы. Амплификацию проводили при следующих температурных условиях: начальная денатурация – 7 мин при 94 °C; 45 циклов в режиме: денатурация – 20 с при 94 °C, отжиг – 20 с при 52 °C, элонгация – 1 мин при 72 °C; и завершающая элонгация – 7 мин при 72 °C. Качество продуктов амплификации определяли с помощью электрофореза. Обе цепи секвенировали с помощью BigDye Terminator Cycle Sequencing Kits в четырехкратном разведении. Последовательности нуклеотидов определяли на автоматическом секвенаторе ABI 3130 (Applied Biosystems, Foster City, Калифорния). ABI-хроматограммы совмещали и редактировали с помощью пакета программ Staden 1.53 (Staden et al., 2000). Все последовательности сданы в GenBank (идентификационные номера MH460239–MH460385).

Филогению реконструировали по методу присоединения соседей Neighbour-Joining (NJ) (Saitou, Nei, 1987) в программе MEGA 5.3 (Tamura et al., 2011). В качестве внешней группы использовали серую утку *Anas strepera* (DQ449148.1). Генетические дистанции вычисляли на основании двухпараметрической модели Кимуры (Kimura, 1980), выбранной с помощью программы Modeltest v. 3.06

(Posada, Crandall, 1998). Медианную сеть гаплотипов реконструировали с помощью программы Network 4.6.1 (Bandelt et al., 1999). Вставки/делеции учитывались как дополнительное пятое состояние признака. Нуклеотидное (π) и гаплотипическое (H) разнообразие, значения теста на селективную нейтральность (Fu's Fs) вычисляли для каждой популяции с помощью программы ARLEQUIN ver. 3.5 (Excoffier, Lischer, 2010). Значения параметров генетической подразделенности Φ_{ST} и анализ молекулярной изменчивости AMOVA также выполнили с помощью ARLEQUIN 3.5. Для оценки экспансии были построены гистограммы распределения попарных нуклеотидных различий в ARLEQUIN 3.5 (Harpending, 1994). Для вычисления максимально правдоподобных оценок параметра роста численности популяции (g) и его стандартного отклонения применяли программу Fluctuate 1.4 (Kuhner et al., 1995).

Результаты

В настоящей работе использованы 195 последовательностей 5′-конца контрольного региона мтДНК длиной 661 п. н., включая 80 уже опубликованных последовательностей (Peters et al., 2005, 2014; Куликова, Журавлев, 2010) (см. табл. 1). Из 661 гомологичной позиции 36 (5.4 %) были вариабельными и 19 (2.9 %) информативными с точки зрения максимальной экономии. Всего было идентифицировано 34 гаплотипа, 23 гаплотипа были уникальными, 7 встречались у 2–10 особей, гаплотип E-3 обнаружен у 13 особей, E-4 – у 16, E-6 – у 28 и самый распространенный гаплотип, E-2, – у 79 особей. Гаплотип E-6 обнаружен во всех выборках, E-2 и E-4 – в шести и пяти из семи выборок соответственно. Среднее число нуклеотидных различий (K) между гаплотипами было 1.87, гаплотипическое разнообразие (H) составило 0.802 ± 0.025. Один гаплотип свиязи из западной Берингии значительно отличался от остальных (K = 21.76) и был идентичен таковым американской свиязи *Mareca americana* из базы данных GenBank/NCBI (AY881737, KJ824074).

Показатели нуклеотидного и гаплотипического разнообразия варьировали от 0.00101 до 0.00531 и от 0.6667 до 1.000 соответственно (табл. 2). Самое высокое значение π отмечено в европейской популяции и западной Берингии, самое низкое – в атлантической североамериканской выборке. Однако после удаления гаплотипа американской свиязи из выборки западной Берингии значение параметра нуклеотидного разнообразия снизилось почти в два раза (см. табл. 2). Впоследствии этот гаплотип был изъят из популяционно-генетического и демографического анализов, поскольку он попал в популяцию европейской свиязи в результате интрогрессии от американской свиязи (Куликова, Журавлев, 2010) и из-за большого числа нуклеотидных отличий искажал результаты анализа. Гаплотипическое разнообразие также оказалось самым высоким в европейской и самым низким в атлантической популяциях (см. табл. 2). Примерно половина значений Φ_{ST} были статистически достоверными (табл. 3).

Table 2. Genetic diversity

Population	*n*	Nh	S	K	H	π
EUR	8	8	9	3.50	1.0000 ± 0.0625	0.005311 ± 0.003442
SIB	22	10	15	2.07	0.8658 ± 0.0519	0.003142 ± 0.002038
FE	106	19	16	1.38	0.7513 ± 0403	0.002095 ± 0.001440
WB	30	9	29	2.91	0.7655 ± 0.0728	0.004413 ± 0.002650
WB*	29	8	10	1.55	0.7488 ± 0.0728	0.002351 ± 0.001610
ALA	8	5	5	1.67	0.8571 ± 0.1083	0.002385 ± 0.001801
PAC	17	8	7	1.53	0.7794 ± 0.0985	0.002321 ± 0.001637
ATL	4	2	1	0.67	0.6667 ± 0.2041	0.001012 ± 0.001135

Notes: *N*, sample size; Nh, number of haplotypes; S, number of segregating sites; K, the average number of nucleotide differences; H, haplotype diversity; π, nucleotide diversity. * population analyzed without the *M. americana* haplotype. Population codes follow Table 1.

Table 3. Genetic differentiation coefficients F_{ST}

Population	EUR	SIB	FE	WB	ALA	PAC
EUR						
SIB	0.04863					
FE	**0.22160**	**0.10870**				
WB	**0.19491**	**0.15413**	0.00986			
ALA	0.09841	0.01606	–0.02456	0.02149		
PAC	**0.17928**	**0.16701**	**0.02193**	–0.01825	0.03381	
ATL	–0.01073	–0.06208	**0.23085**	**0.29133**	0.19691	**0.32412**

Notes: Population codes follow Table 1. Statistically significant differences ($p < 0.05$) are shown in bold.

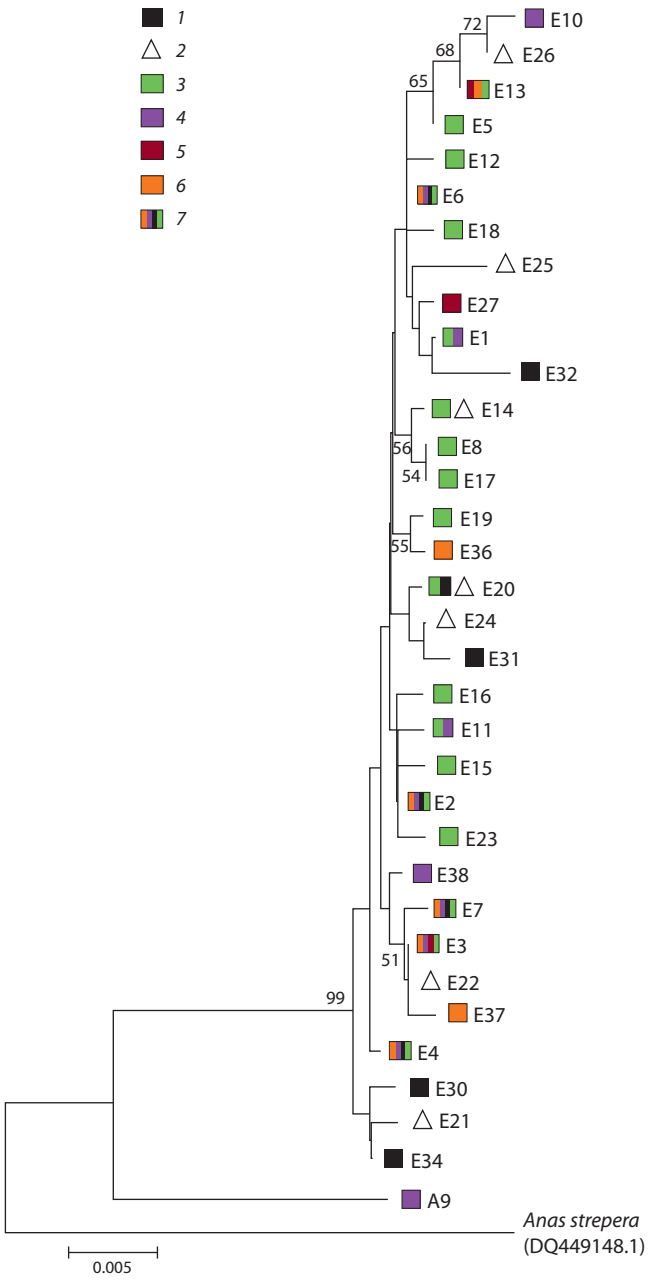

Fig. 1. Unrooted Neighbour Joining tree illustrating mtDNA control region haplotypes.
Bootstrap values (500 replicates) exceeding 50 % are shown next to the branches. *1*, Europe; *2*, Siberia; *3*, Far East; *4*, western Beringia; *5*, Alaska/Aleutians; *6*, Pacific coast of North America; *7*, haplotypes found in five or more populations.

Самые высокие значения индекса генетической дифференциации были получены при сравнении выборки из Атлантического побережья Северной Америки с выборками из Тихоокеанского побережья Северной Америки (0.324), западной Берингии (0.291), Дальнего Востока (0.231). Значительные различия были также обнаружены между популяциями европейской и дальневосточной (0.222), европейской и западной Берингии (0.195), европейской и тихоокеанской Северной Америки (0.179), а также сибирской и тихоокеанской Северной Америки (0.167), сибирской и западной Берингии (0.154). Умеренно дифференцированными были дальневосточная и сибирская популяции (0.109).

Анализ молекулярной изменчивости (AMOVA) показал, что 91.85 % генетического разнообразия обусловлено внутрипопуляционной изменчивостью, тогда как на изменчивость между популяциями приходится 8.15 %. Максимально снизить внутрипопуляционную изменчивость и получить самое высокое значение межгрупповой изменчивости удалось с помощью группировки популяций по географической близости. При объединении популяций дальневосточной, тихоокеанской, западной Берингии и алеутской в одну группу, европейской, сибирской и атлантической – в другую доля межгрупповой изменчивости составила 13.89 %, внутрипопуляционной – 84.73 %, а на межпопуляционную изменчивость внутри групп пришлось 1.38 %.

Консенсусное NJ-древо объединило все гаплотипы *M. penelope* в один кластер с 99 % бутстреп-поддержкой, исключив гаплотип A-9, обнаруженный у одной особи из Анадыря (рис. 1). Воспроизводимость ветвления внутри кластера была низкой, и лишь несколько узлов ветвления имели значения бутстреп выше 50 %. Медианная сеть гаплотипов имела структуру, близкую к звездообразной (рис. 2). Центральные гаплотипы встречались с самой высокой частотой и были обнаружены во всех или почти во всех исследованных популяциях *M. penelope*. Гаплотипы отстояли друг от друга в большинстве случаев на одну и единично на две или три нуклеотидные замены, за исключением гаплотипа A-9 из Анадыря, отделенного от основной клады 19 заменами. Гаплотипы не формировали кластеров в соответствии с их географической принадлежностью.

Результаты теста Fu's Fs на селективную нейтральность свидетельствовали в поддержку гипотезы роста численности популяций в прошлом (табл. 4). Так, значения Fu's Fs были отрицательными, за исключением выборки из североамериканской Атлантики и достоверно отличались от нуля для дальневосточной, сибирской, европейской и тихоокеанской выборок. Отрицательные значения теста Fu's Fs могут трактоваться и как свидетельство действия направленного отбора. Однако распределение попарных нуклеотидных различий между парами гаплотипов во всех выборках было унимодальным и соответствовало ожидаемому, согласно модели увеличения численности популяции в прошлом (данные не приведены). Низкие и статистически недостоверные значения суммы квадратных отклонений SSD между наблюдаемым и ожидаемым распределениями подтвердили достоверность соответствия. По данным коалесцентного анализа, максимально правдоподобные значения параметра роста численности (g) исследуемых популяций имели положительные значения, варьируя от 725.8 до 6528.6 (см. табл. 4). Ввиду большого значения стандартного отклонения параметра g для атлантической североамериканской выборки положительная оценка роста численности оказалась недостоверной (99.9 % CI: –581.8 – 2715).

Обсуждение

Генетическое разнообразие оказалось высоким во всех исследованных выборках: средние значения нуклеотидного

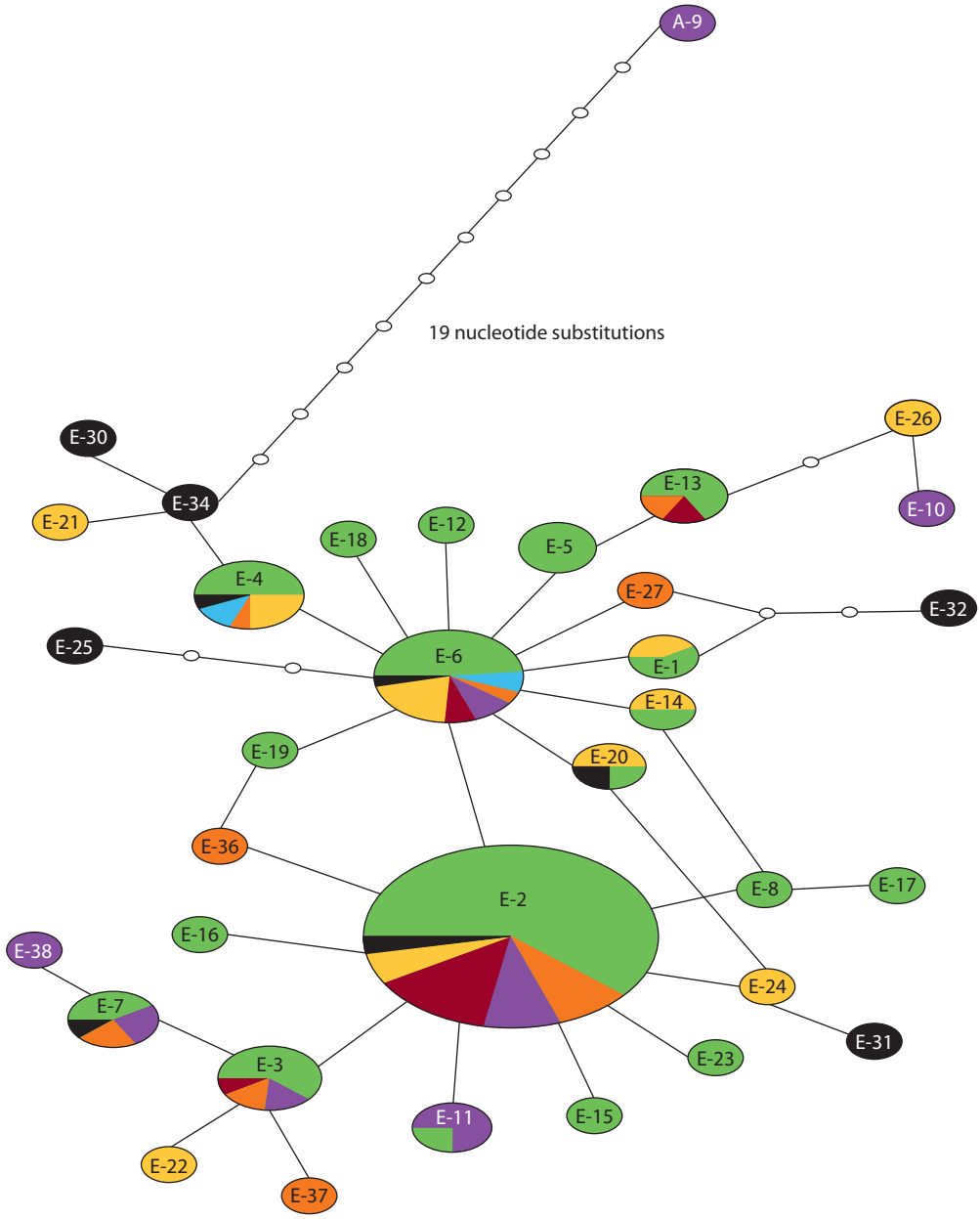

Fig. 2. Unrooted network illustrating the phylogenetic relationships of the mtDNA haplotypes.

The size of each ellipse is proportional to the number of individuals with the corresponding haplotype. Small ellipses indicate intermediate ancestral haplotypes not found in the samples.

1, Europe; *2*, Siberia; *3*, western Beringia; *4*, Far East; *5*, Alaska/Aleutians; *6*, Pacific coast of North America; *7*, Atlantic coast of North America.

Table 4. Demography parameters

Population	SSD	*p*-value	Fu's Fs	*p*-value	G	SD (G)
EUR	0.0242	0.57	–5.063	0.004	2180.42	173.352
SIB	0.0109	0.22	–4.0281	0.01	2369.77	321.473
FE	0.0076	0.21	–13.14	0	1863.11	83.2869
WB	0.0033	0.56	–2.26	0.078	725.802	241.624
ALA	0.0101	0.67	–1.69	0.071	6528.63	518.407
PAC	0.005	0.64	–3.6915	0.003	3576.6	467.931
ATL	0.0898	0.41	0.34	0.473	1066.66	1648.45

Note: Population codes follow Table 1.

и гаплотипического разнообразия составили 0.00295 ± ± 0.00200 и 0.8123 ± 0.0912 соответственно, что укладывается в пределы изменчивости других видов утиных птиц (π = 0.0020–0.0130; H = 0.68–0.987; (McCracken et al., 2001; Kulikova et al., 2004, 2005; Kraus et al., 2011). Доля внутрипопуляционной генетической изменчивости составила 91.85 %, тогда как межпопуляционная компонента изменчивости оказалась равной 8.15 %. Филогенетические реконструкции продемонстрировали отсутствие кластеризации по географическому признаку (см. рис. 1 и 2). Аналогичные результаты были получены в исследованиях других видов утиных (Cronin et al., 1996; McCracken et al., 2001; Kulikova et al., 2005; Peters, Omland, 2007; Flint et al., 2009; Kraus et al., 2011; Peters et al., 2014). Значительная натальная дисперсия, симпатрия на зимовке или в местах гнездования, смена мест гнездования и сезонные миграции способствуют перераспределению особей в пределах ареала (Павлов и др., 1997; Flint et al., 2009), что и объясняет высокое внутрипопуляционное генетическое разнообразие и низкую межпопуляционную дифференциацию.

Тем не менее анализ молекулярной изменчивости AMOVA позволил выявить некоторую генетическую структурированность наших данных. Самые высокие показатели генетической подразделенности были получены после объединения популяций в две группы, европейско-сибирскую и восточноазиатскую. В первую вошли выборки из Европы, Сибири и североамериканской Атлантики, во вторую – выборки из Дальнего Востока, западной Берингии, Аляски, Алеутских островов и Тихоокеанского побережья Северной Америки. Учитывая, что на изменчивость между группами приходится 13.9 % генетического разнообразия, можно предположить, что популяции внутри одной группы чаще обмениваются мигрантами, чем с популяциями из другой группы. Известно, что формирование пар у утиных птиц часто происходит во время зимовки (Rohwer, Anderson, 1988). Ареалы зимовок сибирской и европейской популяций свиязи накладываются друг на друга на значительной территории, которая, начинаясь от севера Испании, простирается в северо-восточном направлении через Францию, Германию и дальше на восток до Урала (Monval, Pirot, 1989). Общими местами зимовок для свиязей из западной Берингии и Дальнего Востока являются юг Корейского полуострова и Япония (Sonobe, Usui, 1993; Павлов и др., 1997).

С середины XX в. евразийская свиязь стала часто встречаться во время зимовки на восточном и западном побережьях Северной Америки (Edgell, 1984; Johnsgard, 2010; Bogiatto et al., 2015). В последние годы отмечены находки свиязей и в летнее время, причем это были не одиночные самцы, а птицы парами. Предполагают, что свиязь даже гнездится в небольших количествах на Аляске и в северо-западной части Канады (Fournier, Hines, 1996; Johnsgard, 2010). В то же время появились сведения о находках представителей этого вида и в Южной Америке (Williams, Beadle, 2003; Johnson, 2018). Согласно полученным в нашей работе результатам, выборка свиязи западного побережья Северной Америки генетически не дифференцирована от популяции западной Берингии и слабо дифференцирована от дальневосточной популяции (см. табл. 3). Птицы из выборки восточного побережья США и Канады относятся к выделенной по данным AMOVA европейско-сибирской группе. Таким образом, на восточном побережье Северной Америки зимуют птицы из Европы и Сибири, а на западном – из Северо-Восточной Азии.

Факт обнаружения гаплотипа американской свиязи в выборке из Анадыря (Чукотка) у особи, фенотипически не отличимой от евразийской свиязи, уже описан в нашей предыдущей работе (Куликова, Журавлев, 2010). Это неудивительно, учитывая встречи фенотипических гибридов *americana* × *penelope* на Чукотке (Кречмар, Кондратьев, 2006). Известно, что американские свиязи гнездятся в бассейне р. Анадырь и что оба вида контактируют в Евразии и Северной Америке во время зимовки (Peterson et al., 1983; Edgell, 1984). Зимовка и гнездование на одной территории, тесное родство американской и евразийской свиязей (Peters et al., 2005, 2014) могут способствовать межвидовой гибридизации.

Заключение

Таким образом, филогеографическая структура евразийской свиязи *M. penelope* оказалась невыраженной. Однако на основании данных полиморфизма контрольного региона мтДНК нам удалось выделить две группы популяций, восточноазиатскую и европейско-сибирскую, что подтверждают и сведения о кольцевании. Но наши результаты базируются на изменчивости мтДНК, которая отражает только матриархальную сторону изменчивости. Для получения полной картины филогеографической структуры вида необходимо продолжить исследования с применением аутосомных и сцепленных с Z-хромосомой маркеров.

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ORCID ID

I.V. Kulikova orcid.org/0000-0003-4847-2560

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A *Drosophila melanogaster* mitotype may have an adaptive meaning

D.V. Petrovskii[1, 2] ✉, L.P. Zakharenko[1, 3] ✉

[1] Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia
[2] Institute of Systematics and Ecology of Animals, SB RAS, Novosibirsk, Russia
[3] Novosibirsk State University, Novosibirsk, Russia
✉ e-mail: dm_petr@ngs.ru; zakharlp@bionet.nsc.ru

Several different mitochondrial clades have been found in natural populations of *Drosophila melanogaster*. Most often, the difference is in single nucleotide substitutions, some of which are conservative. Some clades are rare, and others dominate. It has been reported that clade III dominates over clades V and VI in seven populations of *D. melanogaster*. We compared *D. melanogaster* strains with different mitotypes by locomotor activity (using TriKinetics Drosophila Activity Monitor), energy expenditure (by indirect calorimetry, based on measuring oxygen consumption) and life span (under extreme conditions at 29 °C). The nuclear genomes of these strains were aligned for several generations by backcrosses. According to our data, individuals with the mitotype from clade III had a higher level of locomotor activity and longer life span. In terms of energy expenditure, the strains studied did not differ. However, the same level of energy expenditure may be differently distributed between the state of activity and the state of rest or sleep. If the energy expenditure during the sleep in flies with different locomotor activity is the same, then an individual with the same overall energy expenditure can move a greater distance or be active longer. This can be interpreted as an advantage of the strain with the mitotype from clade III compared to the other two mitotypes studied. If individuals have different energy expenditure values at rest, the strains with lower energy expenditure at rest spend less energy during forced inactivity. In this case, the mitotype from clade III should also be advantageous. What nucleotide substitutions in the mitotype from clade III can provide an adaptive advantage is not clear yet. We assume that individuals with widespread clade M(III) may have adaptive advantages compared to other mitotypes due to their greater locomotor activity even with the same energy expenditure. Further studies are required, for mitotypes are polymorphic for single nucleotide polymorphism not only between but also within the clades.
Key words: *Drosophila melanogaster*; mitotype; life span; locomotor activity; energy expenditure.

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Митотип *Drosophila melanogaster* может иметь адаптивное значение

Д.В. Петровский[1, 2] ✉, Л.П. Захаренко[1, 3] ✉

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Институт систематики и экологии животных Сибирского отделения Российской академии наук, Новосибирск, Россия
[3] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
✉ e-mail: dm_petr@ngs.ru; zakharlp@bionet.nsc.ru

В природных популяциях *Drosophila melanogaster* обнаруживают несколько митохондриальных клад, отличающихся друг от друга по первичной последовательности. Чаще всего это однонуклеотидные замены, часть из них консервативна. Одни клады встречаются редко, другие доминируют. В семи исследованных на сегодняшний день популяциях *D. melanogaster* клада III преобладает по сравнению с кладами V и VI. Мы сравнивали линии *D. melanogaster* с разными митотипами, но с выровненными в течение нескольких поколений беккроссами ядерными геномами, по двигательной активности (с использованием TriKinetics Drosophila Activity Monitor), энергообмену (методом непрямой калориметрии, на основе измерения потребления кислорода) и по длительности жизни (в экстремальных условиях содержания при 29 °C). По нашим данным, у особей с митотипом, относящимся к кладе III, выше уровень локомоторной активности и больше продолжительность жизни. По энергопотреблению исследованные линии не различаются. Однако один и тот же уровень энергообмена может быть по-разному распределен между состоянием активности и состоянием покоя. Если энергообмен в состоянии покоя у мух с разной локомоторной активностью одинаков, то особь при одинаковых тратах суммарной энергии может перемещаться на большее расстояние или дольше проявлять активность. Это можно интерпретировать как преимущество линии

с митотипом, относящимся к кладе III, по сравнению с двумя другими исследованными митотипами, относящимися к кладам V и VI. Если особи имеют разный энергообмен в покое, то линии с наименьшим энергообменом в покое потратят меньше энергии при вынужденном бездействии. И в этом случае митотип, относящийся к кладе III, будет иметь преимущества. Какие нуклеотидные замены в этом митотипе могут обеспечивать адаптивное преимущество, пока остается непонятным. Мы предполагаем, что особи из широко распространенной клады III могут иметь адаптивные преимущества по сравнению с другими митотипами благодаря большей локомоторной активности даже при одинаковом энергообмене. Требуются дальнейшие исследования, поскольку митотипы полиморфны по набору однонуклеотидных замен не только между кладами, но и внутри клады.
Ключевые слова: *Drosophila melanogaster*; митотип; продолжительность жизни; локомоторная активность; энергообмен.

Introduction

The mitochondrial genome is responsible for supplying cell with energy. In particular, it encodes a number of the proteins involved in the Krebs cycle, β-oxidation of fatty acids, and oxidative phosphorylation. Many studies indicate that there is a link between the mtDNA structure and life span (Lehmann et al., 2008; Muradian et al., 2010). For example, it is thought that the mutation frequency in mtDNA of placental mammals enables lower production of reactive oxygen species (ROS) (Rottenberg, 2007). Different mito-haplotypes of *Drosophila simulans* vary in ATPase activity, mitochondrial cytochrome C content, hydrogen peroxide content, and other bioenergetic parameters (Katewa, Ballard, 2007). Though the mentioned authors used *D. simulans* from sympatric populations, the effect of mitonuclear interactions on the observed differences can hardly be ruled out (Stuart, Brown, 2006). Among numerous ageing theories, the free-radical theory of ageing takes a special place. According to this theory some ROS, specifically those produced in mitochondria, impair the cells (Harman 1956; Halliwell, 2012).

Due to the significant mtDNA variability, the difference between the mitochondria that belong to different strains may exceed one hundred SNPs (Zhu et al., 2014). The researchers studying mtDNA polymorphism in *D. melanogaster* identify several mitochondrial clades. One classification divides them into the M and S clades different by one conservative single-nucleotide substitution 37C/T (position 2187 in the sequence with GenBank accession number NC001709) (Richardson et al., 2012; Ilinsky, 2013; Ilinsky et al., 2013). Another classification defines eight clades grouping into M(I–V, VIII) and S(VI–VII). The clades are different by their SNP sets, which are partly conservative (Richardson et al., 2012; Early, Clark, 2013; Ilinsky, 2013). Clade M(III) prevails in such natural populations as Raleigh, North Carolina (United States) (Richardson et al., 2012), Beijing (China), Ithaca (NY, United States), Netherlands, Tasmania, and Zimbabwe (Early, Clark, 2013) if compared to clades M(V) and S(VI), and it is highly polymorphic with regard to its SNP set. According to (Maklakov et al., 2006), the intrapopulation variability may affect life span and other physiological parameters.

In the present study we compare strains with different mitotypes, such as a mito-haplotype from clade III (M(III)), a mito-haplotype from clade V, and a mito-haplotype from clade IV (S(VI)), by their locomotor activity, energy expenditure, and life span to understand whether any of these strains have physiological advantages over others.

Materials and methods

***Drosophila* strains.** Mitochondria are considered to be an important factor affecting life span and other physiological parameters. However, the variability of a mitochondrial genome is influenced by the genetic background determined by the nuclear genome (Clancy, 2008). While the mitochondrial genome is inherited from the mother, the nuclear genome is contributed by both parents. By means of backcrosses, this feature allows scientists to obtain strains with different mitotypes but the same nuclear DNA. Saturation backcrosses during at least 10 generations for male flies (strain Bi90) produced *Drosophila* strains having similar nuclear genomes and three different mitochondrial genomes: M(III), M(V), and S(VI). The *Drosophila* strains used in this study were provided by N.E. Gruntenko. Two generations of flies were treated with tetracycline (0.25 mg/ml) to make a *Wolbachia*-free strain and prevent the possible effect the bacteria may have on important physiological parameters (Gruntenko et al., 2017). It should be noted that this antibiotic does not affect *Drosophila*'s life span (Min, Benzer, 1997). The strains were partly duplicated to avoid the possible effect of incomplete genome substitution (Gruntenko et al., 2017). The following strains based on the strain Bi90 nuclear genome were studied: M(III) – a mitotype from clade III (initial genome of strain W304, two lineages), M(V) – a mitotype from clade V (initial genome of strain B90, two lineages), and S(VI) – a mitotype from clade VI (initial genomes of strains W1118 (two lineages), W153 and W181 (one lineage each)).

The energy expenditure of the tested flies was studied by indirect calorimetry, measuring oxygen consumption in a hermetically sealed chamber with alkali. When breathing, animals consume oxygen and exhale carbon dioxide and water. The exhaled CO_2 reacts with the alkali (KOH) to form a solid matter (K_2CO_3) so the initial pressure in the chamber falls proportionally to the amount of the consumed oxygen. The apparatus to carry out energy expenditure measurements was designed to be similar (with slight modifications) to that described in (Diarra et al., 1999). It consisted of ten thermostatic chambers (eight measuring chambers and two reference ones) of 3 cubic ml each and a single pressure sensor. Ten male flies (3–7 days old) were placed into each chamber. The experiment lasted from 11 a.m. to 4 p.m., the measurements starting one hour after acclimation of the flies to a temperature of 29 °C. The pressure drop in each chamber was measured twice, 6 min each time.

To estimate the energy expenditure, the inclination angle of the approximation curve on the pressure measurement

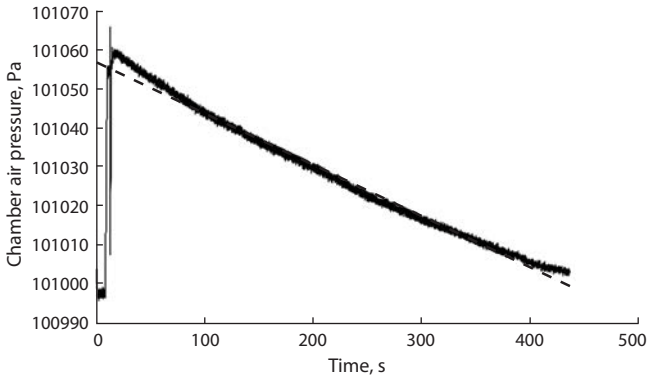

Fig. 1. Example of oxygen consumption measurement.
The solid line is the pressure in the chamber; the dotted line is the approximating line.

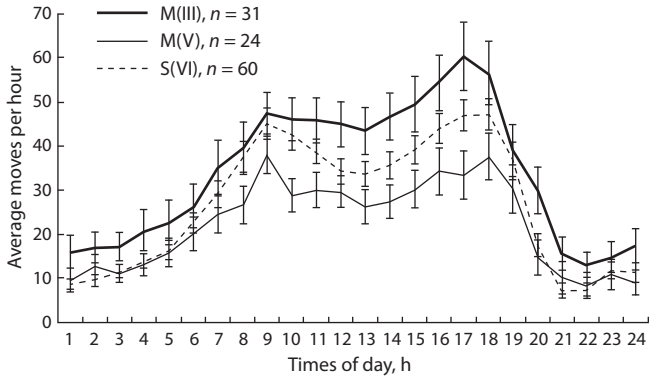

Fig. 2. The daily dynamics of the locomotor activity in different *Drosophila* mitotypes (mean ± SE).

graph was measured (Fig. 1). For the purposes of statistical analysis, the average value for two repeated measurements was considered. The flies were weighed immediately after the experiment. Four experiments were carried out in total.

The locomotor activity in the male flies of the tested strains was monitored using TriKinetics Drosophila Activity Monitor ("Trikinetics", Waltham, MA, United States). To perform the monitoring, each male was placed in a glass tube containing its standard feed. The locomotor activity of the fly was determined as the number of times the fly crossed an infrared beam per minute within five days. For each fly, its daily average locomotor activity was calculated based on its average hourly activity from 11 a.m. to 4 p.m. from day 2 to day 4 of the experiment. The flies that did not survive until the end of the experiment were excluded from the analysis. For each strain, 12 to 16 flies (3–7 days) were analyzed. The experiment was conducted at 29 °C.

The life span of the tested flies was determined at the same temperature (29 °C), each tube containing 20 male flies. For each strain, five tubes were analyzed.

Statistical analysis. The results were evaluated with Statistica 6.0. To compare the mean values, Fisher's least significant difference method (LSD) was applied. The life span was estimated by the logrank test (Bland, Altman, 2004) with the χ^2 statistics.

Results

In daytime hours, the maximum locomotor activity was recorded in M(III) – mitotype flies (Fig. 2). Their average daily activity was 51.2 ± 5.9 crossings per hour, which was statistically higher ($p < 0.02$) than in S(VI) (39.9 ± 2.9) or M(V) (35.0 ± 4.0, $p < 0.05$). The difference between the M(V) and S(VI) mitotypes in average daily activity was insignificant ($p > 0.4$).

The average oxygen consumption per fly was 2.76 µl/h. In the studied flies, this parameter statistically correlated with body weight (R = 0.44, $p \approx 0.01$) (Fig. 3). When averaged over strain, the values of oxygen consumption correlated with the average daily activity (R = 0.61, $p \approx 0.1$). However, when calculated as oxygen consumption per mg of fly body weight, they statistically correlated with neither body weight ($p > 0.2$) nor average daily activity ($p > 0.8$). The performed experiments demonstrated statistically significant differences

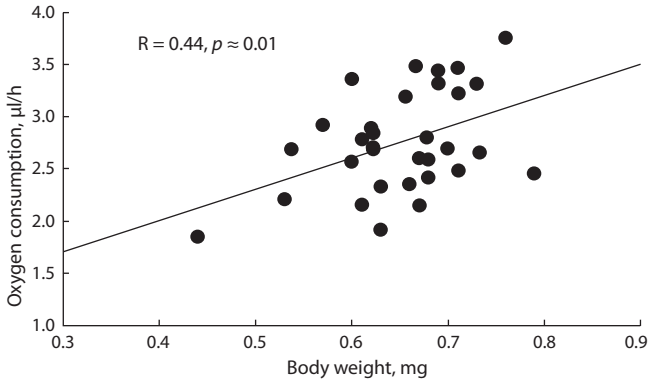

Fig. 3. Correlation of the average values of oxygen consumption with the average body weight of flies.

Energy expenditure in different *Drosophila* mitotypes

Mitotype	*n*	Body weight, mg	Average oxygen consumption	
			per fly, µl/h	per gram body weight, ml/g/h
M(III)	8	0.662 ± 0.014	2.83 ± 0.19	4.27 ± 0.25
M(V)	8	0.653 ± 0.019	2.79 ± 0.19	4.31 ± 0.35
S(VI)	12	0.644 ± 0.023	2.70 ± 0.11	4.21 ± 0.12

Notes: *n* – number of flies; the data are presented as mean±SE.

between the mitotypes in neither body weight nor oxygen consumption (Table).

The life span in the M(III) mitotype flies was statistically higher than that in the S(VI) ($\chi^2 = 9.24$, $p < 0.01$) and M(V) ($\chi^2 = 4.83$, $p < 0.05$) mitotypes. No statistically significant differences between the S(VI) and M(V) mitotypes were found (Fig. 4).

Discussion

The studied M(III) – mitotype flies demonstrated the higher level of locomotor activity, but their energy expenditure was close to that in flies of M(V) and S(VI) mitotypes, which can be considered an adaptive advantage of M(III) flies. The

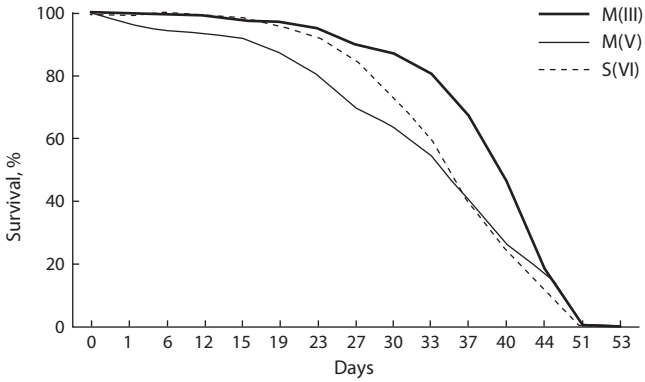

Fig. 4. Life spans of males with different mitotypes.

dependence of oxygen consumption on locomotor activity can be linearly approximated (Schmidt-Nielsen, 1982), so the inclination angle of the line can be interpreted as price of activity or efficiency, while its vertical intercept reflects the metabolic rate at rest (metabolic rate at zero activity). This is important, because the same level of energy expenditure in flies with different genotypes can be distributed differently between the rest and active states.

Since in our study we had only one activity/energy expenditure point on the plane for each mitotype, and since through a single point a myriad of lines can be drawn, we could not unambiguously estimate the energy distribution between the rest and active states. Nevertheless, we can consider two extreme cases.

If in flies with different mitotypes the energy expenditure values at rest are similar, the approximating lines for these mitotypes would have different inclination angles, hence different prices of activity (Fig. 5, *a*). The least steep line (for M(III) mitotype) means that these individuals can move further distance or remain active longer spending the same amount of energy, which can be interpreted as an advantage of the mitotype if compared to the others. However, if individuals have different energy expenditures at rest (See Fig. 5, *b*) and similar prices of activity (similar approximation curve inclination angles), then the M(III) strain would get an advantage over the strains of the other studied mitotypes, because the least energy expenditures at rest would help these flies spend less energy during inevitable inactivity under unfavorable conditions.

From all viewpoints, the actual pattern seems to be a combination of the two extreme cases, and in order to study the way energy expenditure depends on the activity parameter it is strongly recommended to investigate both parameters simultaneously. In this case, a series of energy expenditure and activity values can be obtained for every strain, which makes it possible to trace the true effect of a mitotype.

In *D. simulans*, the life span does not correlate with exhaled CO_2 (Melvin et al., 2007). The same was confirmed in our experiments with *D. melanogaster*. However, the life span of the M(III) mitotype male flies kept under extreme conditions (29 °C) turned out to be higher than those of the M(V) and S(VI) mitotypes, whose life expectancies were similar.

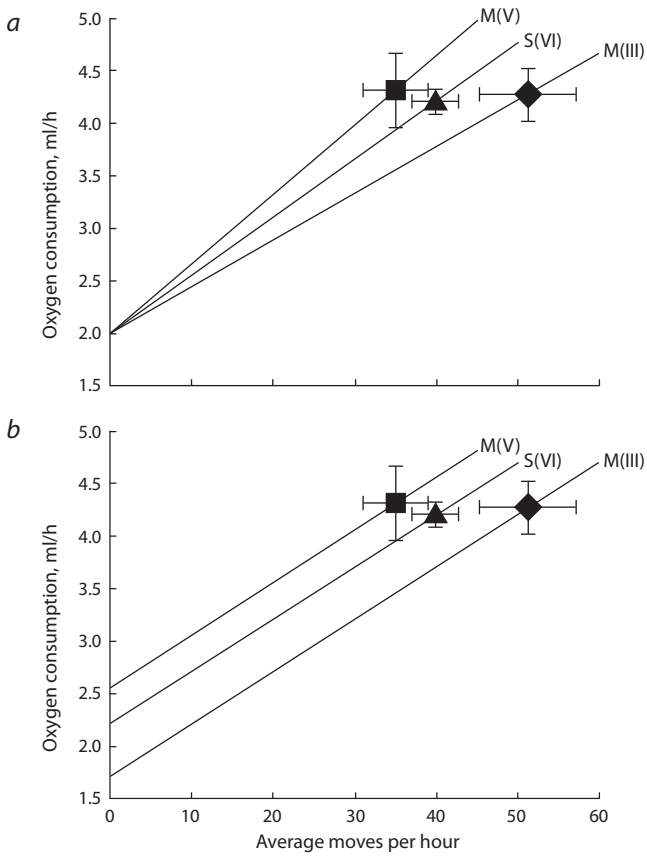

Fig. 5. Hypothetical variants of energy expenditure dependence on the locomotor activity in flies with different mitotypes.

a, flies with different mitotypes have the same level of oxygen consumption at zero activity, but the angles of inclination of the approximating straight lines are different; *b,* flies with different mitotypes have the same angles of inclination of the approximating straight lines, but the levels of oxygen consumption differ at zero activity. The lower straight line is M(III), the upper is M(V), and the middle is S(VI).

Conclusion

We assume that individuals of the M(III) mitotype strain, belonging to the most widespread clade, have adaptive advantages as compared to the other mitotypes due to their higher locomotor activity. Which nucleotide substitutions in M(III) are responsible for this advantage remains an open issue, and further investigation should be performed with more strains related to the same clade and to other mitotic clades.

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ORCID ID

D.V. Petrovskii orcid.org/0000-0002-0623-0363
L.P. Zakharenko orcid.org/0000-0002-6341-8522

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