Genomics-Based Diagnostic Marker Development for *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*

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ABSTRACT

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A computational genomics pipeline was used to compare sequenced genomes of *Xanthomonas* spp. and to rapidly identify unique regions for development of highly specific diagnostic markers. A suite of diagnostic primers was selected to monitor diverse loci and to distinguish the rice bacterial blight and bacterial leaf streak pathogens, *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. A subset of these primers was combined into a multiplex polymerase chain reaction set that accurately distinguished the two rice pathogens in a survey of a geographically diverse collection of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, other xanthomonads, and several genera of plant-pathogenic and plant- or seed-associated bacteria. This computational approach for identification of unique loci through whole-genome comparisons is a powerful tool that can be applied to other plant pathogens to expedite development of diagnostic primers.

Expansion of global trade in agricultural seed and produce increases the difficulty in controlling the movement of plant pathogens into new agroecosystems. Rapid detection and accurate identification of pathogens are critical steps to guide responses for containment or elimination of pathogens. However, robust and inexpensive diagnostic tools are not available for identification and classification of many plant pathogens. Historically, the primary hurdle in developing highly specific, easily used diagnostic tools for any pathogen has been the difficulty in finding unique features, whether they be cell surface antigens or DNA sequences, that have been validated against an extensive collection representative of a pathogen population (38). Due to this rate-limiting step, diagnostic tools often target only one feature (i.e., a single cell surface component or genic region). The reliance on one or a few unique features is risky because of the high degree of genotypic diversity and plasticity among many microbial pathogens. Testing large numbers of strains, both pathogens and nonpathogens, can overcome this limitation; however, for many pathogens, particularly those whose movement is regulated by governments, it is difficult to get access to collections of geographically diverse, validated strains.

Our goal was to demonstrate the utility of a computational comparison of whole-genome sequences to guide decisions for rapid development of precise diagnostic tools. Using this computational approach, we address two major issues in the development of diagnostic tools (i.e., the ability to rapidly identify many unique pathogen features and the inclusion of multiple unique target features in a diagnostic suite to add confidence to diagnosis). We show that comparative genomics can reveal sequence similarities and dissimilarities of closely related organisms, and this sequence information can be used to create diagnostic primers for polymerase chain reaction (PCR). For this proof-of-concept study, we used comparative genomics to develop a suite of diagnostic primers that distinguished two closely related pathogens, *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola.*

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* are important pathogens of rice, causing bacterial blight and bacterial leaf streak of rice (*Oryza sativa* L.), respectively (26–28). Apart from the importance of accurate identification and distinction of these two pathogens for efficient control practices and research purposes, the need for precise diagnostic tools is acutely important for regulatory reasons. Importation and interstate movement of these pathogens within the United States has long been regulated by the United States Department of Agriculture (USDA) Plant Protection and Quarantine (PPQ) program within the Animal and Plant Health Inspection Service (APHIS) and, recently, both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* were designated as select agents (http://www.aphis.usda.gov/programs/ag_selectagent/) according to the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (Public Law 107-188; June 12, 2002). From the global seed trade perspective, phytosanitary regulations enforced in many countries impose perhaps the most critical need for accurate and reliable diagnostic tools for *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*, although the relevance of seed transmission and spread of these pathogens is still controversial (13,27,34,41). Unfortunately, incorrect diagnoses can result in large financial losses.

Several diagnostic tools are currently available for identification of *X. oryzae* pv. *oryzae* and, to a lesser extent, *X. oryzae* pv. *oryzicola*. A set of monoclonal antibodies developed in the late 1980s has been widely used for diagnosis and distinction of several *Xanthomonas* spp. and pathovars, including *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* (5,6,8,9,11,13,24). These antibodies continue to be of value and, for some pathovars, more than one

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pathovar-specific antibody is available (9,13). The major shortcoming with this approach is that the development and testing of antibodies that target unique features is expensive and time consuming.

Early DNA-based approaches to distinguish *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* involved amplification of the 16S rDNA followed by digestion with restriction enzymes (20,40). However, because 16S rDNA sequences exhibit 98.6% similarity within the genus *Xanthomonas* (15), this approach does not reliably distinguish or differentiate these two *X. oryzae* pathovars. The approach is only useful if supported by other sequence information such as the 16S-23S rRNA internal transcribed spacers. Primers based on the 16S-23S rDNA spacer region were designed for *X. oryzae* pv. *oryzae* but their design and testing were based on *X. oryzae* pv. *oryzae* isolates from only one country and *X. oryzae* pv. *oryzicola* isolates were not screened (1); therefore, the reliability of these primers for accurate identification of geographically diverse collections is unknown. Repetitive DNA sequences, usually insertion sequence (IS) elements (22,33), differentiate *X. oryzae* pathovars from each other and from other *Xanthomonas* spp. by hybridization (22) or PCR-amplification strategies (3,34,37). The distinction is based on polymorphic hybridization or amplification patterns. However, the high degree of diversity of *Xanthomonas* isolates within and between countries, partially driven by movement of these mobile elements, complicates the analysis of patterns for diagnosis. DNA-based assays developed for single gene targets (e.g., a membrane fusion protein [17], a putative siderophore receptor, and an *hrpF* gene in *X. campestris* species [10,41]), although potentially reliable, were not validated on a diverse and wide array of strains. Thus, although the DNA-based approaches have high potential, finding unique target sequences can be slow, and the success and adoption by the diagnostic community have been limited because the tests are not easy to use (based on size polymorphism rather than plus/minus), are dependent on one genome feature, or are not validated against a large number of strains.

In this study, we exploited the available genome sequence information in public databases to develop specific PCR primers for accurate diagnosis of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Our

Table 1. Bacterial strains used in this study. Region, state, province or island of isolation is listed after each strain where applicable

Species	Strains	Country	Source
Xanthomonas oryzae pv. *oryzae*	HUB1 #32 (Hubei, Wuxue), Janling 691 #35 (Hubei, Shiaogan), FJI#30 (Fujian), NX42 (Ningxia Wuzhong), HN35 (Heuan Kaifung), HB18 (Hebei Fungren), HB21 (Fungning), RS25, RS50, RS61, 6-1 - 6-30 (30 strains), Xoc China	China	Z. Qi, C. Song, and J. S. Wang
	CIAT1185, CIAT1186	Columbia	V. Verdier
	A3842, A3872, A3846, A3875	India	J. E. Leach
	XPUKPT0092, XPUKPT0639 and XPUKPT0640 (Kapurthala, Punjab), A3857(I10), (Pautnagar, Uttar Pradesh), A3872(I13) (Gorgal, Uttar Pradesh), XAPHYD0221 (Muttakpally, Andhra Pradesh), XAPRPM0099 (Ramchandrapuram, Andhra Pradesh), XGJNWG0518 (Nawagam, Gujrat), XHRKKR0151 (Kurukshetra, Haryana), XKAMDY0192 (Mandya, Karnatka), XKAMYS0053 (Mysore, Karnatka), XORBGD0608 (Bargad, Orissa), XORSBP0222 and XORSBP0493 (Sambalpur, Orissa), XORSNP0620, XORSNP0635 and XORSNP0644 (Sonepur, Orissa), XUPFZB0090 and XUPFZB0093 (Faizabad, Uttar Pradesh), XUPPBT0278 (Pilibhit, Uttar Pradesh), XUPSKN0599 (Sant Kabir Nagar, Uttar Pradesh), XUTPNT0089 (Pantnagar, Uttaranchal)	India	D. Mishra
	IXO16 (Ciranjans, West Java), IXO56 (Pusakanegara Exp. Station, West Java), IXO58 (Kuniugan Exp. Station, West Java), IXO60	Indonesia	…
	MAFF311018	Japan	A. Bogdanove
	KACC10331, XOO197 (Jeonnam, Seung jn), XOO212 (Kyunggi Icheon), XOO214, KOO367	Korea	S. H. Choi
	MXO90 (Mardi, S. Perai), MXO92 (Mada, Kedah), MXO99 (Jln. Kg Paya, Pulau Sayap, Kedah), MXO101 (Kota K. Muda, Kedah), MXO300 (Mada, Kodiang, Kedah)	Malaysia	…
	R13	Myanmar	I. Buddenhagen
	NXO537 and NXO544 (Dhanusa), NXO588 (Jhapa), NXO604 (Chitwan), NXO619 and NXO622 (Syanja), NXO624 (Tanahua), NXO638 (Rupendhi)	Nepal	T. Adhikari
	PXO20, PXO39, PXO40, PXO61, PXO68, PXO69, PXO70, PXO71, PXO74, PXO78, PXO79, PXO80, PXO83, PXO84, PXO86, PXO99A, PXO105, PXO110, PXO111, PXO112, PXO113, PXO115, PXO116, PXO117, PXO121, PXO126, PXO128, PXO132, PXO145, PXO164, PXO171, PXO172, PXO173, PXO177, PXO180, PXO183, PXO186, PXO187, PXO188, PXO191, PXO280, PXO339, PXO 340, PXO341, PXO344, PXO347, PXO349, PXO363	Philippines	C. Vera Cruz
	CL-1	Sri Lanka	I. Buddenhagen
	R-7, XOO3, XOO4, XOO5, XOO8, XOO10, XOO11, XOO12	Thailand	J. E. Leach
	R-3	Australia	I. Buddenhagen
	BAI1, BAI2, BAI3 and BAI4 (Bagre)	Burkina Faso, Africa	V. Verdier
	CFBP1947, CFBP1948	Cameroon, Africa	V. Verdier
	MAI1, MAI2 and MAI3 (Niono), MAI9 (Molodo), CFBP1949, CFBP1951	Mali, Africa	V. Verdier
	NAI2, NAI5, NAI6 and NAI7 (Toula), NAI8 and NAI9 (Bonfeba)	Niger, Africa	V. Verdier
X. oryzae pv. *oryzicola*	Xoc China, RS60, RS85, RS105, 8-1 through 8-12, 8-14 through 8-16, 8-18 through 8-32	China	J. S. Wang, C. Song
	BXOR1	India	R. Sonti
	MAI3, MAI4, MAI5, MAI6, MAI7, MAI8, MAI10, MAI11, MAI12 and MAI13 (Niono)	Mali	V. Verdier
	CFBP2287 (NCPPB2921)	Malaysia	V. Verdier

(*continued on next page*)

approach was feasible because genome sequences in varying stages of completion (draft to finished) were available for over 50 plant-pathogenic or plant-associated bacteria (http://cpgr.plantbiology.msu.edu). This included the genomes of three different *X. oryzae* pv. *oryzae* strains (23,29,35) and one *X. oryzae* pv. *oryzicola* strain (A. Bogdanove, *personal communication*; http://cmr.jcvi.org). We used this analysis to develop an arsenal of oligonucleotide primers that distinguished *X. oryzae* pathovars of diverse geographic origin from other plant-pathogenic bacteria, and differentiated *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola* using PCR.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. Bacterial strains used in this study and their geographic origin (where known) are listed in Table 1. These included 164 strains of *X. oryzae* pv. *oryzae* and 89 of *X. oryzae* pv. *oryzicola* from diverse geographic locations as well as 40 strains of other plant-pathogenic xanthomonads. Also included were bacteria isolated from rice in the United States and originally identified as *X. campestris* pv. *oryzae* (16), as well as unclassified bacteria isolated from rice seed or leaves in India, China, the Philippines, and the United States. Due to importation restrictions, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from collaborators were provided as DNA or heat-killed cells. Either bacterial cells or isolated DNA were used in PCR reactions. DNA extractions at Colorado State University (CSU) were performed using the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's recommendations, except that DNA was eluted in 30 µl of water in the final step. All samples were diluted to 20 ng/µl in water. At the International Rice Research Institute (IRRI), DNA extractions were performed according to Cottyn et al. (12).

Isolation of bacteria from seed and pathogenicity assays. Seed samples from asymptomatic rice grown in IRRI fields in the Philippines were provided by the IRRI Seed Health Unit. A second set of leaves and seed samples from plants that exhibited atypical bacterial blight symptoms, also from IRRI fields, were used for direct-detection assays. Grains and sheath samples were disinfected with 70% ETOH for 30 s, decanted, and then rinsed with sterile distilled water three times. Phosphate-buffered saline (PBS) with Tween 20 (0.1 ml/liter, pH 7.0) was added, and the samples were crushed using a mortar and pes-

Table 1. (*continued from preceding page*)

Species	Strains	Country	Source
X. oryzae pv. *oryzicola* (*continued*)	BLS51 (Pili, Camarines Sur), BLS54, BLS 99, BLS 101, BLS 102, BLS 103, BLS 105, BLS 106, BLS 111, BLS 123, BLS 145, BLS 159, BLS 175, BLS 179, BLS 187, BLS 220, BLS 256 (IRRI), BLS 276, BLS 280, BLS 281 (IRRI), BLS 285 (IRRI), BLS 289 (Talisay, Camarines Norte), BLS 291 (Iloilo), BLS 294 (Camaysihay, Palo, Leyte), BLS 295 (Midsayap, North Cotabato), BLS 298 (IRRI), BLS 303 and BLS 305 (San Dionisio, Iloilo), BLS 333, BLS 338 (Talavera, Nueva Ecija), BLS 348, BLS 354, BLS 356 (San Fabian, Isabela), BLS 357, BLS 365 (Burgos, Isabela), BLS 377, BLS 404, BLS 413, BLS 415 (Laong Lupa, Lapaz,Tarlac), BLS 417, BLS 421, BLS 468 and BLS 483 (IRRI)	Philippines	C. Vera Cruz
X. axonopodis pv. *alfalfae*	KX-1, FX-1		L. E. Claflin
X. axonopodis pv. *allii*	0177 (Colorado)	United States	H. Schwartz
X. axonopodis pv. *holcicola*	3, 66 (Kansas), 93, 93e, 107, 114, 116 and 124 (Machache, Lesotho), 123 (Siloe, Lesotho), 429	Africa, United States	L. E. Claflin
X. axonopodis pv. *malvacearum*	535, 2919	United States	
X. axonopodis pv. *phaseoli*	454 (Colorado)	United States	H. Schwartz
X. axonopodis pv. v*asculorum*	515	…	L. E. Claflin
X. axonopodis pv. *vesicatoria*	82-8, 85-10,1123	United States	A. Bogdanove
X. campestris pv. *campestris*	Xcc X1g10	…	L. E. Claflin
X. campestris pv. *carotae*	Xcc01, 9933	United States	R. Bostock
X. campestris pv. *dieffenbachiae*	G278	…	C. M. Vera Cruz
X. campestris pv. *pelargonii* (*geranium*)	4486	…	L. E. Claflin
X. campestris pv. *gummisudans*	…	…	L. E. Claflin
X. campestris pv. *glycines* (*sojense)*	4455	…	L. E. Claflin
X. cucurbitae	N22299	…	L. E. Claflin
X. fragariae	462, Brazil	…	L. E. Claflin
X. horotum pv. *pelargonii*	X-1, X-5	…	L. E. Claflin
X. translucens	1381, 1589 and Divide (North Dakota), XtKS	United States	T. Adhikari, N. Tisserat
X. translucens pv. *cerealis*	4546, NCPPB1943	United States	B. Cunfer, L. E. Claflin
X. translucens pv. *hordei*	2181	…	L. E. Claflin
X. translucens pv. *phleipratensis*	PDDCC#5744	…	L. E. Claflin
X. translucens pv. *secalis*	…	…	L. E. Claflin
Acidovorax avenae	BPJ1188	…	C. Vera Cruz
Burkholderia andropogonis	…	…	L. E. Claflin
B. glumae	BPJ1622	…	C. Vera Cruz
Erwinia herbicola		…	L. E. Claflin
Pseudomonas syringae pv. *syringae*	…	…	L. E. Claflin
Ralstonia solanacearum	…	…	J. E. Leach
Unknown, isolated from rice cv. Lemont or rice seed	X207-A-1, X200-1, X1-8 , X4-4C, X11-5A and X1-10 X13-5C (Texas), X8-1A, RU87-17 and X8-1B (Louisiana), X44-D1, X57-5, X100-1, X211-1 X4-2C, X4-4D, X7-5A, X54-A1, X7-2D, JBG1, JBG2, JBG5, JBG8, X212-3-1, X37-2	United States	C. Gonzalez (8,15,18,27,29)
Unknown, isolated from rice	Ven	Venezuela	V. Verdier
Unknown, isolated from rice seed	XOAPHYD1011, XOAPHYD1012, XOAPHYD1013, XOAPHYD1014, XOAPHYD1015	India	D. Mishra
	(Seed isolates tested only with primers in Table 2) SHU 98, SHU83, SHU104, SHU110, SHU111, SHU113, SHU114, SHU115, SHU116, SHU117, SHU118, SHU120, SHU122, SHU123, SHU130, X54)	Philippines	C. Vera Cruz

tle. The extract was shaken for 2 h at 30°C at 130 rpm. For direct isolation, 1 ml of extract was transferred to a 2-ml microtube and centrifuged briefly to precipitate debris. An aliquot of the supernatant was diluted and appropriate dilutions were spread evenly onto Suwa's (30) and WF-P media (18). A 1-ml aliquot was transferred to a 1.5 ml-microtube and centrifuged for 10 min at 13,000 rpm, and the supernatant was decanted. The pellet was washed twice with 70% ETOH, allowed to air dry, and resuspended in 50 µl of 10 mM Tris-HCl and 1 mM EDTA buffer (pH 8.0). This solution was used directly as the source of DNA for PCR.

To enrich for bacteria from seed, 100 µl of seed extract was added to 900 µl of nutrient broth and incubated at 30°C with shaking (130 rpm) for 24 h. Bacteria from a 1-ml aliquot were pelleted by centrifugation for 10 min at 13,000 rpm. The supernatant was decanted and the pellet was washed twice with 70% ETOH. The pellet was resuspended in 50 µl of 10 mM Tris-HCl and 1 mM EDTA buffer, and this solution was used directly as the source of DNA for PCR.

Pathogenicity of all bacteria isolated from rice seed was assessed using the leaf-clip inoculation technique (19) for bacterial blight and the spray-inoculation technique (2) for bacterial leaf streak on 21-day-old rice cvs. IR24 and IR50, respectively. Nonpathogenic xanthomonads (X25, X36, X54, and X61) and water were included as controls.

Primer design. Diagnostic primers were designed using a custom computational pipeline (Fig. 1) implemented in Perl. The first step identified unique *X. oryzae* pv. *oryzae* (23,29,35) and *X. oryzae* pv. *oryzicola* loci using a BLASTN (E = 1e – 1) search of the loci against the loci of all sequenced *Xanthomonas* genomes (4). Primers were developed from the unique chromosomal loci using Primer3 (32). Predicted primer pairs were searched against sequences of all available *Xanthomonas* genomes using reverse PCR (re-PCR; n = 2, g = 1) (36). Primer sequences that were detected in genomes other than the target genome were discarded from the set. The primers were searched separately against all *Xanthomonas* genomes with publicly available sequences using BLASTN (E = 10), and primer sequences detected in genomes other than a target genome were again discarded. The initial primers were designed to produce amplicons of about 300 bp. After testing in conventional PCR reactions, primers that were specific were redesigned to amplify different-sized fragments for use in the multiplex PCR primer set (Table 2, underlined primers).

Primer screening. Candidate primer sets were screened in conventional PCR reactions against the sequenced strains of *X. oryzae* pv. *oryzae* (PXO99A) and *X. oryzae* pv. *oryzicola* (BLS256). Primers that produced amplicons of predicted sizes were then screened with a panel of six bacterial strains representing different countries. This panel included *X. oryzae* pv. *oryzae* strains PXO99A (Philippines), KXO85 (Korea), and MAFF311018 (Japan) and *X. oryzae* pv. *oryzicola* strains BLS256 (Philippines), BXOR1 (India), and Xoc-China (China). For single primer pair reactions, 20 ng of template bacterial genomic DNA was used whereas, for multiplex reactions, 40 ng was used. For amplification of fragments directly from bacterial cells, 1 µl of a cell suspension (10^7 CFU/ml) was used per reaction. The conventional PCR reaction mixture contained 0.5 µl of 10 mM dNTPs, 2.5 µl of 10× buffer, 2.0 µl of 25 mM $MgCl_2$, 0.02 units of *Taq* polymerase (New England BioLabs, Ipswich, MA), and 0.5 µl of each 10 µM primer in a total volume of 25 µl. The PCR protocol included an initial denaturing step at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min 30 s; and a final extension at

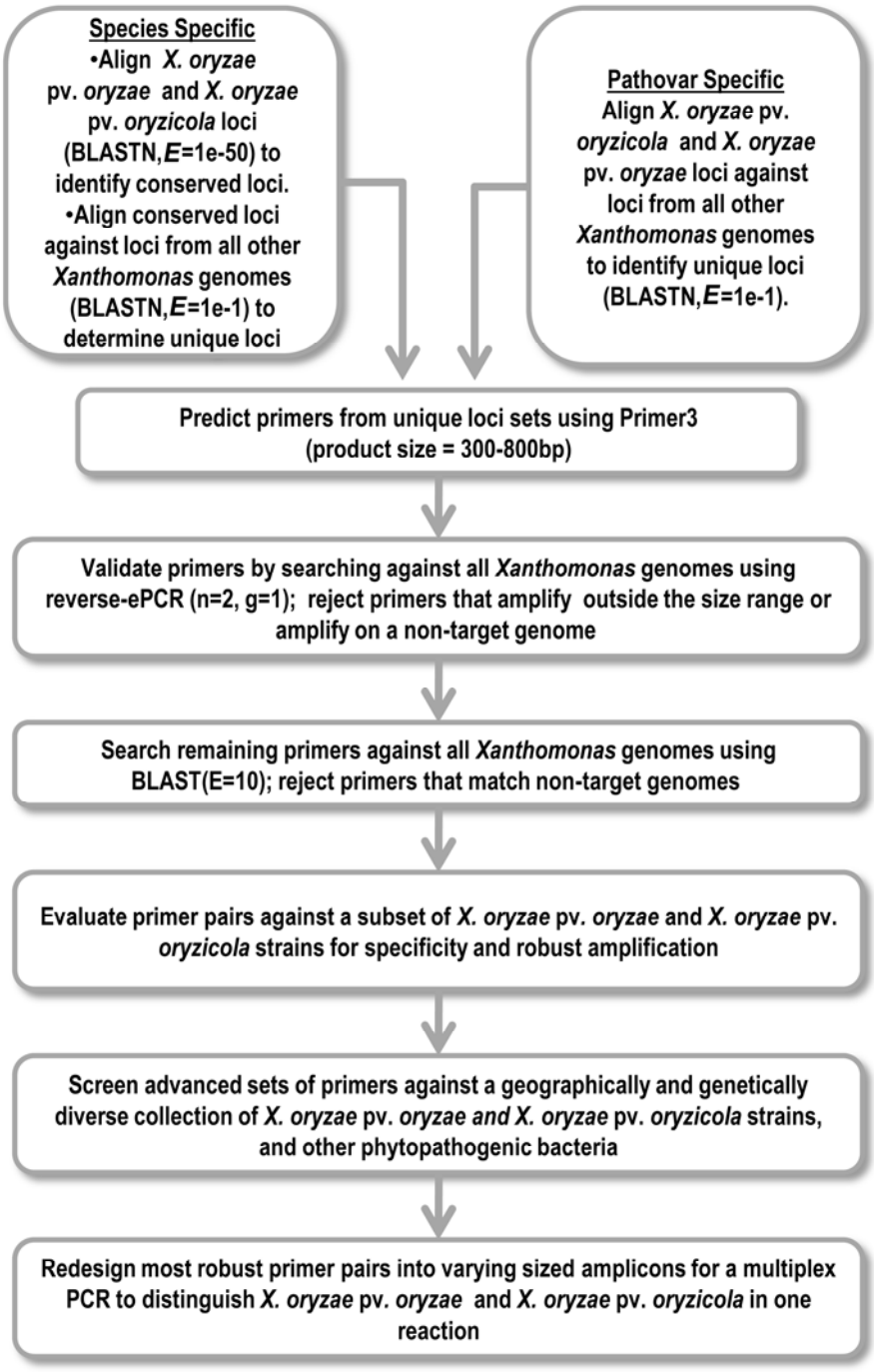

Fig. 1. Computational pipeline that uses genome-wide comparisons to design species- and pathovar-specific markers for *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. E values refer to the statistical significance threshold for reporting matches. Primers, genome tools, and other information are located at http://cpgr.plantbiology.msu.edu.

72°C for 7 min. Products were analyzed by separation in 1% agarose gels (1% Tris-acetate-EDTA [TAE] buffer), stained with ethidium bromide, and visualized under UV light.

A subset of amplicons was sequenced and primers that produced fragments with sequences matching targeted loci were advanced for further screening. Ultimately, all primer pairs listed in Table 2 were screened against all strains listed in Table 1.

Multiplex development and testing. A set of primers was redesigned to produce amplicons of different sizes for a multiplex PCR system with the goal of distinguishing *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from each other and from other bacteria in a single reaction. Criteria for advancement to the multiplex were that primers exhibited appropriate specificity, amplified sequences from all strains of the target pathovar, and robustly amplified sequences from the target pathovar. For each locus, Primer 3 was used to predict primers that would generate amplicons of between 50 and 1,500 bp. Each redesigned primer pair was tested individually as well as with a group of primers destined for the multiplex set. The final multiplex set, which included the four primer pairs underlined in Table 2, was screened against all bacteria in Table 1.

A 25-µl reaction mixture was used for the multiplex reaction. This contained 0.5 µl of 10mM dNTPs, 2.5 µl of 10× buffer, 2.0 µl of 25 mM $MgCl_2$, 0.06 units of *Taq* polymerase (New England BioLabs), and 0.5 µl of each 10 µM primer in a total volume of 25 µl. PCR was carried out in a

Table 2. Computationally designed polymerase chain reaction primers specific for *Xanthomonas oryzae* species and for the two pathovars *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola*

Target, name[a]	Locus	Annotation[b]	Sequence	Product size (bp)
X. oryzae				
Xo2207F	XOOORF2207	Endoproteinase Arg-C	TGTACCCGGAGAATTTCAGC	324
Xo2207R	…	…	AATACGTTTGCAGCGTTTCG	…
Xo3756F	XOOORF3756	Hypothetical protein	CATCGTTAGGACTGCCAGAAG	331
Xo3756R	…	…	GTGAGAACCACCGCCATCT	…
Xo2967F	XOOORF2967	Conserved hypothetical protein	CGGTTCGTTCAAGACACCTT	336
Xo2967R	…	…	CCCTCCTTGACATAGCTGGA	…
Xo1321F	XOOORF1321	Type IV pilus modification protein PilV (*pilV*)	TATCGTCGTTCTCGCATTTG	304
Xo1321R	…	…	ATTGAGAACGGTTGCACTGG	…
X. oryzae pv. *oryzae*				
Xoo4009F	XOOORF4009	Hypothetical protein	CCTTCATTTCCGTCGTCATC	302
Xoo4009R	…	…	ATGCATGAAGAACCACCACA	…
Xoo2976F	XOOORF2976	Dual specificity phosphatase, catalytic domain protein	GCCGTTTTTCTTCCTCAGC	337
Xoo2976R	…	…	AGGAAAGGGTTTGTGGAAGC	…
Xoo80F	XOOORF0080	Hypothetical protein	GCCGCTAGGAATGAGCAAT	162
Xoo80R	…	…	GCGTCCTCGTCTAAGCGATA	…
Xoo3350F	XOOORF3350	ABC transporter permease	GCAAGCTGATCGGTATCCTC	300
Xoo3350R	…	…	GCGAGACCTTGAACTGGAAC	…
X. oryzae pv. *oryzicola*				
Xoc2071F	XOCORF2071	Cysteine synthase (O-acetylserine sulfhydrylase)	GGGATCCATCAAGCTCAAGA	329
Xoc2071R	…	…	CGTATTGGTTCAGCCAGACC	…
Xoc3866F	XOCORF3866	LPS O-antigen biosynthesis protein	ATCTCCCAGCATGTTGATCG	691
Xoc3866R	…	…	GCGTTCAATCTCCTCCATGT	…
Xoc3864F	XOCORF3864	*wxocB*; putative glycosyltransferase (39)	GTGCGTGAAAATGTCGGTTA	945
Xoc3864R	…	…	GGGATGGATGAATACGGATG	…
Xoc3863F	XOCORF3863	Methyltransferase	GCGGTACGCTAGTGATGACA	360
Xoc3863R	…	…	GTTTCCGTGCTATCCGTTGT	…

[a] Specificity refers to the species or pathovar each primer is targeted to amplify: both pathovars (*X. oryzae*) or individual pathovars (*X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola*). Underlined primers indicate those designed for the multiplex analysis.
[b] Annotation is reported from http://cmr.jcvi.org.

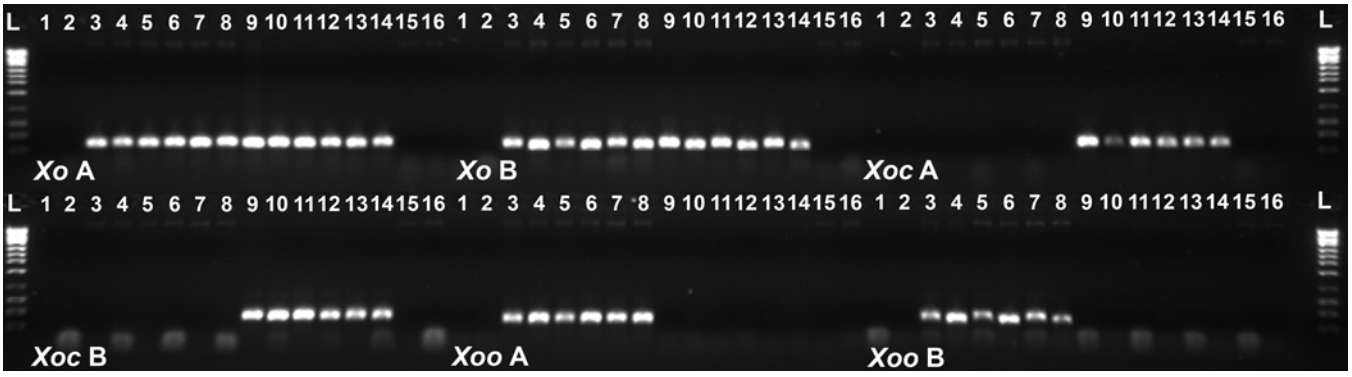

Fig. 2. Conventional polymerase chain reaction screen showing specificity of 12 computationally designed primers to amplify *Xanthomonas oryzae* (panels *Xo*A and *Xo*B), *X. oryzae* pv. *oryzicola* (*Xoc*A and *Xoc*B), or *X. oryzae* pv. *oryzae* (*Xoo*A and *Xoo*B). Primers screened in panel *Xo*A were Xo2207 (324 bp) in odd lanes and Xo3756 (331 bp) in even lanes; panel *Xo*B: Xo2967 (336 bp) odd lanes and Xo1321 (304 bp) even lanes; panel *Xoc*A: Xoc3866 (335 bp, later redesigned to produce 691-bp fragments for the multiplex) odd lanes and Xoc2071 (329 bp) even lanes; *Xoc*B: Xoc3864 (377 bp, later redesigned to produce 945-bp amplicons for the multiplex) odd lanes and Xoc3863 (360 bp) even lanes; *Xoo*A: Xoo80 (162 bp) odd lanes and Xoo4009 (302 bp) even lanes; *Xoo*B: Xoo2976 (337 bp) odd lanes and Xoo3350 (300 bp) even lanes. The bacterial strains screened are in this order: lane L, 1-kb Plus DNA Ladder; lanes 1 and 2, negative control; lanes 3 and 4, *X. oryzae* pv. *oryzae* MAFF311018; lanes 5 and 6, *X. oryzae* pv. *oryzae* NXO588; lanes 7 and 8, *X. oryzae* pv. *oryzae* A3857 (I10); lanes 9 and 10, *X. oryzae* pv. *oryzicola* BLS256; lanes 11 and 12, *X. oryzae* pv. *oryzicola* BLS99; lanes 13 and 14, *X. oryzae* pv. *oryzicola* BLS483; and lanes 15 and 16, *X. campestris* pv. *phleipratensis.* Primer sequences are listed in Table 2.

PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA), or a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The multiplex cycle program was an initial denaturing step at 94°C for 3 min; followed by 31 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 2 min; and a final extension at 68°C for 10 min. Products were analyzed either by separation in 2% agarose gels (1% TAE buffer) (Fig. 2) or by using microfluidic technology with an Experion Automated Electrophoresis Station (Bio-Rad Laboratories) and a 1K DNA chip according to the manufacturer's recommendations. Negative controls contained only water in the reaction mixture.

RESULTS

Identification of unique features by whole-genome comparisons. Using the computational pipeline diagramed in Figure 1, 85 *X. oryzae* pv. *oryzae*, 86 *X. oryzae* pv. *oryzicola*, and 1,762 *X. oryzae* unique loci with a length greater than 300 bp were identified. From these unique loci, 947 *X. oryzae* pv. *oryzae,* 626 *X. oryzae* pv. *oryzicola*, and 124 *X. oryzae* primer pairs were predicted and listed at http://cpgr.plantbiology.msu.edu. The *X. oryzae* pv. *oryzae* and, more substantially, the *X. oryzae* pv. *oryzicola* markers are enriched in hypothetical proteins, consistent with our computational pipeline to identify unique loci to these two pathovars.

Sets of primers with different predicted specificities (36 *X. oryzae*, 51 *X. oryzae* pv. *oryzae*, and 36 *X. oryzae* pv. *oryzicola*) that amplified correct fragment sizes from the sequenced strains of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* were then screened against a panel of six bacterial strains. A subset of 34, 21, and 26 primer pairs were advanced for testing with a larger panel of six geographically diverse strains each of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* and a subset of other xanthomonads and plant-pathogenic bacteria, respectively. A set of four *X. oryzae* pv. *oryzae*, four *X. oryzae* pv. *oryzicola*, and four *X. oryzae* primer sets that amplified their targets accurately and robustly (Table 2) were then tested against all strains listed in Table 1. These primers amplified all expected targets and did not produce amplicons for other species tested. The most robust pairs were included in a multiplex set. Note that many primer pairs were designed but not tested; these untested primers, which are listed at the CPGR site (http://cpgr.plantbiology.msu.edu), may target other unique features in the bacterial genomes and, therefore, may be valuable resources.

Development of a multiplex set of primers that distinguish *X. oryzae* pathovars. A subset of primers were redesigned for a multiplex PCR system with the goal of distinguishing *X. oryzae* pv. *oryzicola* from *X. oryzae* pv. *oryzae* and from other bacteria in a single reaction. Initial multiplex sets included up to eight different primer pairs. However, in our hands, consistent and universal amplification was only achieved with pools of four primer pairs. The final multiplex set included primers that produce a 162-bp product unique to *X. oryzae* pv. *oryzae,* a 331-bp species-specific product in both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola,* and 691- and 945-bp products unique to *X. oryzae* pv. *oryzicola* (Fig. 3; Table 2). All *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains in Table 1 produced the expected size of amplicons, including representative strains from diverse geographic locations. No other known species of *Xanthomonas* or other genera tested produced amplicons with the multiplex primers (Fig. 4). The reliability of the multiplex primers in distinguishing the *X. oryzae* pathovars was confirmed in labs at CSU and at IRRI.

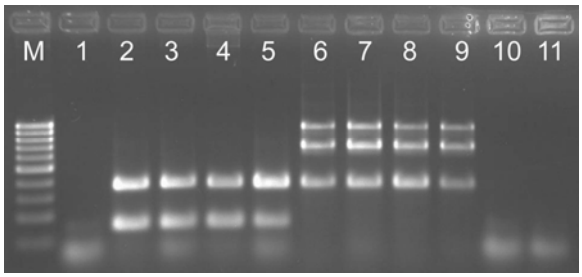

Fig. 3. Multiplex polymerase chain reaction primers differentiate *Xanthomonas oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola.* Lane M, 100-bp marker (Fisher Exactgene); lane 1, negative control, water; lanes 2–5, *X. oryzae* pv. *oryzae* PXO99A (positive control), MAFF311018, KXO85, and N5; lanes 6–9, *X. oryzae* pv. *oryzicola* BLS256, RS85, BXOR1, and M13; lane 10, *X. axonopodis* pv. *holcicola* 107; and lane 11, *Erwinia herbicola.* Sequences for primers Xoo80 (162 bp), Xoc3864 (945 bp), Xoc3866 (691 bp), and Xo3756 (331 bp) are shown in Table 2. Products were separated in a 2% agarose gel at 60 mV for 90 min.

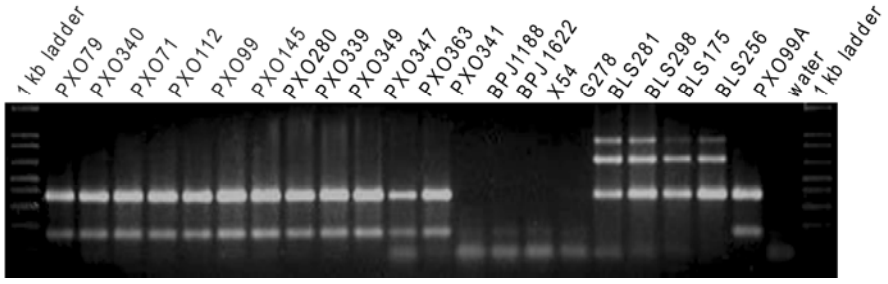

Fig. 4. Multiplex polymerase chain reaction primer set specifically amplifies and distinguishes strains of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola. X. oryzae* pv. *oryzae* strains are PXO79, PXO340, PXO71, PXO112, PXO99, PXO145, PXO280, PXO339, PXO349, PXO347, PXO363, and PXO341. Other pathogenic bacteria originally isolated from rice seed are BPJ1188 (*Acidovorax avenae*), BPJ 1622, BPJ1622 (*Burkholderia glumae)*, and the nonpathogens X54 (*Xanthomonas* sp.) and G278 (*X. campestris* pv. *dieffenbachiae*). *X. oryzae* pv. *oryzicola* strains are BLS281, BLS298, BLS175, and BLS256. Water = no DNA template, molecular weight marker = 1-kb ladder.

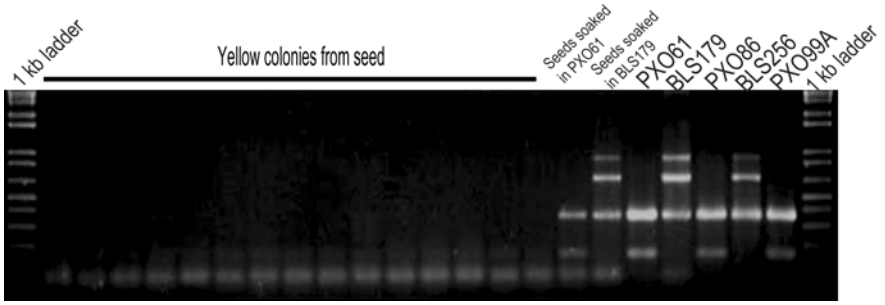

Fig. 5. Multiplex polymerase chain reaction primer set does not amplify nonpathogenic seed bacteria and is not inhibited by seed extracts. Lanes 1–15 are representatives of 174 seed bacterial isolates from the International Rice Research Institute Seed Health Unit (SHU) that are not pathogenic to rice: SHU 98, SHU83, SHU104, SHU110, SHU111, SHU113, SHU114, SHU115, SHU116, SHU117, SHU118, SHU120, SHU122, SHU123, and SHU130, respectively. Next lanes are DNA amplified from extracted from seed soaked in *Xanthomonas oryzae* pv. *oryzae* PXO61 and seed soaked in *X. oryzae* pv. *oryzicola* BLS179. *X. oryzae* pv. *oryzae* strains PXO61 and PXO86 and *X. oryzae* pv. *oryzicola* strains BLS179 and BLS256 are positive controls. Molecular weight marker = 1-kb ladder.

The multiplex primers accurately amplified fragments diagnostic for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in extracts from seed soaked in bacteria (Fig. 5). No amplification occurred in DNA extracts from uninoculated or asymptomatic plant tissues (*data not shown*). Of 174 bacterial isolates from seed tested, none showed typical bacterial blight symptoms on rice cv. IR24 (grayish, water-soaked lesions) or bacterial leaf streak symptoms on IR50 (water-soaked, streaked lesions along leaf veins) in comparison with *X. oryzae* pv. *oryzae*- and *X. oryzae* pv. *oryzicola*-inoculated and water controls (*data not shown*). Consistent with their inability to cause disease on rice, none of these 174 isolates produced amplicons with the *X. oryzae*-, *X. oryzae* pv. *oryzae*-, or *X. oryzae* pv. *oryzicola*-specific primers in the multiplex (Fig. 5). These data support the specificity of the multiplex set for the two *X. oryzae* pathovars and indicate that the assay is not adversely affected by seed extracts.

***Xanthomonas* spp. from rice in the United States.** The *X. oryzae*-specific primer Xo3756 and the *X. oryzae* pv. *oryzae*-specific primer Xoo80 in the multiplex set were selected because they amplified all reported *X. oryzae* pv. *oryzae* strains, including strains weakly pathogenic to rice from the United States (Texas and Louisiana; 16). Although primer Xoo80 amplified the U.S. strains, the product was consistently less intense in gels compared with other *X. oryzae* pv. *oryzae* strains (*not shown*). In addition to the primers in the multiplex set, all rice-pathogenic strains from the United States produced amplicons with Xo1321, an *X. oryzae* species-specific primer (Tables 3 and 4). However, apart from Xoo80, conventional primers designed to be specific for *X. oryzae* pv. *oryzae* and that consistently amplified *X. oryzae* pv. *oryzae* from other parts of the world did not produce amplicons in the U.S. strains (Tables 3 and 4, subset). Although the primers shown here did not distinguish between monoclonal antibody groups (9), a screen of additional primers indicated that it would be possible to distinguish among the U.S. strains (*data not shown*). Only one *X. oryzae* pv. *oryzicola*-specific primer pair, Xoc76, amplified a few U.S. strains; this primer pair did not produce amplicons with any other *X. oryzae* pv. *oryzae* strains tested.

The 16S rDNA of this subset of U.S. strains was amplified and sequenced (*data not shown*). Based on alignments, the U.S. strains, except JBG1 and JBG2, most closely resembled *X. oryzae* pv. *oryzae*. JBG1 and JBG2 aligned closely with *X. translucens*, consistent with their lack of amplification with our primer sets. Overall, these results are consistent with previous reports that the weakly virulent U.S. strains may be *X. oryzae* species, but they are clearly distinct from Asian and African *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola*.

Table 3. Amplification of rice isolates from the United States with selected *Xanthomonas oryzae* species- and pathovar-specific primers

			Primer pair[a]								
Strain[b]	**Origin**	**Pathogenic[c]**	**Xo1321 304 bp**	**Xo2967 336 bp**	**Xoo80 312 bp**	**Xoo2976 337 bp**	**Xoo3350 300 bp**	**Xoo4009 302 bp**	**Xoc76 407 bp**	**Xoc2071 329 bp**	**Group[d]**
X. oryzae pv. *oryzae*											
PXO99A	Philippines	+	+	+	+	+	+	+	–	–	NA
X. oryzae pv.*oryzicola*											
BLS256	Philippines	+	+	+	–	–	–	–	+	+	NA
U.S. isolate											
X1-8	Texas	+	+	–	+	–	–	–	–	–	I
X1-10	Texas	+	+	–	+	–	–	–	–	–	I
X4-4D	Texas	+	+	–	+	–	–	–	–	–	IV
X8-1A	Louisiana	+	+	–	+	–	–	–	–	–	I
X11-5A	Texas	+	+	–	+	–	–	–	(+)	–	III
JBG-1	Texas	–	–	–	–	–	–	–	–	–	NA
JBG-2	Texas	–	–	–	–	–	–	–	–	–	NA

[a] Specificity of each primer is designated by prefix: both pathovars (Xo), *X. oryzae* pv. *oryzae* (Xoo), or *X. oryzae* pv. *oryzicola* (Xoc); + = amplification with a primer pair, (+) = weak or unreliable amplification, – = no amplification.
[b] *X. oryzae* pv. *oryzae* PXO99A and *X. oryzae* pv. *oryzicola* BLS256 are the sequenced strains of each pathovar. U.S. isolates X1-8, X1-10, X4-4D, X8-1A, and X11-5A are from rice and were reported to be *X. campestris* pv. *oryzae* (later renamed *X. oryzae* pv. *oryzae*) (16). JBG-1 and JBG-2 were isolated from rice but are not pathogenic to rice.
[c] Pathogenicity to rice was determined for *X. oryzae* pv. *oryzae* by leaf clipping (19) and for *X. oryzae* pv. *oryzicola* by the spray inoculation technique (2).
[d] Monoclonal antibody group assignment according to Benedict et al. (9) monoclonal antibody reaction; NA = not applicable.

Table 4. Polymerase chain reaction primers that differentiate the U.S. strains of *Xanthomonas oryzae* from Asian and African strains

Primer specificity, name[a]	**Locus**	**Annotation[b]**	**Sequence**	**Product size (bp)**
X. oryzae				
Xo1321F	XOOORF1321	*PilV*, type IV pilus modification protein	TATCGTCGTTCTCGCATTTG	304
Xo1321R	…	…	ATTGAGAACGGTTGCACTGG	…
Xo2967F	XOOORF2967	CDP—alcohol phosphatidyltransferase	CGGTTCGTTCAAGACACCTT	336
Xo2967R	…	…	CCCTCCTTGACATAGCTGGA	…
X. oryzae pv. *oryzae*				
Xoo80F	XOOORF0080	Hypothetical protein	TCAACCGGAGGAACATGATTA	312
Xoo80R	…	…	GCGTCCTCGTCTAAGCGATA	…
Xoo2976F	XOOORF2976	*fhaB*—filamentous hemagglutination activity domain	GCCGTTTTTCTTCCTCAGC	337
Xoo2976R	…	…	AGGAAAGGGTTTGTGGAAGC	…
Xoo4009F	XOOORF4009	Ubiquinone biosynthesis protein	CCTTCATTTCCGTCGTCATC	302
Xoo4009R	…	…	ATGCATGAAGAACCACCACA	…
X. oryzae pv. *oryzicola*				
Xoc76F	XOCORF0076	*fni*—Isopentenyl-diphosphate delta-isomerase, type 2	CGCTGCTGATAAGTTCGATG	407
Xoc76R	…	…	CGATCCCACTTCCTTGACC	…

[a] Specificity refers to species or pathovar that each primer is targeted to amplify: both pathovars (Xo), *X. oryzae* pv. *oryzae* (Xoo), or *X. oryzae* pv. *oryzicola* (Xoc).
[b] Annotation is reported from http://cmr.jcvi.org.

DISCUSSION

Development of specific diagnostic tools is hampered by the difficulty in identifying unique and distinguishing features among the target organisms. We used a bioinformatic comparative genome pipeline to rapidly identify unique and conserved loci in *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, and to predict and design highly specific diagnostic primers. Primers were designed to provide a simple confirmation of identity by presence or absence in either organism. In our first sweep of the genome comparative data, a large number (n = 1,933) of potentially unique regions were identified. By applying increasingly stringent computational filters, 1,697 primer pairs were selected that distinguished *X. oryzae* from other species, and *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola*. In sequential step-wise PCR tests with increasing numbers of strains from a geographically diverse collection of *X. oryzae* pathovars as well as other bacteria, a subset of primers (12 total) was validated for target specificity in PCR reactions with a geographically diverse collection of *X. oryzae* pathovars as well as other bacteria (http://cpgr.plantbiology.msu.edu). To ensure a robust assessment of the bacterial genomes, the final subset of primers included primers that amplified loci scattered around genomes of both pathovars. Finally, a subset of these primers were computationally redesigned to produce different-sized amplicons that were easily distinguished after separation in agarose gels, and the primers were validated for use in a multiplex PCR test to expedite diagnosis and reduce costs.

Acceptance and application of diagnostic tests requires that the tests be validated with many strains of the target pathogen and other microbes. Access to large collections of strains for validation can be problematic, particularly if the organisms are regulated, as are the *X. oryzae* pathovars. We are grateful to our many colleagues worldwide who provided heat-killed bacteria or DNA of validated strains or who validated our primers in their labs. For example, collaborators have screened primers Xoo80, Xoo4009, Xo1321, Xo2207, Xo2967, Xoc3866, and Xoc3864 in PCR assays against additional confirmed *X. oryzae* strains. Fourteen confirmed *X. oryzae* pv. *oryzae* strains and five confirmed *X. oryzae* pv. *oryzicola* strains from India were correctly identified using these primer pairs (D. Mishra, Bayer Crop Science, *personal communication*). The primers did not amplify 21 yellow bacteria isolated from rice seed that did not cause any symptoms upon re-inoculation onto rice (D. Mishra, Bayer Crop Science, *personal communication*). In testing the primers in different labs, however, we noted the importance of calibrating PCR machines for accurate results.

Fluidity and plasticity of microbial genomes complicates taxonomic classification of organisms which, in turn, complicates development of diagnostic tools. Comparisons of genome sequence data is providing insights into relationships among the xanthomonads, and will help taxonomic reclassification (25,35). Some computationally designed primers that were not advanced to the multiplex set distinguished the subgroups of *X. oryzae* pv. *oryzae*. Asian populations consistently amplified expected products, most likely because the only genome sequences in the databases for primer design were from Asian strains. A few primers distinguished African strains from Asian strains (*data not shown*). The most distinct group of strains was the weakly pathogenic U.S. strains. These strains were originally isolated from rice showing symptoms similar but not identical to bacterial blight in the early 1980s in Texas and Louisiana, and were classified as *X. campestris* pv. *oryzae* (16). Although species-specific and pathovar-specific primers were identified that amplified the U.S. and Asian strains, most advanced primers did not produce amplicons from DNA of the U.S. strains.

The 16S rDNA of pathogenic U.S. strains most closely resemble but are not identical to *X. oryzae* pv. *oryzae.* Thus, our studies associate the U.S. strains with the species *X. oryzae*, which is consistent with previous reports (14,16). However, similar to observations with other technologies (9,16,21,31,33), our pathovar-specific primers indicate that the U.S. strains are distinct from the two pathovars *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola,* and confirm that the strains were not recently introduced from Africa or Asia. The origin of the U.S. strains and their relationships to other *Xanthomonas* pathogens of cereals remains an intriguing mystery. Large-scale genome sequencing to allow comparisons of selected U.S. strains is in progress and will help to clarify the identity of these organisms and their relationship to African and Asian *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*.

Our goal of developing robust and specific primers to diverse genomic loci was successful because of the availability of quality genome sequences for not only the target organisms, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, but also many other plant-pathogenic and plant-associated bacteria (http://cpgr.plantbiology.msu.edu). Many of the loci identified putatively encoded hypothetical proteins. However, it is not surprising that the whole-genome screen also detected loci predicted to be involved in pathogen virulence. For example, one of our primer pairs specific to *X. oryzae* pv. *oryzicola* targets a previously described virulence locus, *wxocB*, a putative glycosyltransferase that is involved in the rhamnose-glucose polysaccharide assembly protein F (39). This gene, along with an LPS O-antigen biosynthesis protein upstream from *wxocB*, is unique to *X. oryzae* pv. *oryzicola* and may be implicated in an innate immune response in host plants (39), and might elucidate the fundamental difference between *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* via their infection strategies.

Our purpose was to demonstrate the utility of a computational approach to rapidly develop specific primers that would distinguish *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola*. In diagnostic laboratories, distinction and diagnosis of these rice pathogens usually follows isolation and culture from tissues or seed. Thus, most of our testing of the primers and assays was with extracted DNA or heat-killed bacterial cells from pure cultures. The primers we report did produce amplicons directly from leaves and extracts of seed soaked in bacteria. However, we did not assess sensitivity or amplification from mixed cultures or directly from seed. Seed-testing labs usually isolate the bacteria to pure cultures before performing diagnoses. Additional filtering steps in the computational pipeline may allow selection of primers that can detect low numbers of bacteria in seed which may improve existing rice-seed-testing regimes (7,12).

Quality diagnostic tools that are fast, robust, low cost, and reliable are the foundation for plant disease detection, epidemiological studies, and control strategies. As a proof-of-concept, we have demonstrated the power of comparative genomics for developing diagnostic primers for two important pathogens. As genome sequence information becomes available for more diverse groups of plant pathogens, the application of this approach across kingdoms will be possible. Comparative genomics is an important tool enabling a new era of precise and confident diagnoses.

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