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# Molecular genetic detection and differentiation of *Xanthomonas oryzae* pv. *oryzicola*, bacterial leaf streak agents of rice

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**Abstract.** The genus *Xanthomonas* comprises phytopathogenic bacteria which infect about 400 host species, including a wide variety of economically important plants. *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957) Swings et al., 1990 is the causal agent of bacterial leaf streak (BLS) being one of the most destructive bacterial diseases of rice. BLS symptoms are very similar to those of bacterial blight caused by closely related *Xanthomonas oryzae* pv. *oryzae*. *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* and often occur in rice fields simultaneously, so separate leaves may show symptoms of both diseases. The quarantine status and high severity of the pathogen require a highly efficient, fast and precise diagnostic method. We have developed an assay for *Xanthomonas oryzae* pv. *oryzicola* detection using real-time polymerase chain reaction (qPCR) and PCR amplicon sequencing. The DNA samples of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* were obtained from the collection of CIRM-CFBR (France). To evaluate the analytical sensitivity of the assay, a vector construct based on the pAL2-T plasmid was created through the insertion of *X. o.* pv. *oryzicola* target fragment (290 bp). Primers and a probe for qPCR were selected for the *hpa1* gene site. They allowed identifying all the strains the sequences of which had been loaded in the GenBank NCBI Nucleotide database before November 11, 2021. The SeqX.o.all sequencing primers were selected for the *hrp* gene cluster sequence, namely for the nucleotide sequence encoding the Hpa1 protein, the sequencing of which allows for efficient differentiation of *X. oryzae* species. The analytical specificity of the system was tested using the DNAs of 53 closely related and accompanying microorganisms and comprised 100 % with no false-positive or false-negative results registered. The system's analytical sensitivity was not less than 25 copies per PCR reaction. Its efficacy has been confirmed using five different qPCR detection systems from different manufacturers, so it can be recommended for diagnostic and screening studies.
Key words: *Xanthomonas oryzae* pv. *oryzicola*; *Xanthomonas*; polymerase chain reaction; qPCR; bacterial leaf streak; specificity; sensitivity; species diagnostics.

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# Молекулярно-генетическое выявление и дифференциация возбудителей бактериальной полосатости листьев риса *Xanthomonas oryzae* pv. *oryzicola*

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**Аннотация.** Бактерии рода *Xanthomonas* Dowson, 1939 поражают около 400 видов растений, в том числе важные сельскохозяйственные культуры. Бактериальная полосатость риса – одно из самых разрушительных заболеваний, вызвано бактериями вида *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957) Swings et al., 1990. Сильное сходство симптомов поражения с другим карантинным близкородственным патовариантом – *Xanthomonas oryzae* pv. *oryzae* (Ishiyama, 1922) Swings et al., 1990, а также возможность совместного заражения делают визуальную идентификацию невозможной. Карантинный статус и высокая вредоносность патогена требуют высокоэффективного, быстрого и точного метода его диагностики. Целью исследования были разработка и апробация наборов реагентов для выявления бактерии *Xanthomonas oryzae* pv. *oryzicola*, вызывающей бакте-

риальную полосатость листьев риса, методом полимеразной цепной реакции в реальном времени (ПЦР-РВ), а также ПЦР с последующим секвенированием ампликонов. В работе изучены образцы ДНК *X. o.* pv. *oryzae* и *X. o.* pv. *oryzicola*, полученные из коллекции CIRM-CFBR (Франция). Для проверки аналитической чувствительности была создана конструкция на основе вектора pAL2-T с целевой вставкой 290 п. н. Были подобраны и апробированы праймеры и зонд для специфической амплификации фрагмента гена *hpa1* методом ПЦР-РВ, позволяющие обнаруживать ДНК *X. o.* pv. *oryzicola*. Показана способность с помощью разработанных праймеров обнаруживать все штаммы *X. o.* pv. *oryzicola*, последовательности которых находились в базе данных GenBank NCBI на 11.11.2021. Аналитическая специфичность набора реагентов протестирована на выборке из ДНК, выделенных из 53 близкородственных и сопутствующих организмов, и составила на исследованной выборке 100 %. Ложноположительных и ложноотрицательных результатов не обнаружено. Проверка аналитической чувствительности показала, что стабильный специфичный сигнал ПЦР-РВ наблюдался при разведении контрольной плазмиды до 25 копий на реакцию. Работоспособность полученного набора реагентов была подтверждена тестированием на пяти приборах для ПЦР-РВ разных производителей, что дает возможность рекомендовать его для проведения диагностических и скрининговых исследований. Праймеры для секвенирования seqX.o.all были подобраны на последовательность кластера генов *hrp*, а именно на нуклеотидную последовательность, кодирующую белок Hpa1. Секвенирование выбранного участка позволяет эффективно дифференцировать бактерии вида *X. oryzae*.
Ключевые слова: *Xanthomonas oryzae* pv. *oryzicola*; *Xanthomonas*; полимеразная цепная реакция; ПЦР-РВ; бактериальная полосатость риса; специфичность; чувствительность; видовая диагностика.

## Introduction

Bacteria *Xanthomonas* Dowson, 1939 are spread worldwide and able to infect at least 400 kinds of plants including those of high economic importance (Bogdanove et al., 2011; Ryan et al., 2011; Fang et al., 2019). Currently, 27 species of this family have been known, many of which demonstrate high levels of virulence and specificity in certain kinds of plants (Leyns et al., 1984; Ryan et al., 2011; An et al., 2020). Bacterial leaf streak (BLS) is considered to be one of the most devastating diseases caused in rice by *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957), Swings et al., 1990 (Soto Suárez et al., 2010).

The disease results in 8 to 32 % of yield loss and is regarded as a serious problem in rice-producing countries (Liu et al., 2014; Jiang et al., 2020). Since the damage done by BLS can seriously threaten the world's food security (Tang et al., 2000; Lang et al., 2014), *Xanthomonas oryzae* pv. *oryzicola* was included in List 1 of harmful quarantine organisms that are not present in the EEU territory as well as in List A1 of the European and Mediterranean Plant Protection Organization (EPPO) that considers the bacteria as quarantine ones[1]. Despite the fact that BLS is believed to have been detected for the first time in 1918 in the Philippines, its pathogen was identified only in 1957 in China (Nino-Liu et al., 2006). For the time being, BLS spread is limited to the tropical and subtropical parts of Asia, Northern Australia and a part of Western Africa (EPPO, 2007; Xie et al., 2014; Jiang et al., 2020). The pathogen is absent in the Russian Federation despite cases of infection[2] in the southern part of the country and the Russian Far East (EPPO, 2007, 2018). According to the EPPO Reporting Service, no cases of *X. o.* pv. *oryzicola* infection have been registered since 1994[3].

*Oryza sativa* L., 1753, commonly known as Asian rice, is a typical host plant for *X. o.* pv. *oryzicola.* In addition, it affects some weed cereals and several other cultivated plants such as *Poaceae* including *Leersia* spp., *Leptochloa* spp., *Oryza* spp., *Paspalum scrobiculatum*, *Zizania*, *Zoysia* spp. (Ou, 1985; Saddler, Bradbury, 2005; EPPO, 2007). The bacteria mainly spread through infected seeds as well as due to mechanical damage. In case of small plants, infection occurs through wind, raindrops, watering or after contacting infected plant material (Mew et al., 1993).

In plants, *X. o.* pv. *oryzicola* reproduce in the substomatal cavity where they get through the stomata to affect the intercellular space of the parenchyma. However, they do not get as far as the xylem and their spread is limited by the mesophyll tissue's apoplast (Nino-Liu et al., 2006; Triplett et al., 2011; Jacques et al., 2016). The early stage of infection is characterized by small watery interveinal strokes that later transform into bacterial effusion (Mew et al., 1993). The veins act as barriers preventing the pathogen's further spread and extending a leaf's affected areas along its length, so they can merge later. In case of severe infection, BLS becomes difficult to differ from the bacterial burn caused by *Xanthomonas oryzae* pv. *oryzae*, another quarantine bacterial species. Visual identification can also be complicated by favorable environmental conditions and plant resistance (Swings et al., 1990; Poulin et al., 2014), since both species can infect rice fields at the same time (Mew et al., 1993; Nino-Liu et al., 2006).

The objective of the presented study was to develop and test an assay for genetic detection and diagnostics of the

[1] EPPO for the EU under Contract 90/399003. Data Sheets on Quarantine Pests. https://gd.eppo.int/download/doc/530_ds_XANTOR_en [Accessed: 23.11.2021].

[2] Cabi Invasive Species Compendium. Datasheet *Xanthomonas oryzae* pv. *oryzicola* (bacterial leaf streak of rice). https://www.cabi.org/isc/ [Accessed: 23.11.2021].

[3] EPPO Global Database. https://gd.eppo.int [Accessed: 23.11.2021].

*Xanthomonas oryzae* pv. *oryzicola* pathogen using real time polymerase chain reaction (qPCR) and PCR amplicon sequencing.

## Materials and methods

The presented study was carried out at the Biotechnology Collective Use Center of the All-Russian Research Institute of Agricultural Biotechnology and Syntol LLC. As qPCR positive controls, the DNA samples of the Indian typical strain of *X. o.* pv. *oryzae* (2532) and Malaysian pathotype of *X. o.* pv. *oryzicola* (2286) from the French Collection of Plant Associated Bacteria (CIRM-CFBP, France) were used. For the last pathotype, a draft whole genome assembly was obtained (Wilkins et al., 2015). *X. o.* pv. *oryzae*'s geographic distribution is limited to the territories of Asia, Africa and North America, while that of *X. o.* pv. *oryzicola* – to the countries of Asia and Africa. Selecting the target strains, we relied upon the customs statistics of rice import to Russia and according to their data 31.7 % of imported rice in 2018 was supplied by India, followed by Thailand, Pakistan and Kazakhstan[4].

In design of oligonucleotides for qualitative detection of *X. o.* pv. *oryzicola* DNA, the *hpa1* gene region was used. As many other gram-negative pathogens, *X. o.* pv. *oryzicola* has the type III secretion system (T3SS) being a molecular syringe with which the bacteria deliver effector proteins directly into the host cell cytosol (Zhu et al., 2000; Furutani, 2003; Li et al., 2011). The T3SS and its secreted components promote a hypersensitive response (HR) in resistant plants and plants not being the main host for the pathogen. The system is coded as *hrp*, a hypersensitivity and pathogenicity gene (Cho et al., 2008; Fan et al., 2017), the main operon of which is composed of more than 20 genes in several transcription units that contain the *hrp*, *hrc* and *hpa* genes (Zou et al., 2006; Cho et al., 2008). The oligonucleotides were synthesized by Syntol LLC using their expendables. To design the qPCR and PCR amplicon sequencing reactions, reaction buffer B-009 (Syntol LLC, Russia) was used.

The buffer had the following component concentrations: 3 mmol of $MgCl_2$, 0.25 mmol of dNTP, and 2.5 e. a. of polymerase with antibodies to inhibit ferment activity (Syntol LLC). When designing the oligonucleotides, we made sure the annealing temperature was 60–62 °C for the primers and 64–67 °C – for the probe with 3′-GC-clamp.

The multiparameter analysis of the properties of the selected primers was performed using such online applications as Thermofisher Multiple Primer Analyzer (https://www.thermofisher.com), Promega Biomath Calculator – Tm for Oligos Calculator (https://worldwide.promega.com), Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu). The qPCR fluorescence-labelled probe incorporated a FAM dye attached to the probe's 5′ end. The RTQ-1 dye attached to the probe's 3′ end served as a quencher. The primer concentration in reaction mixture was 800 nM, and 400 nM – in the probe. The qPCR reaction's repeatability and reproducibility was assessed using the following detection systems: ANK-M (IAI RAS, Russia), QuantStudio 5 (Thermo Fisher Scientific, USA), CFX-96 (Bio-Rad, USA), DTprime 5 (DNA-Technology, Russia), Rotor-Gene 6000 (Qiagen, USA). The obtained results were considered positive if the fluorescence signal level exceeded the threshold of 10 % module difference of the lowest and highest signals.

To verify the analytical sensitivity of the assay, a vector pAL2-T-based structure (Eurogen, Russia) with a 290 bps inclusion of *X. oryzae* pv. *oryzicola* was designed. Ligation was carried out after the PCR product was purified using the ColGen DNA purification kit (Syntol LLC). To design the vector-based structure, a T4 DNA ligase buffer (Thermo Fisher Scientific) was used. The plasmid DNA was impregnated into *Escherihia coli* bacteria (Migula 1895) through thermal shock. The vector's presence was attested using the PCR-colony method with the standard M13 primers followed by 1.5 % agarose-gel visualization. Plasmid DNA separation was carried out using a PlasGen reagent kit (Syntol LLC). The obtained circular plasmid was processed with the *NotI* restriction enzyme (ThermoFisher Scientific), its concentration measured in a Quantus fluorometer (Promega Corporation, USA). To test the analytical sensitivity of the designed assay, qPCR to dissolve the plasmid were replicated 2 and 4 times. The analytic specificity of the designed primers and probes was tested using the DNAs of 53 closely related and accompanying microorganisms (Alyapkina et al., 2018).

Bioinformatic analysis and data processing were performed using the UGENE (Unipro, Russia) and AliView (Sweden) software solutions.

To sequence *X. o.* pv. *oryzicola*'*s* DNA, a primer couple including seqX.o.all_F 5′-TCTTTGAACACACAATTC GGCGG-3′ and seqX.o.all_R 5′-TGG AGAATCTCTC CGACGATA-3′ was designed. The amplification program of PCR amplicon sequencing reaction included primary denaturation (5 min at 95 °C); cyclic denaturation (15 s at 95 °C); annihilation (40 s at 60 °C); cyclic elongation (36 cycles of 30 s at 72 °C); final elongation (5 min at 72 °C). The sequencing was carried out using a Nanofor 05 genetic analyzer (IAI RAS).

## Results and discussion

The search for nucleotide sequences in GenBank NCBI found 208 of them to belong to the Xanthomonas family including 20 strains of *X. o.* pv. *oryzicola*. During sequence alignment performed in AliView, qPCR oligonucleotides were selected for the regions of the *hpa1* target gene conservative to *X. o.* pv. *oryzicola* in such a way that the selected

[4] Agrobusiness Think Tank. https://ab-centre.ru/articles/analiz-importa-risa-v-rossiyu-v-2001-2019-gg-

**Table 1.** Primer and probe sequencies selected for the *hpa1* gene region of *X. o.* pv. *oryzicola* and their amplification programs

| Name | Sequence | Amplification program |
|---|---|---|
| Sva3X.o.cola_F | F 5′-ATTCGAGCCAGGGCRGCAATG-3′ | 5 min at 95 °C<br>15 s at 95 °C<br>40 s at 60 °C<br>50 cycles |
| Sva3X.o.cola_R | R 5′-ACCAAAGTCGCCGCCGCTGCT-3′ | |
| Sva3X.o.cola_FAM | Pb 5′-(FAM) AATCAGCAGGCCGGGAAGGAGAA (RTQ1)-3′ | |

Note. F – forward primer; R – reverse primer; Pb – probe.

primers' attachment sites were strictly specific and enabled the detection of all the target's stains the DNA sequences of which had been loaded in the GenBank NCBI database[5] before 11.11.2021. Table 1 demonstrates the primer and probe sequencies for *X. o.* pv. *oryzicola* diagnostics, selected for its *hpa1* gene region.

The analytic specificity of the assay was tested using 53 DNA samples of closely related and accompanying microorganisms from different collections that included 9 DNA samples of the bacteria belonging to the Xanthomonas family. The samples were obtained from collections:

– of the All-Russian Center of Plant Quarantine and Federal Service for Veterinary and Phytosanitary Surveillance's divisions: *Ralstonia solanacearum* 0023, 0027, 0029, 0030, *Erwinia amylovora*, *Clavibacter michiganensis* subsp. *sepedonicus* 0140, 0028, 0244, *C. m.* subsp. *michiganensis* 0240, 0241, 0242, 0243, *X. o.* pv. *oryzae* 0227, *X. phaseoli*, *Pectobacterium carotovorum* subsp. *carotovorum* 0141, 0168, *P. atrosepticum* 0142, *Dickeya solani*, *Xylophilus ampelinus* 0124, *Pantoea stewarti*, *P. st.* subsp. *indologenes*, *P. aglomerance*, *Candidatus Liberibacter*, *Acidovorax citrulli*;
– CIRM-CFBP collection, France: *P. st.* subsp. *indologenes* CFBP 3614, *C. m.* subsp. *nebraskensis CFBP 2405*, *CFBP 3491*, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens CFBP 3418*, *C.* fl. pv. *poinsettiae CFBP 2403*, *C. fl.* pv. *oortii CFBP 1384*, *X. axonopodis* pv. *phaseoli CFBP 2534*;
– Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany: *X. gardneri* DSM 19127, *X. perforans* DSM 18975, *P. wasabiae* DSM 18074, *X. euvesicatoria* DSM 19128, *X. vesicatoria* DSM 22252, *X. translucens* pv. *translucens* DSM 18974, *P. cacticida* DSM 21821, *P. betavasculorum* DSM 18076, *D. dadantii* subsp. *dieffenbachiae* DSM 18013, *D. d.* subs. *dadantii* DSM 18020, *D. paradisiaca* DSM 18069, *D. chrysanthemi* DSM 4610, *D. zeae* DSM 18068, *P. c.* subsp. *odoriferum* DSM 22556;
– Singerta Company's collection (Russia): *C. m.* subsp. *michiganensis*, *Agrobacteria* spp., *X. campestris* pv. *campestris*, *X. translucens* pv. *translucens*;

[5] National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov [Accessed: 23.11.2021].

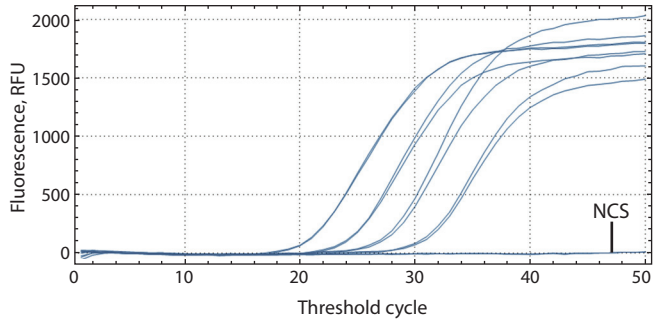


**Fig. 1.** qPCR results, a series of dilutions of a plasmid containing *X. o.* pv. *oryzicola* DNA target insertion (0376), FAM detection channel.
CFX-96 (Bio-Rad) interface; NCS – negative control sample.

– All-Russian Microorganism Collection of G.K. Skryabin Institute of Microorganism Biochemistry and Physiology (Pushchino, Moscow region, Russia): *C. m.* subsp. *insidiosus* BKM Ac-1402$^T$, *C. m.* subsp. *nebraskensis* BKM Ac-1404$^T$, *Pseudomonas savastanoi* BKM B-1546;
– All-Russian Collection of Industrial Microorganisms of Kurchatov National Research Center – GosNIIgenetika (Moscow, Russia): *C. albidum* ВКПМ B-1834.

The primers and probe's analytic specificity for the abovementioned sample set was 100 %. All the samples containing *X. o.* pv. *oryzicola* DNA came positive, which was confirmed by sequencing. No false-positive results were registered including those for the DNA of *X. o.* pv. *oryzae*, which is a closely related variant of the target pathogen.

For testing the assay's analytical sensitivity, the initial concentration of the plasmid with *X. o. pv. oryzicola* insertion of 13 ng/µl or $3 \times 10^9$ copies/µl was used. qPCR in a series of seven dilutions was performed as four 10-time dilutions, first in double repeat, and then in quadruple repeat starting from the fifth series (Fig. 1). After the first dilution, the plasmid concentration reduced to $3 \times 10^5$ copies/µl. Starting from 150 copies, all the following dilution series were additionally titrated as $2 \times 10^n$, $5 \times 10^n$, $7 \times 10^n$ in quadruple repeat. For a series of seven 10-time dilutions, the kinetic curve slope comprised A = –2.671, and the correlation ratio, $R^2 = 0.989$. A stable specific signal was observed down to 25 copies in the reaction mixture. In case of 10-time dilution of *X. o.* pv. *oryzicola* DNA, the

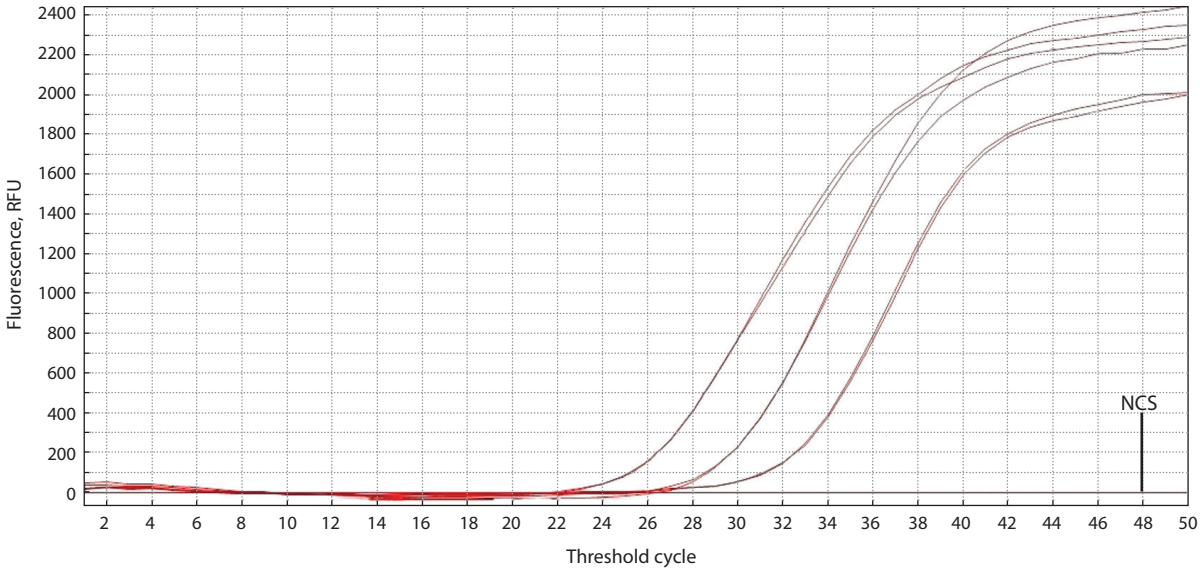


**Fig. 2.** qPCR results, a series of dilutions of a plasmid containing *X. o.* pv. *oryzicola* DNA target insertion (0376), FAM detection channel. ANK-M (IAI RAS) interface. NCS – negative control sample.

**Table 2.** qPCR results obtained in different detection systems using the assay to detect *X. o.* pv. *oryzicola*'s *hpa1* gene region

| Samples, 10-time dilution | Threshold cycle, Ct | | | | |
|---|---|---|---|---|---|
| | ANK-M (IAI RAS) | QuantStudio 5 (Thermo Fisher Scientific) | CFX-96 (Bio-Rad) | DTprime 5 (DNA-Technology) | Rotor-Gene 6000 (Qiagen) |
| $10^{-3}$ | 25.84 | 25.52 | 26.43 | 25.40 | 25.54 |
| $10^{-3}$ | 25.99 | 25.37 | 26.43 | 25.40 | 25.45 |
| $10^{-4}$ | 29.36 | 29.32 | 29.48 | 29.10 | 29.02 |
| $10^{-4}$ | 29.53 | 29.33 | 29.43 | 29.00 | 28.95 |
| $10^{-5}$ | 32.27 | 32.68 | 32.56 | 32.20 | 32.46 |
| $10^{-5}$ | 32.35 | 32.88 | 32.29 | 31.80 | 32.26 |
| Negative control | Not available | Undetermined | Not available | Not available | Not available |
| Negative control | Not available | Undetermined | Not available | Not available | Not available |
| Kinetic curve slope, A | 3.20 | 3.67 | 3.00 | 3.30 | 3.43 |
| Correlation ratio, $R^2$ | 0.998 | 0.999 | 0.998 | 0.997 | 1.000 |
| Efficiency, E % | 105 | 87 | 116 | 101 | 96 |

assay showed lower sensitivity – down to 43 copies in the reaction mixture.

The designed assay was tested using five qPCR detection systems from Russian and foreign manufacturers (Fig. 2, Table 2). As a matrix, a series of 10-time dilutions of the pathogen's DNA was applied. The kinetic curve slope comprised A = 3.00–3.67, the correlation ratio, $R^2 = 0.997$–1.000, and the efficiency, E = 87–116 %. The threshold value difference comprised ± 1 cycle, which was due to the features of the systems' design and their threshold cycle computation algorithms.

To test the primer pair (seqX.o.all_F and seqX.o.all_R) enabling for Sanger sequencing diagnostics, direct PCR was performed. As a matrix, the DNAs of *X. o.* pv. *oryzae* (2532) and *X. o.* pv. *oryzicola* (2286) were used as well as a 1:1 bacterial DNA mixture to imitate joint infection.

To differentiate the two closely related bacterial species, a region from 2 288 483–2 288 778 bps characterized by a large number of nucleotide changes relative to the reference sequence CP050113.1 from the NCBI GenBank database was used. Comparison of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola*'s nucleotide sequences and their mixture

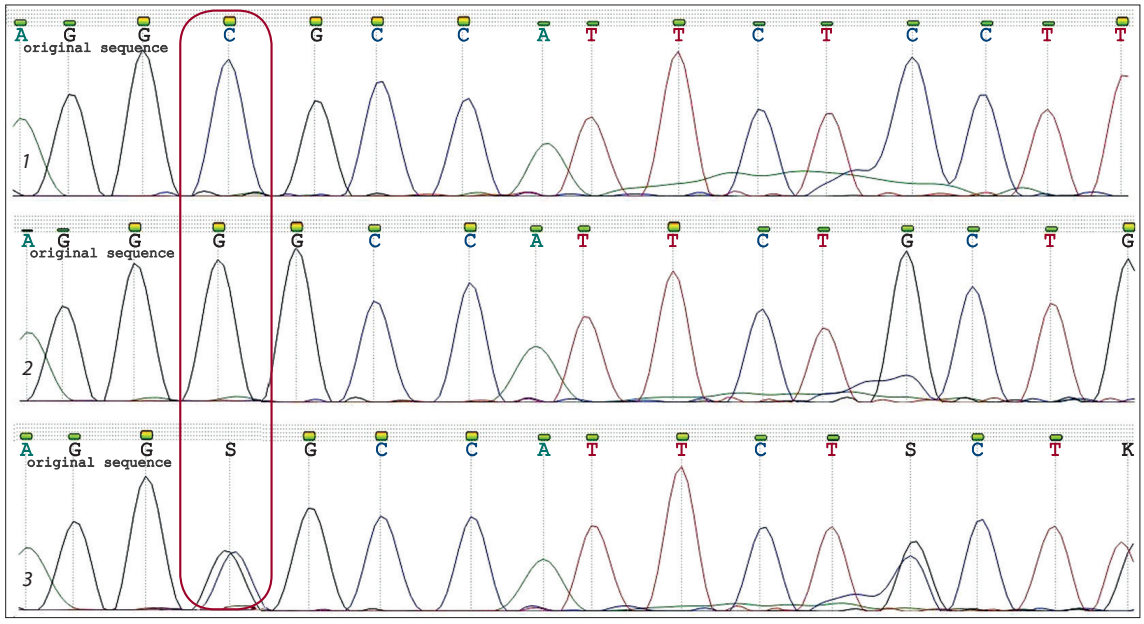


**Fig. 3.** DNA sequence fragments of *X. oryzae* pv. *oryzicola* (*1*), *X. oryzae* pv. *oryzae* (*2*), and a DNA mixture of the two agents (*3*) that resulted from the sequencing using the seqX.o.all primer pair.

The box marks peak matching. Data processed in UGENE v. 38.1 (Unipro).

can be seen in Fig. 3 where peaks C (*X. o.* pv. *oryzicola*) and G (*X. o.* pv. *oryzae*) match unlike the sequencing results for each of the agents. Bioinformatic analysis of the obtained sequences confirmed they could infect a host both individually and jointly.

Alignment of the obtained nucleotide sequences in the mixture of DNA *X .o.* pv. *oryzae* and *X. o.* pv. *oryzicola* detected 19 nucleotide changes relative to the reference genome CP050113.1 (Table 3).

Apart from the nucleotide changes listed in the Table 3, a three-nucleotide insertion in *X. o.* pv. *oryzicola* (in position 2288667 bps) and a three-nucleotide deletion in *X. o.* pv. *oryzicola* (in position 2288702 bps) were found relative to the reference *X. o.* pv. *oryzae* genome (Fig. 4).

The specific primer placement on *X. oryzae* enables analyzing pathovariant sequences to detect joint infection by two closely related bacteria and indicate their species.

The obtained nucleotide sequences made it possible to confirm the cultures' relation to the collected strains in relation to the genome data base. Alignment of the sequences from the data base enabled us to understand certain intraspecific diversity of the *X. o.* pv. *oryzae* strains that came from Africa. At the same time, *X. o.* pv. *oryzicola*'s diversity was not that high and limited to a single SNP per studied region of the *hpa1* gene cluster. Despite the genetic polymorphism of the population of bacterial infections in rice, the designed sequencing and qPCR primers make it possible to detect all isolates irrespectively of the origin of the material included in the Nucleotide NCBI database.

Most of the procedures to detect *X. o.* pv. *oryzae* are applicable for *X. o.* pv. *oryzicola* as well. Their identifica-

**Table 3.** Detected nucleotide changes in the amplified fragment resulted from the sequencing using the seqX.o.all primer pair

| No. | Nucleotide change's position relative to CP050113.1, bps | Nucleotide change's type | |
|---|---|---|---|
| | | *X. o.* pv. *oryzae* | *X. o.* pv. *oryzicola* |
| 1 | 2288729 | G | C |
| 2 | 2288720 | G | C |
| 3 | 2288717 | T | G |
| 4 | 2288677 | A | C |
| 5 | 2288656 | C | T |
| 6 | 2288640 | C | T |
| 7 | 2288636 | A | G |
| 8 | 2288622 | G | T |
| 9 | 2288621 | A | G |
| 10 | 2288578 | G | A |
| 11 | 2288571 | T | C |
| 12 | 2288566 | G | A |
| 13 | 2288565 | C | T |
| 14 | 2288564 | C | T |
| 15 | 2288563 | C | G |
| 16 | 2288562 | T | C |
| 17 | 2288561 | G | T |
| 18 | 2288559 | T | C |
| 19 | 2288558 | T | C |

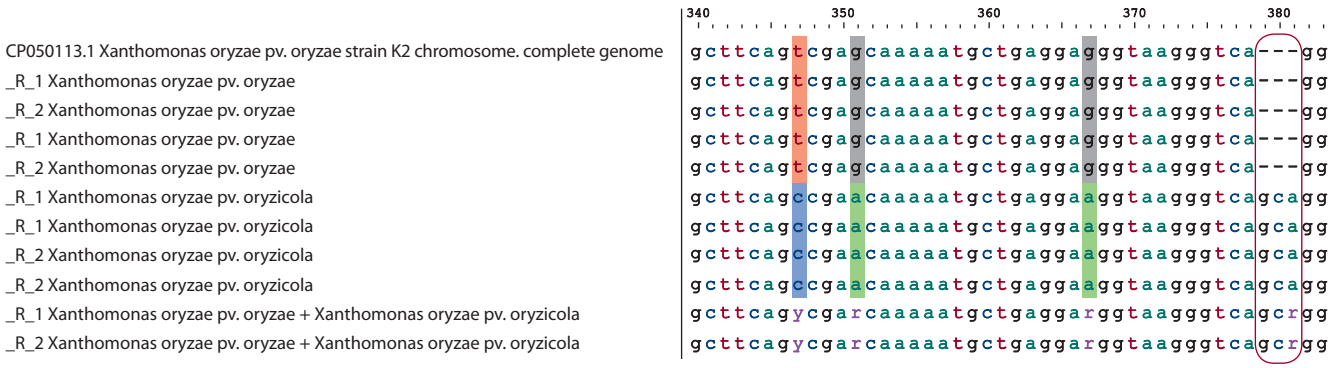


**Fig. 4.** Alignment of the DNA sequencies of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* and that of their mixture resulted from the sequencing using the seqX.o.all primer pair.

The box marks the deletion. The alignments were obtained in AliView v. 1.27 (Sweden).

tion begins with selecting the samples with clear infection symptoms for further cultivation in a nutrient solution. The method has its drawbacks since the colonies of both bacteria grow slowly in isolation media. Another problem is the presence of dominating kinds of bacteria and bacterial antagonists that prevent proper observation of the target ones. Division of *X. oryzae*'*s* two pathovariants is possible due to their phenotypical features, induction symptoms, serological test, fingerprinting (polyacrylamide gel electrophoresis) and phagotyping results (Vera Cruz et al., 1984; Benedict et al., 1989; EPPO, 2007).

Restriction fragment length polymorphism changes allow one to observe the almost compete genetic diversity of isolates and their origin (Gonzalez et al., 2007). However, this technique has a number of significant disadvantages such as insufficient sensitivity and specificity; high labor intensity that prevents the method from being used in diagnostic and industrial laboratories. For that reason, PCR has become the key method for detection of the *X. oryzae* bacteria (Sakthivel et al., 2001).

Current assays allow for *X. oryzae* detection in general, and further separation into pathovariants requires a standard PCR assay with species-specific primers recommended by the EPPO and All-Russian Center of Plant Quarantine, the results of which are to be sequenced. The EPPO protocol for *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* identification recommends the TXT/TXT-4R primers (Sakthivel et al., 2001; EPPO, 2007; Lang et al., 2010) to be used. To detect *X. oryzae* DNA using qPCR, it is recommended to use the X.o.F/X.o.R primers devised by the All-Russian Center of Plant Quarantine (Egorova et al., 2014). To separate the two pathovariants, qPCR can be performed using the PF/PR primers and the TaqMan probe that have been specifically designed to detect *X. o.* pv. *oryzae* (Zhao et al., 2007).

In 2021, the All-Russian Center of Plant Quarantine carried out an interlaboratory comparison to detect BLS in rice (21XOO). 16 reference centers and interregional vet laboratories took part and successfully passed a test to detect the disease using the *Xanthomonas oryzae* pv. *oryzicola*-RT assay we have designed.

## Conclusion

The designed assay enables the detection of BLS agents in rice. Being a reagent kit for qPCR, is also equipped with the seqX.o.all_F/R primers for PCR amplicon sequencing that detect *X. oryzae* in cases of individual and joint infection. The system allows for robust screening of quarantinable products and confirms obtained qPCR results with DNA sequencing. The assay has been successfully tested using five qPCR diagnostic systems from different manufactures and can be recommended for diagnostic and screening analysis in research and diagnostic laboratories.

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