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Genetic differentiation in *Oryza meridionalis* Ng based on molecular and crossability analyses

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Abstract

Molecular and hybridization studies were carried out to detect variation patterns in *O. meridionalis*. A total of 119 polymorphic RAPD markers were produced from 12 10-mer operon primers while 67 alleles were detected using 11 SSR primers. Cluster analysis based on RAPD and SSR markers identified distinct clusters for accessions coming from Irian Jaya (Indonesia) and Queensland, the Northern Territory, and Western Australia. Intraspecific hybrids showed pollen stainability and panicle fertility ranging from 0 to almost 97%. Fertile hybrids were obtained from crosses between accessions of the same geographic origin, specifically those involving the Irian Jaya accessions that showed greater than 70% pollen stainability and panicle fertility. In crosses involving accessions with different origins, partially fertile to sterile hybrids were obtained between accessions coming from Irian Jaya and Queensland. In contrast, most crosses between the Northern Territory and Irian Jaya accessions and Northern Territory and Queensland accessions produced sterile hybrids. The study proves that *O. meridionalis* is a good taxonomic species undergoing gradual speciation corresponding to its geographic distribution in northern Australia and Irian Jaya.

Introduction

In this paper, we present the results of our genetic diversity analysis of *Oryza meridionalis* signifying the completion of our study on the biosystematics of the AA genome *Oryza* species (Naredo et al. 1997, 1998; Lu et al. 1997, 1998; Martin et al. 1997; Juliano et al. 1998). These studies aimed at better conservation and use of rice and its closest wild relatives that have been an important focus of the International Rice Genebank in recent years.

O. meridionalis is an annual diploid wild species ($2n = 24$) of the Ser. *Sativae* (=*O. sativa* complex of Vaughan 1989) that includes the cultivated species *O. sativa* L. and *O. glaberrima* Steud. and five other wild species, *O. nivara* Sharma et Shastry, *O. rufipogon* Griff., *O. glumaepatula* Steud., *O. barthii*, and *O. longistaminata* A. Chev. et Roeht. (Lu 1999a). *O. meridionalis* was previously believed to be endemic to the northern parts of Australia but was recently collected in Irian Jaya, Indonesia (Lu and Silitonga 1999). It inhabits seasonal wetlands, swamps, and depressions with shallow water, and locations with black soil. In Irian Jaya, *O. meridionalis* was found in deserted fields or in farmers' fields (Lu 1999b).

O. meridionalis was formerly referred to as the Australian form of *O. perennis* (Oka and Morishima 1967; Morishima 1969, 1986). It is morphologically distinct, however, from the Asian

species *O. rufipogon* and *O. nivara* and the African species *O. barthii* A. Chev. (Ng and Chang 1981) and was recognized as a taxonomic species by Ng et al. (1981). This classification is supported by results from RFLP analysis (Wang et al. 1992) and RAPD analysis (Martin et al. 1997).

Hybridization studies revealed that *O. meridionalis* is isolated by an F_1 sterility barrier from *O. nivara* and *O. rufipogon* (Naredo et al. 1997) and the New World diploid *O. glumaepatula* (Naredo et al. 1998). However, it shares the AA genome with species in Ser. *Sativae* as revealed by high bivalent formation and normal meiosis in interspecific hybrids indicative of high genomic affinity (Lu et al. 1997, 1998).

Although morphological variation in *O. meridionalis* has been observed from field surveys, herbarium specimens, and routine characterization of germplasm collections, the genetic variation patterns of the species across its distribution range in northern Australia and Irian Jaya remain unknown. Morphological characterization is a straightforward tool for the study of variation in plants that has remained an important part of genetic diversity studies of germplasm materials, and it has been used to estimate variation in wild rice populations (Morishima 1969; Juliano et al. 1998). The use of molecular tools such as PCR-based markers like RAPD (random amplified polymorphic DNA) and microsatellite or SSR (simple sequence repeats) involves a quick and simple procedure requiring small amounts of DNA to detect high levels of polymorphism. In rice conservation research, the RAPD technique has provided useful insights into variation patterns, thus leading to a better estimate of diversity in *O. sativa* (Mackill 1995; Virk et al. 1995; Fuentes et al. 1999) and its wild relatives (Martin et al. 1997; Buso et al. 1998; Ge et al. 1999; Qian et al. 2001). SSR analysis that involves the use of co-dominant markers with known map positions has been used to detect DNA polymorphism and genetic diversity in *Oryza* (Akagi et al. 1997; Olufowote et al. 1997; Davierwala et al. 2000; Ni et al. 2002). However, its use in detecting intraspecific diversity in wild *Oryza* species has yet to be established.

The objectives of this study were to detect variation patterns in *O. meridionalis* using RAPD and SSR markers and to determine the genetic relationships among the *O. meridionalis* populations from intraspecific crosses.

Materials and methods

Establishment of plant materials

Seeds of 40 accessions of *O. meridionalis* (Table 1) from the International Rice Genebank Collection at IRRI covering the geographical distribution of the species in northern Australia and Irian Jaya (Figure 1) were used in this study. Except for the materials from Irian Jaya (represented by original seeds), the other accessions had all been rejuvenated at the International Rice Genebank. The taxonomic identity of each accession was confirmed following Vaughan (1989) and a comparison was made with isotype herbarium sheets made from the genebank accession (IRGC 101147) from which the original description of *O. meridionalis* by Ng et al. (1981) came. The 40 accessions were morphologically compared with three IRG accessions each of *O. nivara* and *O. rufipogon* (Table 2) and scored at appropriate growth stages for morphological characters including that of the leaf, culm, panicle, and spikelet.

Prior to germination, the seeds underwent hot-air treatment of 50 °C for 5 days to break dormancy. Hulled seeds were germinated on sterilized Petri dishes lined with moist filter paper under controlled conditions of 31 °C, 99% RH, and 12/12 h light. Germinated seeds were transferred to culture solution (Yoshida et al. 1976) and maintained under glasshouse conditions of 21/29 °C and 70% RH. The seedlings were transplanted singly in 12-inch diameter pots at 24 days after seeding and maintained in the IRG screenhouse. The plants were provided with maximum pest and disease control measures throughout the duration of the experiment.

RAPD and microsatellite analyses

DNA was extracted as described by Virk et al. (1995) pooled from four plants per accession, except for those from Irian Jaya, which were represented by one to 10 plants. DNA concentration was estimated by comparing with λ DNA in 0.7% agarose after electrophoresis in 0.5×TBE buffer. For

Table 1. O. meridionalis accessions used in molecular and hybridization studies.

Code	IRGC accession number	Source		Collector's notes on habitat/population structure	RAPD	SSR	Hybridization
		Country	Province				
M1	101145	Australia	Northern Territory		X	X	X
M2	101146	Australia	Northern Territory		X	X	X
M3	101147	Australia	Northern Territory		X		X
M4	101148	Australia	Northern Territory		X		
M5	101466	Australia	Northern Territory		X	X	
M6	103317	Australia	Queensland		X	X	X
M7	103319	Australia	Northern Territory		X		X
M8	103320	Australia	Unknown		X		X
M9	103321	Australia	Unknown		X	X	X
M10	103322	Australia	Northern Territory		X		
M11	104085	Australia	Northern Territory	Open shade, 0.3 m water depth	X		
M12	104086	Australia	Northern Territory	Partial shade, 0.3 m water depth	X		
M13	104089	Australia	Northern Territory	0.3 m water depth	X	X	X
M14	104092	Australia	Northern Territory		X	X	X
M15	104093	Australia	Northern Territory	0.4 m water depth	X		X
M16	104498	Australia	Northern Territory		X	X	X
M17	105279	Australia	Western Australia	Marshy place 0 to 1 m high water	X	X	
M18	105281	Australia	Western Australia	Marshy fringe of Lake Kununurra, under partial shade	X		
M19	105282	Australia	Northern Territory	Large marshy area disturbed by pigs and buffaloes	X	X	
M20	105287	Australia	Queensland	Farm pastures near salty area	X	X	X
M21	105288	Australia	Queensland	Same as M20	X		X
M22	105289	Australia	Queensland	Swampy area	X	X	X
M23	105290	Australia	Queensland	Irrigation canal near an entirely invaded field	X		X
M24	105294	Australia	Queensland	Swampy area; occurs with *O. rufipogon*	X	X	X
M25	105295	Australia	Queensland	Swampy canal, short plants	X	X	X
M26	105298	Australia	Queensland	Large pool in full sunshine; plants intermediate between the annual and perennial type; heterogeneous population	X	X	X
M27	105299	Australia	Queensland	Swampy area half shaded by *Eucalyptus* trees	X		X
M28	105300	Australia	Queensland	Swampy area	X	X	X
M29	105301	Australia	Queensland	Permanent water hole	X		X
M30	105302	Australia	Queensland	Small dried-up, much-disturbed swamp	X	X	X
M31	105304	Australia	Queensland	Swamp under shade	X		X
M33	105306	Australia	Queensland	Small, much-disturbed swamp	X	X	X
M34	105598	Australia	Northern Territory		X		
M35	IJW-003	Indonesia	Irian Jaya	Swamp	X	X	X
M36	IJW-004	Indonesia	Irian Jaya	Deserted rice field; very dry	X	X	X
M37	IJW-005	Indonesia	Irian Jaya	Canal	X	X	X
M38	IJW-007	Indonesia	Irian Jaya	Deserted rice field; dry land	X	X	X
M39	IJW-008	Indonesia	Irian Jaya	Swamp	X	X	X
M40	IJW-013	Indonesia	Irian Jaya	Swamp; near *O. sativa* field	X	X	X
M41	IJW-023	Indonesia	Irian Jaya	Swamp	X	X	X

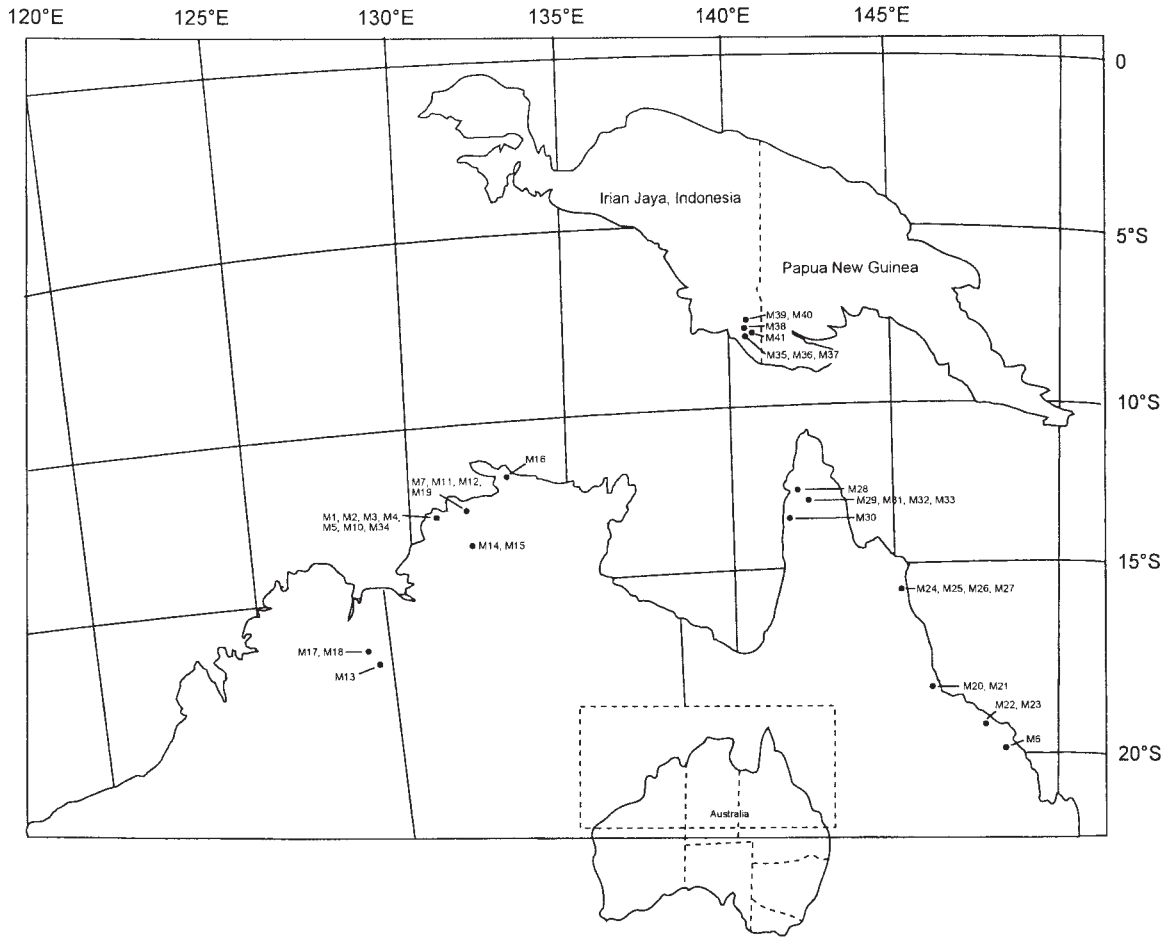

Figure 1. Distribution in northern Australia and Irian Jaya of *O. meridionalis* accessions used in molecular and hybridization studies.

RAPD analysis, PCR amplification was performed in a Hybaid Omnigene thermocycler as described by Martin et al. (1997).

A subset consisting of 25 accessions indicated in Table 1 was included for microsatellite analysis. Primer pairs for 20 microsatellite markers were initially screened for the quality of DNA patterns. PCR reactions were carried out in 25-μL volumes containing 50 ng DNA, 0.2 μM each of the primer (forward and reverse), 1× PCR buffer (100 mM Tri–HCl, 500 mM KCl, 0.1% gelatine), 1 mM $MgCl_2$, 0.1 mM dNTPs, and 1 unit *Taq* polymerase. Amplification was performed under the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and an extension of 5 min at 72 °C. Amplification products were mixed with loading dye, denatured at 95 °C and run for 180 min on ReproGel™ using ALF Express™. A 50–500-bp external sizer and appropriate internal sizer were used for band sizing.

Intraspecific hybridization

Intraspecific crosses, establishment of hybrids, and the derivation of pollen stainability and panicle fertility were achieved as described by Naredo et al. (1998). The hybrid status of the F_1 plants was established by morphological characterization and/or SSR analysis.

Data analysis

Cluster analysis was carried out on the dissimilarity matrix of Euclidian distances based on 23 quantitative morphological characters to generate a dendrogram by the unweighted pair group method

Table 2. *O. nivara* and *O. rufipogon* accessions included as test samples in morphological validation.

Species	Code	IRGC accession number	Source	
			Country	Province
O. nivara	N1	101967	India	Orissa
	N2	103821	China	Guandong
	N3	105391	Thailand	Central Thailand
O. rufipogon	R1	104714	Thailand	Ayutthaya
	R2	105402	China	
	R3	105953	Indonesia	West Java

with arithmetic mean (UPGMA) using NTSYS-pc (Rohlf 1994). The characters were standardized prior to analysis.

RAPD and SSR band patterns were scored for presence or absence in each sample. Cluster analysis was carried out on a similarity matrix of Jaccard coefficient based on the combined data from RAPD and microsatellite markers to produce a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA) using NTSYS-pc (Rohlf 1994). Statistical support of the branches was tested with a bootstrap analysis using the software program WinBoot developed at IRRI (Yap and Nelson 1996) with 5000 data resamples.

Results

Validation of samples as O. meridionalis

Cluster analysis was performed to validate the taxonomic status of the 40 accessions identified as *O. meridionalis* with *O. rufipogon* and *O. nivara* serving as test species. The UPGMA dendrogram in Figure 2 shows that at a dissimilarity level of 5.96, the *O. meridionalis* accessions formed a cluster highly distinct from the *O. rufipogon* and *O. nivara* accessions.

RAPD and SSR analyses

The 12 RAPD primers produced a total of 173 RAPD fragments that ranged from 305 to 3277 bp in size. Table 3 shows that the number of bands scored per primer ranged from eight (OPA-15) to 23 (OPD-10). Band polymorphism ranged from 36.4% (OPA-15) to 85.0% (OPA-11). A total of 119 bands were shown to be polymorphic.

Amplification products for nine SSR primers (RM2, RM3, RM9, RM13, RM14, RM16, RM17, RM122, and RM168) were poorly resolved. The 11 primers listed in Table 3 that cover six of the 12 chromosomes of the rice genome produced clear and distinct bands and produced a total of 67 alleles. The number of alleles per primer ranged from three to 13. The average PIC value was 0.62 and ranged from 0.44 (RM6) to 0.90 (RM1).

The similarity matrix of Jaccard coefficient derived from 119 RAPD bands and 67 SSR alleles showed an average similarity index of 41.2%. No variation was detected in the accession pairs M17 and M18 from Western Australia and M22 and M23 from Queensland, while the lowest similarity index (10.8%) was observed between M1 (Northern Territory) and M40 (Irian Jaya).

Figure 3 shows that at a similarity level of 0.443 distinct clusters were formed by the accessions coming from Irian Jaya (cluster 1), Queensland (cluster 2), and Queensland, the Northern Territory and Western Australia (cluster 3). The high bootstrap *P* values especially for cluster 1 (100.0%) and cluster 2 (94.4%) indicate that *O. meridionalis* has distinct populations defined by their geographic distribution.

It is of interest to note that, at a higher similarity level of 0.551, differences in latitude and longitude resulted in the differentiation of accessions with common origins. For instance, M41 collected at 140°50′E longitude diverged from the other Irian Jaya accessions collected between140°27′E and 140°33′E longitude. The cluster formed by the Queensland accessions collected between 12°40′S and 18°50′S latitudes was distinct from that formed by the accessions M23, M22, and M6 collected from higher latitudes (20°00′S and 20°35′S). Among the accessions from the Northern

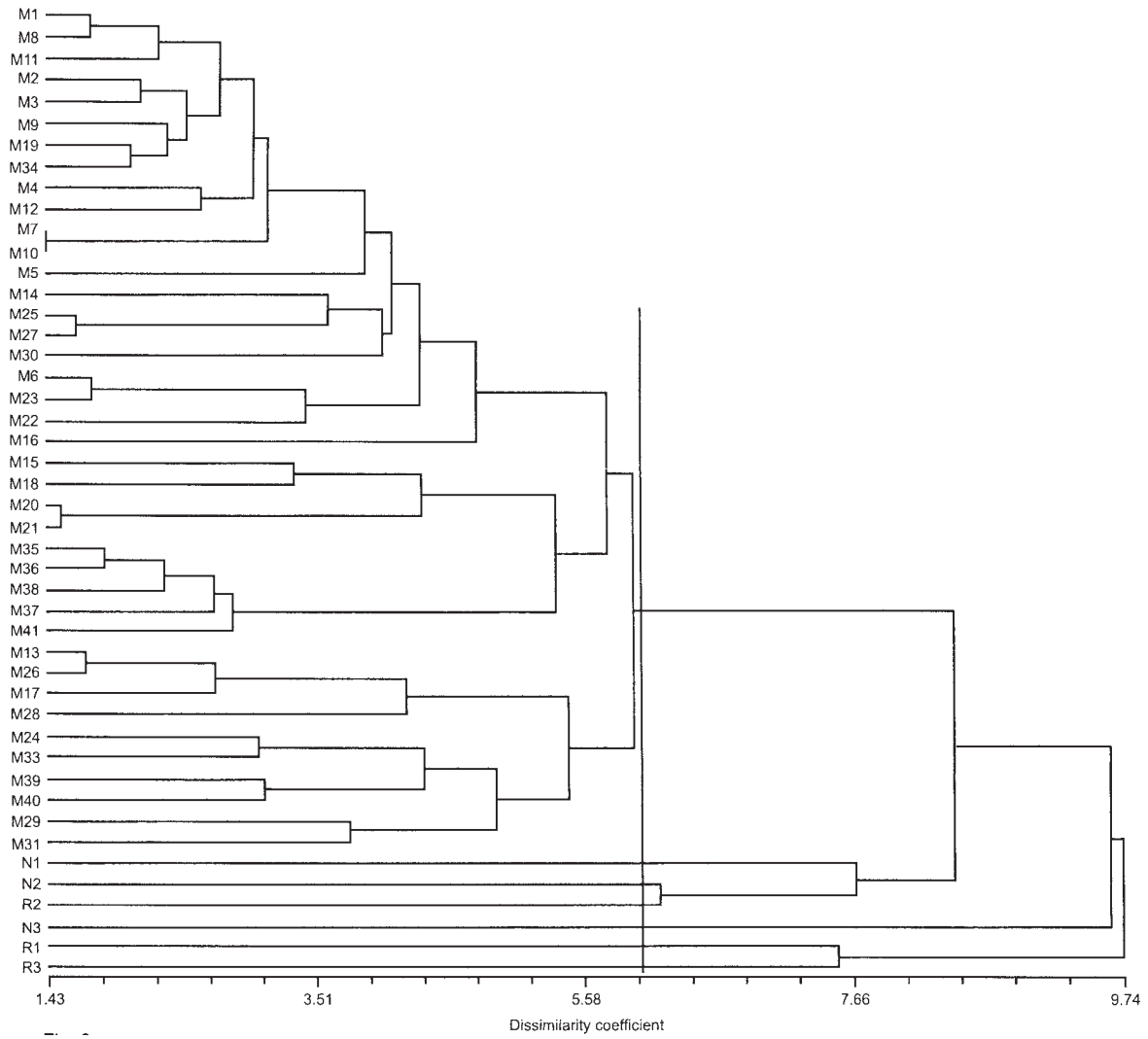

Figure 2. UPGMA dendrogram obtained from a dissimilarity matrix of Euclidian distances based on 23 quantitative characters. *O. nivara* (N1 to N3) and *O. rufipogon* (R1 to R3) were included as test species.

Territory, M14 and M15 collected at 13°34′S latitude diverged from the other accessions collected between 12°23′S to 12°35′S latitude. The accessions from Western Australia, M17 and M18, and the Northern Territory accession, M13, collected at 15°51′S and 15°53′S latitudes, respectively, clustered with M26 from Queensland.

Intraspecific hybridization

A total of 14 588 spikelets were pollinated for 111 crosses involving 31 *O. meridionalis* accessions. About 92% of the crosses produced seeds with a mean seed set of 23.9%. Hybrids were obtained from 64% of the crosses. In general, the intraspecific hybrids developed into normal and vigorous plants, except for those derived from the crosses M2×M35 and M2×M39, which showed weakness at the vegetative stage and died before flowering. M2 is from the Northern Territory while M35 and M39 are from Irian Jaya.

Pollen stainability and panicle fertility of the intraspecific hybrids provided similar measures of hybrid fertility (Table 4). Pollen stainability ranged from 1.0% shown by the Northern Territory–Irian

Table 3. RAPD primers and SSR markers used to detect genetic variation in *O. meridionalis*.

RAPD analysis				SSR analysis				
Primer	Sequence (5′ to 3′)	No. of bands scored	Polymorphic bands (%)	Marker	SSR motif (in IR36)	Chrom. location	No. of alleles detected	PIC[a]
OPA-09	GGGTAACGCC	13	61.5	RM1	$(GA)_{26}$	1	13	0.90
OPA-11	CAATCGCCGT	20	85.0	RM5	$(GA)_{15}$	2	10	0.84
OPA-14	TCTGTGCTGG	9	66.7	RM6	$(GA)_{16}$	1	3	0.44
OPA-15	TTCCGAACCC	8	36.4	RM 11	$(GA)_{17}$	3	4	0.46
OPC-03	GGGGGTCTTT	16	37.5	RM 12		12	3	0.46
OPC-06	GAACGGACTC	21	80.9	RM 18	$(GA)_{15}$	3	8	0.78
OPC-12	TGTCATCCCC	10	70.0	RM 19	$(ATC)_{10}$	5	4	0.45
OPC-13	AAGCCTCGTC	16	62.5	RM 20	$(ATT)_{14}$	11	4	0.51
OPD-09	CTCTGGAGAC	10	70.0	RM148	$CTCTAT(GT)_{12}TTT$	3	3	0.61
OPD-10	GGTCTACACC	23	78.3	RM164	$(GT)_{16}TT(GT)_4GAG$	5	8	0.76
OPD-15	CATCCGTGCT	13	76.9	RM167	$GGAA(GA)_{16}GGGG$	11	7	0.67
OPK-06	CACCTTTCCC	14	64.3					

[a] $PIC = 1-\Sigma(P_i)^2$, where P is the proportion of the population with the ith allele, for each microsatellite locus.

Jaya cross M15×M41 to 96.7% shown by the Queensland–Queensland cross, M25×M28. Panicle fertility varied from 0% shown by the Northern Territory–Queensland crosses M14×M22 and M15×M23 and the Queensland–Queensland cross M26×M28 to 96.7% shown by the Irian Jaya–Irian Jaya cross M40×M37.

In general, crosses between accessions with common origin produced fertile hybrids. For instance, crosses among the Irian Jaya accessions produced only fertile hybrids with >75% pollen stainability and >70% panicle fertility. Fertile hybrids were likewise obtained from the reciprocal crosses between the Northern Territory accessions M1 and M16 and the cross M14×M13 and partially fertile hybrids from the reciprocal crosses between M15 and M13. M15 crossed to M1 and M7, however, produced highly sterile hybrids. Fertile hybrids were obtained from crosses among the Queensland accessions, except for those that involved M26, which produced highly sterile hybrids.

In crosses between accessions with different origins, sterile to fertile hybrids were obtained from the Irian Jaya and Queensland crosses. Reciprocal crosses of the Queensland accession M26 with the Northern Territory accessions M7 and M15 produced partially fertile hybrids. Crosses between accessions from the Northern Territory and Irian Jaya and most of the Northern Territory and Queensland crosses produced sterile hybrids.

The crossing polygon (Figure 4) summarizes the crossability in *O. meridionalis* from the Northern Territory, Queensland, and Irian Jaya based on the panicle fertility of the hybrids.

Discussion

The study revealed the utility of SSR and RAPD markers in detecting genetic variation in wild *Oryza* species. Molecular marker analysis strongly defined population divergence according to geographic distribution as shown by the differentiation of the Irian Jaya accessions from those collected from northern Australia. In northern Australia, an east–west variation pattern was observed among accessions from Queensland, the Northern Territory, and Western Australia. Latitudinal variation was pronounced among the Queensland accessions where populations from 20°00′ to 20°35′S latitudes diverged from those collected from lower latitudes. Among the Northern Territory accessions, those collected at latitude 13°34′S differed from those collected at latitudes 12°00′S to 12°35′S. Both patterns of variation are significant, since, in Australia, rainfall tends to decrease inland with distance from the coast and climatic differences assigned to latitude generally

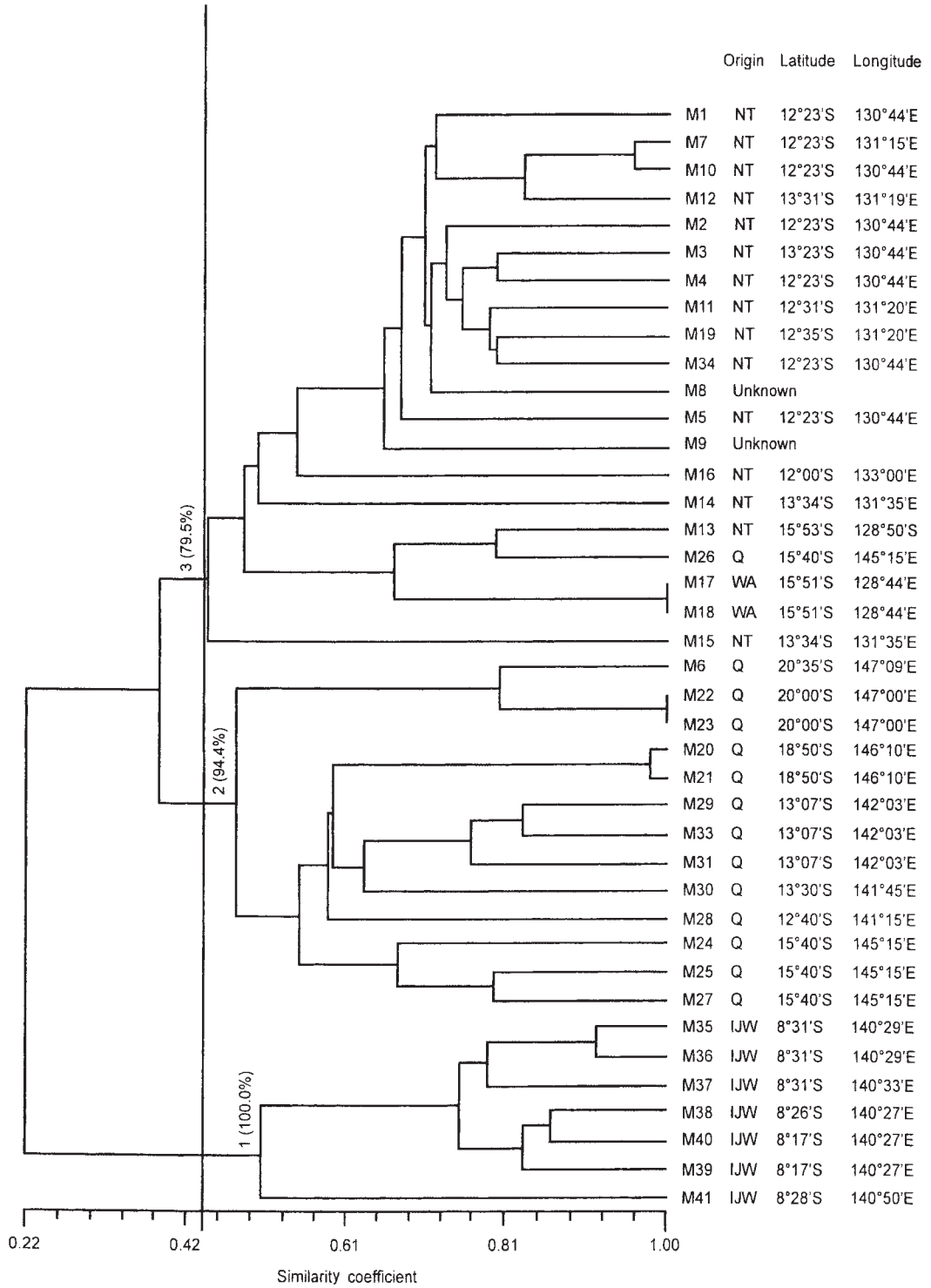

Figure 3. UPGMA dendrogram obtained from a similarity matrix of Jaccard coefficient based on 119 RAPD and 67 SSR markers. Bootstrap *P* values for main clusters are enclosed in parentheses.

Table 4. Pollen stainability and panicle fertility of *O. meridionalis* intraspecific hybrids.

Cross	Pollen stainability (%)	Panicle fertility (%)
Irian Jaya–Irian Jaya		
M35×M38	91.7	87.8
M35×M39	96.0	74.8
M35×M40	90.0	78.8
M37×M40	75.3	70.8
M38×M35	92.3	87.7
M39×M35	95.5	77.2
M39×M37	91.7	94.8
M39×M40	91.6	88.9
M40×M35	91.9	81.5
M40×M37	86.2	96.7
M40×M39	95.4	92.5
Northern Territory–Northern Territory		
M1×M15	6.3	5.3
M1×M16	83.7	82.5
M7×M15	16.2	6.2
M13×M15	56.0	37.4
M14×M13	66.7	59.9
M15×M13	45.2	29.1
M15×M7	8.4	2.8
M16×M1	83.9	85.3
Queensland–Queensland		
M6×M22	96.0	60.5
M6×M23	96.6	55.6
M22×M23	95.9	53.3
M23×M6	94.8	68.8
M25×M28	96.7	63.3
M26×M25	1.8	0.7
M26×M28	–[a]	0
M28×M25	94.9	66.9
M30×M25	38.3	35.0
M30×M26	3.8	2.6
Irian Jaya–Queensland		
M24×M36	16.1	13.1
M24×M38	19.8	6.3
M24×M39	20.5	20.2
M25×M35	10.2	14.5
M29×M37	80.7	72.1
M29×M40	69.1	60.4
M31×M35	35.4	14.6
M31×M38	75.2	30.1
M33×M37	95.6	74.4
M35×M24	–	24.2
M35×M25	14.6	16.5
M36×M24	20.5	15.1
M36×M25	8.7	3.9
M37×M25	56.5	42.6
M37×M29	65.9	55.5
M38×M24	14.9	12.7
M38×M31	49.3	33.4
M40×M29	60.4	45.1
Northern Territory–Irian Jaya		
M1×M36	18.9	3.3
M15×M41	1.0	6.5
Northern Territory–Queensland		
M1×M30	22.5	9.0
M3×M25	9.7	2.4
M3×M30	22.6	4.6
M6×M14	6.2	0.2
M7×M26	29.8	40.2
M14×M22	5.4	0
M14×M23	2.8	1.1
M15×M23	7.8	0
M15×M25	1.6	0.7
M15×M26	48.9	34.3
M16×M25	9.8	4.9
M22×M14	4.0	0.4
M23×M14	3.8	0.9
M25×M15	1.2	0.5
M25×M16	11.4	2.4
M26×M15	54.8	36.6
M26×M7	27.1	40.0
M28×M16	16.5	4.0

[a] Not observed.

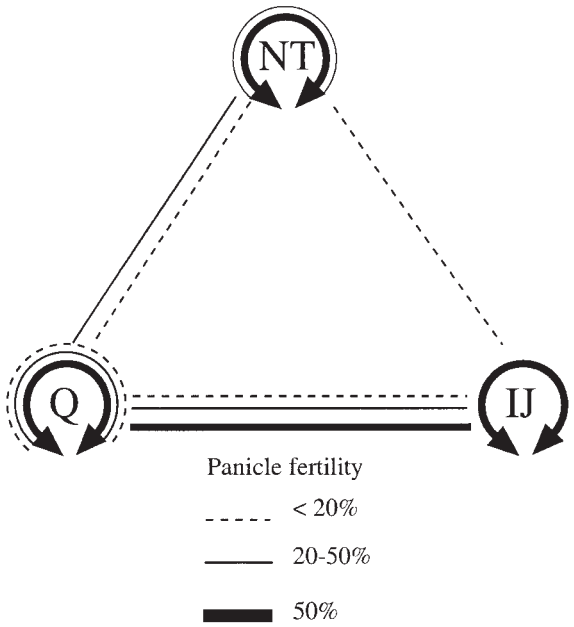

Figure 4. Crossing polygon based on panicle fertility of hybrids summarizing genetic relationships in *O. meridionalis* populations. Note that crosses among the Irian Jaya (IJ) accessions produced only fertile hybrids. In contrast, fertile to sterile hybrids were obtained from among the Northern Territory (NT) or Queensland (Q) accessions.

influence rainfall (McQueen 1990). This also explains the seemingly disjunct distribution of *O. meridionalis* in northern Australia, where population distribution coincides with areas of sufficient rainfall, a habitat requirement. However, Second (1988) suggested that the area around the Kimberly Plateau in Western Australia should be explored more carefully for forms that might be adapted to extreme aridity.

The differentiation patterns in *O. meridionalis* detected by molecular analysis are accompanied by reproductive isolation in the form of F_1 hybrid sterility and, to a lesser extent, hybrid weakness. This is not the case in *O. barthii*, for which intraspecific crosses between the morphologically distinct weedy and truly wild forms produced fertile hybrids (unpublished data). In the case of *O. meridionalis*, the development of reproductive barriers can be the result of the geographical disjunction (both latitudinal and longitudinal) of the populations since those located within a common geographic area (e.g., the Irian Jaya populations) produced only fertile hybrids. The relative fertility of hybrids produced from crosses between accessions from Queensland and Irian Jaya provides evidence for gene flow among these populations possibly effected by the migration of birds that feed on seeds of *O. meridionalis* (Lu 1999b).

The study also revealed the differentiation of populations with common geographic origin. For instance, molecular analysis showed M15 to be highly diverged from the other Northern Territory accessions. M26 diverged from the other accessions from Queensland but closely clustered with M13, M17, and M18 from Western Australia. M26 is described in passport data as a diverse population that was 'a somewhat intermediate type between annual and perennial' (Second 1988). Interestingly, crosses of M15 and M26 produced partially fertile hybrids. This suggests that speciation in *O. meridionalis* appears to proceed with no defined changes in morphology because these populations formed a morphologically cohesive group with the other *O. meridionalis* accessions, distinct from both *O. nivara* and *O. rufipogon*.

Evidence obtained from molecular and hybridization studies, indicates that differentiation in *O. meridionalis* largely corresponds to its geographic distribution. From the genetic variation patterns produced in the study, we propose that *O. meridionalis* be retained as a distinct taxon, composed of populations undergoing gradual speciation.

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