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Development of PCR-Based Detection Methods for the Quarantine Wild Rice Seeds Contaminating Imported and Exported Crops

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ABSTRACT

The establishment and spread of invasive alien weeds in agricultural fields have become a serious problem in Japan. Since the amendment of the Plant Protection Act in Japan, weeds have been included among the target quarantine pests. Therefore, reliable methods are required for the accurate identification and detection of harmful invasive alien weed seeds in international crop trade. In this study, we focused on *Oryza punctata*, a wild rice species native to Africa, which occurs as a weed in paddy fields in its native range and may pose a serious weed risk if introduced into Japan. We developed detection and identification methods for this species based on the polymerase chain reaction (PCR). This species consists of diploid and tetraploid lineages, with the latter being an allopolyploid. One primer pair specific to the diploid lineage and three primer pairs specific to the tetraploid lineage were designed based on multiple sequence alignments of chloroplast genome sequence data obtained from public databases. In addition, PCR conditions were optimized for each primer pair. Electrophoresis confirmed that the expected DNA bands were obtained for *O. punctata* accessions. The primers developed in this study are useful for the quarantine detection of *O. punctata* seeds contaminating imported and exported crops.

1 | Introduction

In recent decades, globalization has accelerated the cross-border movement of agricultural commodities and materials, increasing opportunities for alien weeds to be introduced into new regions and to cause damage to agricultural production and natural ecosystems. One major pathway for the introduction of alien weeds is the unintentional contamination of imported or exported commodities and materials with weed seeds. In Japan, agricultural damage caused by alien weeds has been reported mainly in forage crop fields since the late 1980s, and unintentional introductions associated with grain imports and related trade have been considered an important underlying driver (Kurokawa 2017; Tominaga and Kurokawa 2020). For example, weed seeds presumed to originate from exporting countries have been detected in imported grains (e.g., maize, soybean, and small grains) unloaded at ports with feed-processing facilities and in imported hay transported into dairy production areas (Shimizu 1998; Asai et al. 2007, 2009). Historically, however, many plant species whose impacts were largely confined to agricultural land were less likely to be designated as "invasive alien species," and Japan's Plant Protection Act did not generally treat weed seeds as regulated objects unless they were associated with parasitic plants or with plant parts that could harbor quarantine pests and diseases. As a result, the unintentional introduction of weed seeds through trade has often been overlooked (Asai 2013; Kurokawa 2017).

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In response to these concerns, Japan amended the Plant Protection Act on April 22, 2022; the amendment was promulgated on May 2, 2022, and came into force on April 1, 2023 (Ministry of Agriculture, Forestry and Fisheries of Japan n.d.). The revised law expanded the definition of harmful plants to include "weeds (including their parts, seeds, and fruits)" (e-Gov Law Search n.d.; Japanese Law Translation n.d.), thereby providing an institutional basis for treating weeds (including seeds) as objects of plant quarantine. Consequently, if certain weeds are designated as quarantine-relevant targets based on risk assessment, import inspections may increasingly require the confirmation and identification of contaminant weed seeds. In addition, during export of agricultural products from Japan, importing-country requirements may also demand confirmation and identification of weed seed contamination. Therefore, alongside the selection of target species through risk assessment, it is important to establish technical foundations that support reliable identification and detection of contaminant weed seeds for practical implementation of weed-related quarantine measures.

Potential entry pathways for contaminant weed seeds include contamination of bulk cargoes such as feed grains and contamination of seed for sowing (Tominaga and Kurokawa 2020). Plant quarantine inspections conducted by the Plant Protection Stations in Japan aim to prevent the entry of regulated harmful organisms. In practice, inspectors verify phytosanitary documents submitted upon importation and examine samples taken from consignments by visual inspection and morphological observation to determine whether regulated organisms are suspected; when necessary, confirmatory tests are conducted using serological diagnostics (e.g., ELISA) or molecular diagnostics (e.g., PCR/RT-PCR) (Obara 2005; Nakama 2008). A similar stepwise approach is realistic for weed seeds if they are designated as quarantine targets: first, suspicious seeds are screened and isolated using visual and morphological criteria, and then objective confirmation is performed to determine whether the seeds correspond to regulated target species.

In particular, contaminant weed seeds may be damaged, incomplete, or obscured by adhering debris, making definitive identification by morphology alone difficult. Moreover, when closely related taxa exhibit similar seed morphology, judgments may vary among inspectors and inspection conditions. Accordingly, combining morphological screening with a rapid and objective molecular identification method for suspicious seeds can improve the accuracy and reproducibility of quarantine decisions. Internationally, the International Plant Protection Convention (IPPC) develops International Standards for Phytosanitary Measures (ISPMs); within ISPM 27 (Diagnostic protocols for regulated pests), a diagnostic protocol for Johnsongrass, *Sorghum halepense* (L.) Pers., was adopted in 2017 as the first weed diagnostic protocol. This protocol positions DNA marker testing as a means to support or verify morphological identification after distinguishing the species from other *Sorghum* taxa using seed morphology and thousand-seed weight, and it also highlights utility for cases where seed morphology is unclear, or seeds are partially damaged (International Plant Protection Convention 2017).

A widely used sequence-based approach for species identification is DNA barcoding, which identifies species using predefined genetic regions (DNA barcodes) as biological identifiers (Kishimoto and Ito 2015). In the genus *Oryza*, which includes cultivated rice (*Oryza sativa* L.)—a major crop in Japan—DNA barcoding has been used for species identification and for analyses of phylogenetic relationships. Both nuclear DNA and plastid DNA have been employed as barcode targets. Multiple studies have investigated *Oryza* using complete plastid genome sequences (Kim et al. 2015; Wambugu et al. 2015), and plastome-based analyses have also suggested the feasibility of species discrimination within the genus (Zhang et al. 2021). Because plastid genome sequences for multiple *Oryza* species and accessions are publicly available, it is possible to search for species-specific regions through comparative analyses of existing sequences, design species-specific primers, and implement a practical confirmatory framework in which the presence or absence of PCR amplification is used to verify whether a suspicious seed corresponds to a quarantine target species. The plastid genome of *Oryza* is approximately 150 kb, which is substantially smaller than the nuclear genome and thus facilitates sequence comparison for primer development.

In this study, we aimed to establish a molecular confirmatory framework within genus *Oryza* that can be positioned as a post-screening test following visual inspection and morphological assessment. We focused on the African wild rice *Oryza punctata* Kotschy ex Steud. Wild rice taxa, including *O. punctata*, are recognized as "weedy rice" that can cause problems in paddy rice production. For example, weedy rice populations reported in Malaysia and Thailand have been suggested to originate from *Oryza rufipogon* Griff. and/or from hybrid progeny between cultivated rice and wild rice (Jamjod et al. 2025). In West Africa, invasions of wild rice such as *Oryza longistaminata* A. Chev. & Roehr. and *Oryza barthii* A. Chev. into rice fields have been reported to reduce yields and even lead to field abandonment (Johnson 2002). Although *O. rufipogon* is not native to North America, naturalized populations have been documented in a national park in Florida, and management measures such as hand removal and herbicide application have been reported (Vandiver et al. 1992). In Kenya, *O. punctata* is recognized locally as a problematic weed that can invade and persist in paddy fields, and competition experiments with cultivated rice have shown its potential to be a troublesome species in rice production (Munene et al. 2008). In North America, where certain wild rice taxa have been observed outside cultivation, agricultural invasions have not been confirmed to date; nevertheless, *O. rufipogon*, *O. longistaminata*, and *O. punctata* are designated as Federal Noxious Weeds, and their importation and interstate movement are regulated (US Department of Agriculture, Agricultural Marketing Service 2024).

O. punctata also comprises two cytotypes—diploid accessions with the BB genome and allotetraploid accessions with the BBCC genome (Vaughan et al. 2003)—and these cytotypes differ in physiological and morphological characteristics (Sano 1980). This intraspecific diversity further motivates the development of robust DNA-based identification and detection approaches. *O. punctata* is, therefore, a useful model system for developing quarantine-relevant identification tools because (i) it is considered absent from Japan while publicly curated germplasm resources and reference sequence data are available; (ii) it has

traits associated with problematic weedy rice in paddy systems (Munene et al. 2008); and (iii) it includes genetically and biologically distinct cytotypes (Vaughan et al. 2003).

Here, we compared plastid genome sequences of *O. punctata* and other *Oryza* species using multiple sequence alignment of publicly available sequences to identify *O. punctata*-specific regions and designed primer pairs targeting those regions. We then evaluated PCR amplification using genomic DNA templates from multiple *Oryza* species to determine conditions under which amplification occurs only in *O. punctata*, thereby providing a practical confirmatory method for distinguishing suspicious contaminant seeds in a quarantine context.

2 | Materials and Methods

2.1 | Design of Primer Pairs Specific to *O. punctata*

The accession numbers and strain names of the materials obtained from public databases in this study are shown in Table S1. There are reportedly approximately 23 species in the genus *Oryza* (Vaughan et al. 2003), but the classification of some species is still under discussion. Accordingly, in this study, we obtained complete chloroplast genome sequence data of *Oryza* species from the National Center for Biotechnology Information (NCBI), based on the classification described in Oryzabase, a database operated by the National Institute of Genetics (NIG), Japan, and on the classification by Zhang et al. (2021). Since there were a large number of complete chloroplast genome sequences registered for *O. sativa*, we selected six representative accessions according to Zhang et al. (2021). For the other species, complete chloroplast genome sequences of all registered accessions were used for multiple sequence alignment. Multiple sequence alignment was performed using CLUSTAL W (Thompson et al. 1994). Primer design was based on the report by Oi (2015).

2.2 | Verification of Primer Target Sequences in *O. punctata* Accessions Using Seed-Derived DNA

Seeds of *O. sativa* provided by the Genebank of the National Agriculture and Food Research Organization (NARO) and wild rice seeds provided by NIG were used as plant materials. The species used for DNA extraction are shown in Table 1. Nuclear genome types were determined based on data provided in Oryzabase.

DNA extraction was performed with slight modifications to the method described by Monna et al. (2002). Specifically, one dehulled brown rice grain and a tungsten bead were placed in a microtube and crushed using a mixer mill (Retsch, MM400). Then, 2% cetyltrimethylammonium bromide (CTAB) buffer was added, and the mixture was vortexed and centrifuged at 15,000 rpm for 10 min at room temperature using a centrifuge (KUBOTA, Model 3520). The supernatant was transferred to a new tube, and an equal volume of a 24:1 mixture of chloroform and isoamyl alcohol was added. After vortexing and centrifugation, the supernatant was again transferred to a new tube, and an equal volume of isopropanol was added. The mixture was vortexed and centrifuged. The supernatant was discarded, 70% ethanol was added, and the mixture was centrifuged at 15,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was air-dried, and then dissolved in 100 μL of 1/10 TE buffer.

Next, sequencing primers were designed outside the primer-binding regions described in Section 2.1, and sequencing was

TABLE 1 | *Oryza* species, nuclear genome types, and cultivar or strain names of the accessions used.

Species name	Nuclear genome type	Cultivar/ strain name
O. sativa	AA	Koshihikari (JPNo. 80825)
O. sativa	AA	KASALATH (WRC02)
O. longistaminata	AA	W1508
O. minuta	BBCC	W1213
O. minuta	BBCC	W1328
O. minuta	BBCC	W1342
O. eichingeri	CC	W1519
O. eichingeri	CC	W1522
O. eichingeri	CC	W1526
O. longiglumis	HHJJ	W1218
O. granulata	GG	W0615
O. meyeriana	GG	W1352
O. punctata	BBCC	W0015
O. punctata	BBCC	W0043
O. punctata	BBCC	W1023
O. punctata	BBCC	W1024
O. punctata	BBCC	W1145
O. punctata	BBCC	W1408
O. punctata	BBCC	W1409
O. punctata	BBCC	W1564
O. punctata	BBCC	W1474B
O. punctata	BB	W1514
O. punctata	BB	W1515
O. punctata	BB	W1577
O. punctata	BB	W1582
O. punctata	BB	W1586
O. punctata	BB	W1590
O. punctata	BB	W1592
O. punctata	BB	W1593
O. punctata	—	W2034

Note: Nuclear genome types were compiled based on information in Oryzabase; species for which no information was available in Oryzabase are indicated by "—".

TABLE 2 | Sequences of the petA-psbJ_F1 & R1 primer target regions in *Oryza* species.

Species name	Accession number	Forward target sequence	Reverse target sequence
O. sativa	AY522329	CTATTTTTTGTTTGCGAGA	AAGGCAAAAAAGCCTTTTTCTTG
O. punctata	KM103375	**CCGCTTTTTGTTTGCGAGA**	**AAGGCTTTTTTGCCTTTTTCTTG**
O. punctata	MT726932	CTATTTTTTGTTTGCGAGA	AAGGCAAAAAAGCCTTTTTCTTG
O. minuta	NC030298	CCGCTTTTTGTTTGCGAGA	AAGGCAAAAAAGCCTTTTTCTTG
O. eichingeri	NC034759	CTATTTTTTGTTTGCGAGA	AAGGCAAAAAAGCCTTTTTCTTG
O. granulata	NC034761	CTATTTTTTGTTTGCGAGA	AAGGCTTTTTTGCCTTTTTCTTG
O. longiglumis	NC034763	CTATTTTTTGTTTGCGAGA	AAGGCTTTTTTGCCTTTTTCTTG

Note: Sequences that are completely identical to the designed primer sequences are shown in bold. Sequences that differ from the primer sequences are shaded in gray.

performed to confirm whether each *O. punctata* accession contained the designed primer target sequences. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and the products were analyzed with a 3500 Genetic Analyzer (Thermo Fisher Scientific).

The PCR reaction mixture for sequence amplification consisted of 0.15 μL of each primer (20 μM), 5.0 μL of Takara EX Premier DNA Polymerase (TaKaRa), 1.0 μL of sample DNA (approximately 20 ng/μL), and sterile water, for a total volume of 10 μL. PCR amplification was performed using a Takara Thermal Cycler Dice Touch (TaKaRa). The PCR conditions were 94°C for 1 min, followed by 30 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. ExoSAP-IT (Thermo Fisher Scientific) was added for PCR product purification, and the reaction was carried out at 37°C for 30 min and 80°C for 15 min.

The sequencing reaction mixture was then prepared by adding 2.0 μL of forward primer (0.8 pmol/μL), 2.0 μL of BigDye Terminator mixture, approximately 50 ng of PCR product, and sterile water, in a total volume of 10 μL. The sequencing reaction was carried out at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, for 25 cycles.

2.3 | Primer Design for *O. punctata* Accessions Lacking Sufficient Sequence Information in Databases

In order to detect *O. punctata* in accessions for which primer pairs could not be designed using only sequence information available in databases, we commissioned new complete chloroplast genome sequencing. Among the *O. punctata* accessions provided by NIG, one accession (W1024) was selected. We outsourced the sequencing of the complete chloroplast genome to Fasmac Co. Ltd. For complete chloroplast genome sequencing, the necessary DNA was extracted from a sample of approximately 3 cm of leaf tissue. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN). The newly obtained sequence was subjected to multiple sequence alignment, and one primer pair was designed using the same procedure described in Section 2.1.

Subsequently, as in Section 2.2, sequencing primers were designed outside the primer-binding regions, and sequencing was performed to confirm whether each *O. punctata* accession contained the designed primer target sequences.

The complete chloroplast genome sequence of *O. punctata* accession W1024 was deposited in DDBJ/GenBank under accession number LC907692.

2.4 | Optimization of PCR Conditions

The PCR primers designed in Sections 2.1 and 2.3 were used to determine optimal PCR conditions for detecting *O. punctata*. DNA extracted from seeds of *Oryza* species in Section 2.2 was used as the template, and PCR conditions were examined so as to obtain clear bands in samples of *O. punctata* but not in those of other species. The PCR reaction mixture was prepared using the same kit, and amplification was carried out using the same PCR machine as in Section 2.2.

The PCR conditions consisted of 94°C for 1 min, followed by cycles of 98°C for 10 s, annealing for 15 s, and 68°C for 30 s. The optimal conditions for the number of cycles and annealing temperature were determined for each primer pair.

The PCR products were electrophoresed on 2% agarose gels (PrimeGel Agarose PCR-Sieve, TaKaRa) in 1× Tris-Borate-EDTA (TBE) buffer to confirm amplification. The volume of PCR products loaded onto the gel was 2 μL.

3 | Results

3.1 | Design of Primer Pairs Specific to *O. punctata*

Multiple sequence alignment of the complete chloroplast genome sequences listed in Table S1 confirmed the presence of *O. punctata*-specific nucleotide sequences in the spacer regions between the petA and psbJ genes, the TrnfM-CAU and TrnT-GGU genes, and the ndhF and rpl32 genes (Table S1; Tables 2–4). Using these specific sequences as targets, we designed three primer pairs that specifically amplify *O. punctata* (Tables 2–4).

TABLE 3 | Sequences of the TrnfM-TrnT_F1 & R1 primer target regions in *Oryza* species.

Species name	Accession number	Forward target sequence	Reverse target sequence
O. sativa	AY522329	TACCCAGCCATATGGGTATAT	CGGTCTGAAAAATTAGGGACTTT
O. punctata	KM103375	TACCCAGCCATATGGGTATAT	CGGTCTGCAAAATTAGGGACTTT
O. punctata	MT726932	**TACCCAGCCATATGGGGTATA**	**CGGTCTGAAAAATTAGGGACTTT**
O. minuta	NC030298	TACCCAGCCATATGGGTATAT	CGGTCTGCAAAATTAGGGACTTT
O. eichingeri	NC034759	TACCCAGCCATATGGGTATAT	CGGTCTGAAAAATTAGGGACTTT
O. granulata	NC034761	TACCCAGCCATATGGGTATAT	CGGTCTGAAAAATTAGGGACTTT
O. longiglumis	NC034763	TACCCAGCCATATGGGTATAT	TGGTCTGAAAAATTAGGGACTTT

Note: Sequences that are completely identical to the designed primer sequences are shown in bold. Sequences that differ from the primer sequences are shaded in gray.

TABLE 4 | Sequences of the ndhF-rpl32_F1 & R1 primer target regions in *Oryza* species.

Species name	Accession number	Forward target sequence	Reverse target sequence
O. sativa	AY522329	GGTTTTGTTGGTTAAAGTCAA	AATGATTCAATCTTTTTTTTGTATT
O. punctata	KM103375	GGTTTTGTTGGTTAAAGTCAA	AAATGATTCAATCTTTTTTTGTATT
O. punctata	MT726932	**GGTTTTGTTGGTTAAAGTCAA**	**GATTCAATCTTTCTTTTTTTGTATT**
O. minuta	NC030298	GGTTTTGTTGGTTAAAGTCAA	AAATGATTCAATCTTTTTTTGTATT
O. eichingeri	NC034759	GGTTTTGTTGGTTAAAGTCAA	AAATGATTCAATCTTTTTTTGTATT
O. granulata	NC034761	GGTTTAGTTGGTTAAATTCAA	AAATGATTCAATCTTTTTTTGTATT
O. longiglumis	NC034763	GGTTTAGTTGGTTAAATTCAA	AAATGATTCAATCTTTTTTTGTATT

Note: Sequences that are completely identical to the designed primer sequences are shown in bold. Sequences that differ from the primer sequences are shaded in gray.

The primers were designed so that the amplified PCR product would be approximately 150 bp in size.

The primer sequences were as follows:

- petA-psbJ_F1 & R1 (product size: 173 bp)
  - petA-psbJ_F1: 5′-CCGCTTTTTGTTTGCGAGA-3′
  - petA-psbJ_R1:5′-AAGGCTTTTTTGCCTTTTTCTTG-3′
- TrnfM-TrnT_F1 & R1 (product size: 144 bp)
  - TrnfM-TrnT_F1: 5′-TACCCAGCCATATGGGGTATA-3′
  - TrnfM-TrnT_R1: 5′-CGGTCTGAAAAATTAGGGACTTT-3′
- ndhF-rpl32_F1 & R1 (product size: 103 bp)
  - ndhF-rpl32_F1: 5′-GGTTTTGTTGGTTAAAGTCAA-3′
  - ndhF-rpl32_R1: 5′-GATTCAATCTTTCTTTTTTTGTATT-3′

3.2 | Verification of Primer Target Sequences in *O. punctata* Accessions Using Seed-Derived DNA

We examined whether each *O. punctata* accession from which seeds were obtained contained the primer target sequences designed based on DNA sequence information from databases. Sequencing was performed using DNA extracted from the seeds of accessions listed in Table 1.

The sequencing primers were as follows:

- petA-psbJ_F1 & R1 sequencing primers
  - petA-psbJ_F1S: 5′-TCAGGGGGATGCAGAAATAG-3′
  - petA-psbJ_R1S: 5′-CCACTACATCGCCTGAACCT-3′
- TrnfM-TrnT_F1 & R1 sequencing primers
  - TrnfM-TrnT_F1S: 5′-GTTCCAGAACCCTCCTTTTT-3′
  - TrnfM-TrnT_R1S: 5′-GGGCTTCTTATTCAATTCCAAA-3′
- ndhF-rpl32_F1 & R1 sequencing primers
  - ndhF-rpl32_F1S: 5′-AAGTCCAAACCCCATTGACA-3′
  - ndhF-rpl32_R1S: 5′-ACGGGAGGATAAAAGGAAGG-3′

The sequencing results showed that the diploid *O. punctata* accessions contained the target sequences corresponding to the petA-psbJ_F1 & R1 primer pair (Table 6). In contrast, some of the tetraploid accessions contained the target sequences corresponding to the TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 primer pairs. However, among the tetraploid accessions supplied, only three accessions (W1023, W1408, and W1564) had the target sequences corresponding to the TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 primer pairs (Table 6). Five of the tetraploid accessions (W0015, W0043, W1024, W1145, and W1409) did not contain any of the primer target sequences, including those corresponding to the petA-psbJ_F1 & R1 primer pair. Furthermore, among the tetraploid accessions, W1474B was confirmed to contain the target sequences corresponding to the petA-psbJ_F1 & R1 primer pair.

3.3 | 3. Primer Design for *O. punctata* Accessions Lacking Sufficient Sequence Information in Databases

We determined that it would be difficult to obtain sufficient sequence information from existing databases for five allotetraploid accessions (W0015, W0043, W1024, W1145, and W1409). Therefore, we commissioned complete chloroplast genome sequencing of W1024 and again performed multiple sequence alignment using the obtained sequence together with the sequences listed in Table S1. As a result, a sequence specific to W1024 was identified in the spacer region between the petA and psbJ genes. Using this specific sequence as a target, we designed a primer pair, petA-psbJ_F2 & R2, that specifically amplifies DNA from W1024 (Table 5).

The primer sequences were as follows:

- petA-psbJ_F2 and R2 (product size: 117 bp)
  - petA-psbJ_F2: 5′-CATTGGCGAGGGGTTATAAAT-3′
  - petA-psbJ_R2: 5′-GAAAGCCTTTGCTCTGATGG-3′

TABLE 5 | Sequences of the petA-psbJ_F2 & R2 primer target regions in *Oryza* species.

Species name	Accession number	Forward target sequence	Reverse target sequence
O. sativa	AY522329	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. punctata	KM103375	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. punctata	MT726932	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. punctata	W1024	**CATTGGCGAGGGGTTATAAAT**	**GAAAGCCTTTGCTCTGATGG**
O. minuta	NC030298	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. eichingeri	NC034759	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. granulata	NC034761	CATTGGCGAGGGGGTTATAAA	GAAAGCCTTTGCTCTGATGG
O. longiglumis	NC034763	CATTGGCGAGGGGGGCTATAA	GAAAGCCTTTGCTTTGATGG

Note: Sequences that are completely identical to the designed primer sequences are shown in bold. Sequences that differ from the primer sequences are shaded in gray.

TABLE 6 | Primer target region sequences of *Oryza punctata* accessions used in this study.

Strain name	Nuclear genome type	petA-psbJ_F1 & R1	TrnfM-TrnT_ F1 & R1	ndhF-rpl32_F1 & R1	petA-psbJ_F2 & R2
W0015	BBCC				○
W0043	BBCC				○
W1023	BBCC		○	○	
W1024	BBCC				○
W1145	BBCC				○
W1408	BBCC		○	○	
W1409	BBCC				○
W1474B	BBCC	○			
W1514	BB	○			
W1515	BB	○			
W1564	BBCC		○	○	
W1577	BB	○			
W1582	BB	○			
W1586	BB	○			
W1590	BB	○			
W1592	BB	○			
W1593	BB	○			
W2034	—	○			

Note: Accessions marked with "○" have nucleotide sequences that are completely identical to the corresponding primer target sequences. Accessions without this mark have nucleotide sequence differences and do not exactly match the primer target sequences.

To verify whether each *O. punctata* accession from which seeds were obtained contained the petA-psbJ_F2 & R2 primer target sequence, sequencing was performed using the following sequencing primers:

- petA-psbJ_F2 and R2 sequencing primers
  - petA-psbJ_F2S: 5′-TCTTCCATTGAAGGCTGTTTTT-3′
  - petA-psbJ_R2S: 5′-TTCTGGATTGGGTTCATCTCT-3′

The sequencing results showed that five accessions (W0015, W0043, W1024, W1145, and W1409) had the petA-psbJ_F2 & R2 primer target sequence (Table 6). In contrast, none of the other *O. punctata* accessions had the petA-psbJ_F2 & R2 primer target sequence (Table 6).

3.4 | Optimization of PCR Conditions

PCR conditions were examined using the four primer pairs designed in this study. The optimal number of amplification cycles was determined to be 30 for petA-psbJ_F1 & R1, TrnfM-TrnT_F1 & R1, and ndhF-rpl32_F1 & R1, and 31 for petA-psbJ_F2 & R2. The optimal annealing temperatures were 68°C for petA-psbJ_F1 & R1, 66°C for TrnfM-TrnT_F1 & R1, 59°C for ndhF-rpl32_F1 & R1, and 64°C for petA-psbJ_F2 & R2. Under these conditions, clear bands were obtained in the target accessions (Figures 1–4).

Increasing the number of cycles or lowering the annealing temperature beyond the above values sometimes resulted in false-positive bands in accessions other than the target accessions. Furthermore, when the number of cycles was reduced or the annealing temperature was increased, bands sometimes became faint or were completely invisible, even in the target accessions.

PCR was also performed on wild *Oryza* species other than *O. punctata*, listed in Table 1, to examine amplification specificity. As a result, bands were obtained in one accession of *O. minuta* J.Presl (W1328) using TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1. Sequencing of this accession confirmed that it had nucleotide sequences completely identical to those corresponding to the TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 primer pairs. Furthermore, bands were obtained in two *O. minuta* accessions (W1213 and W1342) using petA-psbJ_F2 & R2. Sequencing of these two accessions confirmed that they had nucleotide sequences completely identical to those corresponding to the petA-psbJ_F2 & R2 primer pair.

4 | Discussion

4.1 | Intraspecific Variation in *O. punctata*

The allotetraploid lineages of *O. punctata* with the BBCC genome are most likely established by hybridization between *O. punctata* (BB genome) as the paternal parent and *O. eichingeri* Peter (CC genome) as the maternal parent; consequently, their chloroplast genomes are considered to be derived from a CC genome species (Zou et al. 2015). In this study, we used chloroplast genome sequence information to develop DNA markers for the identification of *O. punctata*.

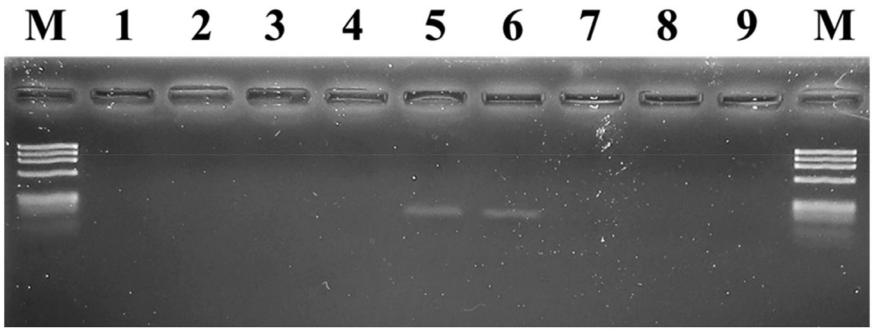


FIGURE 1 | Electrophoresis of PCR products amplified with petA-psbJ_F1 & R1. Annealing temperature was 68°C. Bands of approximately 170 bp were observed in lanes 5 and 6, where amplification was expected. Lanes 1–9: 1, *Oryza sativa* "Koshihikari" (JPNo. 80825); 2, *O. sativa* "KASALATH" (WRC02); 3, *O. punctata* (BBCC) W0015; 4, *O. punctata* (BBCC) W1023; 5, *O. punctata* (BB) W1577; 6, *O. punctata* (BB) W1582; 7, *O. eichingeri* W1519; 8, *O. granulata* W0615; 9, *O. longiglumis* W1218. M, φX174/HaeIII digest (Marker 4; NIPPON GENE).

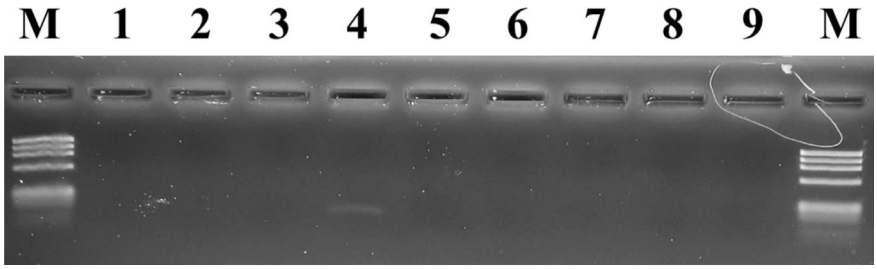


FIGURE 2 | Electrophoresis of PCR products amplified with TrnfM-TrnT_F1 & R1. Annealing temperature was 66°C. A band of approximately 150 bp was observed in lane 4, where amplification was expected. Lanes 1–9: 1, *Oryza sativa* "Koshihikari" (JPNo. 80825); 2, *O. sativa* "KASALATH" (WRC02); 3, *O. punctata* (BBCC) W0015; 4, *O. punctata* (BBCC) W1023; 5, *O. punctata* (BB) W1577; 6, *O. punctata* (BB) W1582; 7, *O. eichingeri* W1519; 8, *O. granulata* W0615; 9, *O. longiglumis* W1218. M, φX174/HaeIII digest (Marker 4; NIPPON GENE).

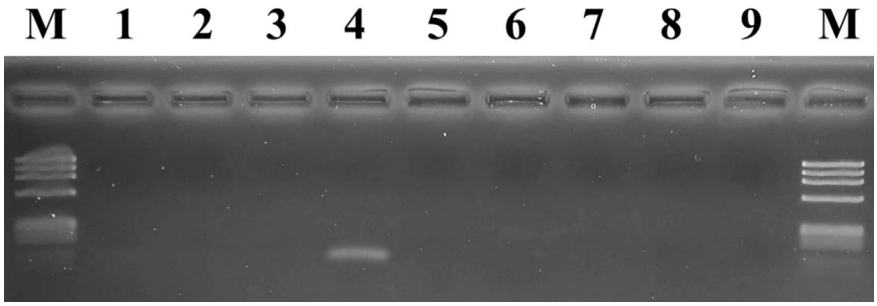


FIGURE 3 | Electrophoresis of PCR products amplified with ndhF-rpl32_F1 & R1. Annealing temperature was 59°C. A band of approximately 100 bp was observed in lane 4, where amplification was expected. Lanes 1–9: 1, *Oryza sativa* "Koshihikari" (JPNo. 80825); 2, *O. sativa* "KASALATH" (WRC02); 3, *O. punctata* (BBCC) W0015; 4, *O. punctata* (BBCC) W1023; 5, *O. punctata* (BB) W1577; 6, *O. punctata* (BB) W1582; 7, *O. eichingeri* W1519; 8, *O. granulata* W0615; 9, *O. longiglumis* W1218. M, φX174/HaeIII digest (Marker 4; NIPPON GENE).

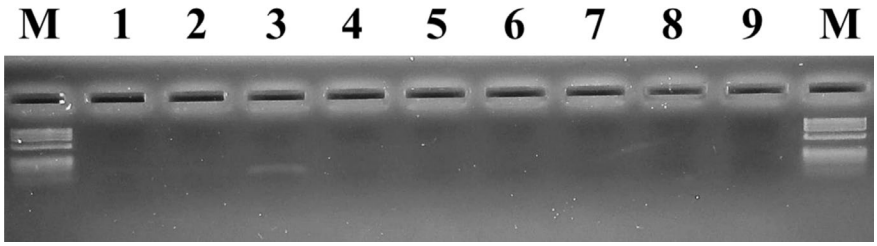


FIGURE 4 | Electrophoresis of PCR products amplified with petA-psbJ_F2 & R2. Annealing temperature was 64°C. A band of approximately 120 bp was observed in lane 3, where amplification was expected. Lanes 1–9: 1, *Oryza sativa* "Koshihikari" (JPNo. 80825); 2, *O. sativa* 'KASALATH' (WRC02); 3, *O. punctata* (BBCC) W0015; 4, *O. punctata* (BBCC) W1023; 5, *O. punctata* (BB) W1577; 6, *O. punctata* (BB) W1582; 7, *O. eichingeri* W1519; 8, *O. granulata* W0615; 9, *O. longiglumis* W1218. M, φX174/HaeIII digest (Marker 4; NIPPON GENE).

Initially, we compared complete chloroplast genome sequences of *Oryza* species, including four sequences from *O. punctata* registered in NCBI (Accession Nos. KF359908, NC027676, KM103375, and MT726932), to determine whether there were any sequences common to *O. punctata* that were not found in other *Oryza* species. However, no sequence that satisfied the above criteria was found (Table S1). Therefore, we attempted to design primers specific to the diploid and tetraploid lineages, respectively, and found that the diploid accessions of *O. punctata* provided by NIG could be specifically detected with petA-psbJ_F1 & R1 (Figure 1). The sequences used to design petA-psbJ_F1 & R1 were present in Accession Nos. KF359908, NC027676, and KM103375. These accessions are considered to represent the diploid lineage. TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 were designed based on Accession No. MT726932, and specific detection of W1023, W1408, and W1564 was achieved among the tetraploid *O. punctata* accessions provided by NIG (Figures 2 and 3). The results suggest that Accession No. MT726932 represents a tetraploid lineage. By contrast, among the tetraploid accessions we received, W0015, W0043, W1024, W1145, and W1409 were not detected with the primer pairs designed from the MT726932 sequence (Figures 2 and 3). To design primers specific to these accessions, we newly determined the complete chloroplast genome sequence of accession W1024, one of the accessions in which no amplification was obtained with the TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 primer pairs. Using the primer pair petA-psbJ_F2 & R2, which was designed based on the chloroplast genome sequence newly obtained for W1024 (Accession No. LC907692), W0015, W0043, W1024, W1145, and W1409 were specifically detected (Figure 4).

Shenton et al. (2020) used genome-wide SNP-based phylogenetic analysis of the *O. officinalis* complex to suggest that the tetraploid lineage of *O. punctata* may have originated from at least two independent hybridizations. Using materials provided by NIG, they treated W1023, W1408, and W1564 as one cluster and W0015, W0043, W1024, W1145, and W1409 as another cluster. In our study, as well, the effective primer pairs differed between these two groups, and this difference can be interpreted as reflecting genetic diversity and phylogenetic group differentiation within the tetraploid lineage.

In recent years, there has been a view that the diploid and tetraploid lineages should be regarded as distinct species, with the tetraploid lineage sometimes being given the species name *O. schweinfurthiana* Prodoehl (Zou et al. 2015). However, although there have been reports of *O. punctata* invading rice fields in Africa, its primary habitat, no description of the genome type of the invading populations has been found. It is therefore believed that the diploid and tetraploid lineages have not been distinguished in these reports. From a quarantine perspective, it is therefore necessary to continue improving detection methods so that both lineages can be reliably detected. On the other hand, given the view that they should be treated as separate species, it is also important to clarify whether the diploid and tetraploid lineages of *O. punctata* pose the same risk of becoming weeds.

4.2 | Database Discrepancies and Challenges in Diagnostic Marker Development

We developed diagnostic primers using the complete chloroplast genome sequences obtained from the NCBI database (as listed in Table S1). However, for *O. minuta*, which has the same BBCC genome as tetraploid *O. punctata*, the nucleotide sequence information in the database sometimes differed from the nucleotide sequences obtained from the seeds we received. Contrary to the database information, we confirmed the presence of sequences corresponding to TrnfM-TrnT_F1 & R1 and ndhF-rpl32_F1 & R1 in *O. minuta* accession W1328 distributed by NIG, as well as sequences corresponding to petA-psbJ_F2 & R2 in *O. minuta* accessions W1213 and W1342. One possible reason for the *O. punctata*–specific sequences in three *O. minuta* accessions is that the species name labels at the genetic resource preservation facility may have been incorrect. Shenton et al. (2020) pointed out the possibility of labeling errors in NIG accessions and proposed that the three *O. minuta* accessions used in this study (W1213, W1328, and W1342) be relabeled as tetraploid *O. punctata*. Our results support this conclusion and show that mislabeling and sequence discrepancies can complicate the development of simple interspecific markers for quarantine use.

Among the tetraploid accessions used in this study, the genome information for accession W1474B was also inconsistent between Oryzabase (NIG) and some literature sources. Although accession W1474B is classified as tetraploid in Oryzabase, we found that it possessed the petA-psbJ_F1 & R1 primer target sequences found in diploid accessions (Table 1). Since W1474B was described as diploid by Sano (1980) and relabeling as diploid was also proposed by Shenton et al. (2020), our results suggest that W1474B may be diploid. This discrepancy between data registered for genetic resources at various institutions and information provided in the literature constitutes one of the challenges when developing interspecific diagnostic markers using database information. Zhang et al. (2021) also reported that approximately 17% of *Oryza* accessions derived from genetic resource preservation facilities and field collections were mislabeled, underscoring the need for careful cross-checking.

4.3 | Robustness of the PCR Primer Set

In this study, we designed *O. punctata* specific primers based on complete chloroplast genome sequences retrieved from public databases. We then assessed the practicality of this primer set by performing PCR using genomic DNA extracted from 18 distributed *O. punctata* accessions as templates and evaluating the presence or absence of amplification products. Among the primer pairs developed here, TrnfM–TrnT_F1 and R1 exploit a difference in a homopolymeric guanine (G) run: the forward primer sequence (TACCCAGCCATATGGGGTATA) contains four consecutive G in *O. punctata*, whereas other *Oryza* taxa have three (Table 3). The petA–psbJ_F2 and R2 pair was designed using a single-nucleotide difference in the forward primer sequence (Table 5). Because these designs rely on a small number of diagnostic bases, false-positive bands may occur when DNA from *Oryza* species other than *O. punctata* is used; indeed, false positives were observed under certain PCR conditions in this study. In a plant quarantine context, false positives are unacceptable. Therefore, when amplification is obtained, it is necessary to verify that the band is truly derived from *O. punctata*. Depending on the situation, this may require sequencing of the PCR amplicon. In environmental DNA surveys, for example, direct sequencing is often performed after real-time PCR amplification to confirm that the amplified sequence originates from the target species (Fukuoka et al. 2016). Similarly, in quarantine applications, when a band is detected by gel electrophoresis, sequencing the PCR product used for electrophoresis and confirming that the amplicon contains the expected *O. punctata*-specific sequence would improve the reliability of the test. If an *O. punctata*-specific sequence is confirmed by sequencing, misidentification of the tested seed as *O. punctata* is highly unlikely.

At the same time, it is also important to pursue the development of more robust primers that reduce the need for sequencing-based confirmation. In establishing an eDNA-based detection system, Fukuoka et al. (2016) suggested that, to lower the risk of detecting closely related species erroneously, primers should be designed so that species-specific bases are concentrated within 5 bases of the 3′ end. In the present study, however, such designs were not feasible, in part because we needed to match the melting temperatures (T_m) of the forward and reverse primers.

Markers that capture intraspecific variation—such as rice cultivar identification markers—have typically been developed using nuclear genome sequence information (Shinmura et al. 2005). Recently, comprehensive genotyping approaches using next-generation sequencing, such as MIG-seq (Multiplexed ISSR genotyping by sequencing), have been developed to detect genome-wide polymorphisms including single-nucleotide polymorphisms (SNPs) (Suyama and Matsuki 2015). Although the present study developed a PCR primer set using chloroplast genome sequence information that is expected to reflect interspecific variation (The Society for the Study of Species Biology 2001), it will also be necessary to explore the development of diagnostic markers based on nuclear genome sequence data by leveraging these newer approaches.

4.4 | Intended Use Cases for the PCR Primer Set Developed in This Study

DNA markers can be broadly classified as dominant or codominant. In developing diagnostic markers applicable to weed quarantine, our group selected a PCR primer set that functions as a dominant marker—that is, one that yields amplification only for the target species, *O. punctata*—as the final deliverable of this study. In quarantine applications, codominant markers can be advantageous because they may be less prone to false-positive or false-negative outcomes. In weed science, codominant markers have been used in practice; for example, dCAPS markers (a codominant marker system) have been applied to identify herbicide-resistant weeds (Yu et al. 2013; Ghanizadeh et al. 2021). In contrast, the IPPC diagnostic protocol for Johnsongrass introduced above presents examples using SCAR and ISSR markers. Although ISSR markers are generally dominant, multiple bands of different sizes are amplified in the genus *Sorghum*, and the number of detectable bands can differ among species. As a result, ISSR can function effectively as a codominant marker system in that context, enabling species identification based on

banding patterns. SCAR markers can be either dominant or co-dominant; in the IPPC protocol, the SCAR marker functions as a dominant marker in which a band is detected when the sample is Johnsongrass (International Plant Protection Convention 2017).

During primer development in the present study, we were able to generate two codominant marker sets that leverage the presence/absence of deletions in the chloroplast genome. However, these codominant markers were effective for distinguishing *O. sativa* and other AA-genome species from *O. punctata*, whereas non-AA wild *Oryza* species produced amplicons of similar size to those of *O. punctata* (data not shown). If *O. punctata* were to be designated as a quarantine target, a practical primer set would need to discriminate the target not only from cultivated rice but also from other wild *Oryza* species. For this reason, we concluded that the codominant markers identified here are not suitable for use in a quarantine setting.

There were additional reasons for choosing a dominant marker as the primary output. Here we assumed a scenario in which a fixed quantity of samples is examined from bulk consignments such as feed grains and, upon detecting a suspect seed that may correspond to a quarantine target, DNA-based confirmation is conducted using that single contaminant seed. In plant quarantine practice for plant diseases, real-time PCR is frequently employed. It is therefore plausible that similar workflows using real-time PCR will be requested for weed quarantine as well. In real-time PCR testing for plant diseases, DNA or RNA is typically extracted from a pooled sample comprising a relatively large number or mass of seeds, and the presence or absence of pathogen DNA/RNA is assessed. If real-time PCR were applied to weed quarantine, the extracted DNA would likely be a mixture derived from the quarantine target (or candidate targets), other weed species, and crop species. Under such conditions, codominant markers would have a high likelihood of detecting both the target and non-target components. Furthermore, existing inspection procedures for genetically modified organisms (GMOs) provide a relevant reference: for flax, wheat, rice, maize, and rapeseed, the number of packages to be opened for sampling is determined by lot size, and a 1-kg sample is collected; 500 g is then ground, and DNA testing is conducted using DNA extracted from a few grams of the homogenized material (Ministry of Health, Labour and Welfare of Japan 2025). While such approaches may be informative for designing real-time PCR workflows in weed quarantine, if only a single quarantine-target weed seed is present in a pooled sample of many seeds or in a large mass, the amount of target DNA in the extract would be extremely low. When marker amplicons are long, an increased number of PCR cycles may be required for detection by real-time PCR. The primer sets designed in this study target short amplicons (approximately 100–200 bp). Shortening the amplicon length is expected to be beneficial not only for testing single seeds but also for potential adaptation to other assays such as real-time PCR. Whether the markers developed here can be directly applied to real-time PCR, however, remains a subject for future investigation.

Author Contributions

Riki Oshima: conceptualization, methodology, investigation, formal analysis, data curation, writing – original draft, writing – review and editing, visualization. **Hiroyuki Kobayashi:** writing – review and editing, supervision. **Maiko Akasaka:** conceptualization, methodology, writing – review and editing, supervision, project administration, funding acquisition, resources, validation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The complete chloroplast genome sequence of *Oryza punctata* accession W1024 has been deposited in DDBJ/GenBank (accession no. LC907692). All other data supporting the findings of this study are available within the article (tables and figures) or from the corresponding author on reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Oryza species, accession numbers, and strain names used for multiple sequence alignment.