Genetic variation and population structure in *Oryza malampuzhaensis* Krish. *et* Chand. endemic to Western Ghats, South India

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Abstract

Oryza malampuzhaensis Krish. *et* Chand. ($2n = 4x = 48$; Poaceae, *Oryza*) is endemic to Western Ghats, South India, and shows a highly localized distribution over a small geographical area in this region. This is the most poorly understood taxon in genus *Oryza* and is often misidentified as *O. officinalis* owing to their close morphology. We assessed the nature and distribution of genetic variation among 11 populations of *O. malampuzhaensis* using random amplified polymorphic DNA markers. The analysis revealed low genetic variation in *O. malampuzhaensis*. Cluster analysis of pairwise genetic distances of populations revealed three distinct clusters and the grouping of populations largely corresponded to their geographical proximity. Restricted gene flow and a geography-dependent differentiation were evident among populations. The altitude-influenced differences in ecological factors among the natural habitats of the populations seem to be the cause of the geography-dependent differentiation. Genetically isolated smaller populations and a narrow genetic base in *O. malampuzhaensis* point to its vulnerability to genetic drift and genetic depauperation. Thus *O. malampuzhaensis* appears to be under the threat of extinction and needs to be conserved by use of suitable methods. The present study also identified molecular markers diagnostic for *O. malampuzhaensis*.

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Introduction

The genus *Oryza* consists of approximately 21 wild and two cultivated species (Khush 1997). The wild *Oryza* species can offer valuable genetic resources, not found in cultigens, for rice improvement programmes (Swaminathan 1986; Xiao *et al*. 1996). A few of them have been introgressed into cultivated backgrounds, and have contributed substantially to increasing rice production (Brar and Khush 1997). Characterization and conservation of wild genetic resources of rice therefore has great importance in future rice improvement.

The Western Ghats region of South India is rich in genetic diversity of *Oryza* and has *O. rufipogon*, *O. nivara*, *O. granulata* and *O. officinalis* (Vaughan 1989a; Vaughan and Sitch 1991). The International Rice Research Institute (IRRI) has identified the Bhoothathankettu, Parambikulam and Karulai forest reserve along the Western Ghats in Kerala for *in situ* conservation of these species (Vaughan 1989a; Vaughan and Sitch 1991). In 1958 a tetraploid wild rice closely resembling the diploid *O. officinalis* was reported from two localities in Malampuzha in Kerala (Krishnaswamy and Chandrasekharan 1958). This taxon is endemic and has a localized distribution along the Western Ghats, particularly in forest reserves (Krishnaswamy and Chandrasekharan 1958; Vaughan 1989b; Joseph *et al*. 1999). There is a lot of disagreement over the phylogenetic affinity of this taxon and it has been treated variously: as a new species, *O. malam-*

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puzhaensis (Krishnaswamy and Chandrasekharan 1958), as a subspecies of *O. officinalis* (Tateoka 1963), and as a tetraploid race of *O. officinalis* (Vaughan 1994). The confusion over the taxonomic affinity of *O. malampuzhaensis* may have partly arisen from the use of limited samples, usually a single accession, in all these studies.

We were attracted to *O. malampuzhaensis* because of the different views on its taxonomy and its importance as the only wild rice endemic to the biodiversity-rich Western Ghats. Besides, *O. malampuzhaensis* is one of the least studied taxa in the genus *Oryza*, and no information is available on its population-genetic structure. The importance of *O. malampuzhaensis* has been strengthened following the recent report that it is a source of resistance to sheath blight disease (Kaushal *et al*. 1998), which causes 10–27% annual crop loss in rice in India (Baker *et al*. 1997). The major hindrance for a thorough study on *O. malampuzhaensis* was the limited collections in international germplasm banks (Vaughan 1989b). In view of this, an extensive expedition was conducted by one of us (Philomena Kuriachan) along the Western Ghats during 1994–96 and a large sample of *O. malampuzhaensis* was collected (Joseph *et al*. 1999). Recently, using these collections, we examined the genetic relationship between *O. malampuzhaensis* and *O. officinalis* and concluded that *O. malampuzhaensis* has diverged enough to deserve a separate species status (Thomas *et al*. 2001).

The goal of this study was to investigate the level of genetic variation within and among natural populations of *O. malampuzhaensis* following random amplified polymorphic DNA marker (RAPD) analysis. Another unresolved problem associated with this taxon is the difficulty in distinguishing it from *O. officinalis*, since the two taxa are close morphologically (Vaughan 1989b; Joseph *et al*. 1999). Therefore the present work also envisaged development of molecular markers diagnostic for *O. malampuzhaensis*.

Materials and methods

***Plant materials*:** *O. malampuzhaensis* is a rhizomatous perennial and propagated through suckers. Its seeds are highly recalcitrant. Material for the study was collected from five locations of the southern Western Ghats (table 1). Distances between the collection sites are given in table 2. Populations from the same collection site were ~ 500 m apart. *O. malampuzhaensis* is represented in scattered smaller populations. A detailed account of the survey conducted earlier for this taxon is reported elsewhere (Joseph *et al*. 1999). The plant materials used in the study are maintained at Rajiv Gandhi Centre for Biotech-

Table 1. Genotypes of *O. officinalis* and *O. malampuzhaensis* used in the study together with their accession number, source and country of origin.

Code	Accession number	Source*	Origin/ collection site	Altitude (m)	Number of samples used
O. officinalis (2*n* = 24; CC)					
1	IRGC 101152	NBPGR, Thrissur, India	Brunei		
2	IRGC 105220	IARI, New Delhi, India	Indonesia		
3	IRGC 104707	IRRI	Gujarat, India		
4	IRGC 100178	NBPGR, Thrissur, India	Thailand		
5	IRGC 105376	NBPGR, Thrissur, India	Thailand		
O. malampuzhaensis (2*n* = 48; BBCC)					
6	KUBOT 10409**	Dept of Botany, Univ. of Kerala	Nilambur	110	1
7	KUBOT 10410	Dept of Botany, Univ. of Kerala	Vellani	400	3
8	KUBOT 10411	Dept of Botany, Univ. of Kerala	Vellani	400	1
9	KUBOT 10402	Dept of Botany, Univ. of Kerala	Peechi	300	1
10	KUBOT 10403	Dept of Botany, Univ. of Kerala	Peechi	250	3
11	KUBOT 10412	Dept of Botany, Univ. of Kerala	Nelliampathy	1100	2
12	KUBOT 10408	Dept of Botany, Univ. of Kerala	Parambikulam	1200	1
13	KUBOT 10406	Dept of Botany, Univ. of Kerala	Parambikulam	1000	3
14	KUBOT 10404	Dept of Botany, Univ. of Kerala	Parambikulam	1000	3
15	KUBOT 10407	Dept of Botany, Univ. of Kerala	Parambikulam	1000	3
16	KUBOT 10405	Dept of Botany, Univ. of Kerala	Parambikulam	1000	1

*IRRI, International Rice Research Institute; IARI, Indian Agricultural Research Institute; NBPGR, National Bureau of Plant Genetic Resources.
**KUBOT, Kerala University Botany Department.

nology, Thiruvananthapuram. Voucher specimens of *O. malampuzhaensis* collections are deposited in the herbarium of the Kerala University Botany Department.

***DNA isolation and PCR analysis*:** Total genomic DNA was isolated from tender leaves following the procedures of Sharp *et al*. (1988). The primers for PCR amplification were obtained from Operon Technologies (Alameda, USA). PCR was carried out in 20-µl reaction volume containing 25 ng of genomic DNA, 1 U *Taq* DNA polymerase (Bangalore Genei, India), 5 pmol primer, 0.2 mM dNTPs, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin. Amplification was carried out in a thermocycler (Perkin Elmer 480) with an initial strand separation at 94°C for 4 min, followed by 40 cycles of 1 min at 94ºC, 1 min at 37°C and 1.5 min at 72°C. After 40 cycles, there was a final extension step of 5 min at 72°C. Amplification products were fractionated on 1.4% agarose gels and were detected by staining with ethidium bromide.

***Data collection and statistical analysis*:** Amplification with each primer was repeated thrice and clearly resolved reproducible fragments were considered in data collection. Each RAPD fragment was treated as a unit character and was scored as 1 (present) or 0 (absent). The 1/0 matrix was prepared for all the fragments scored and was used to calculate pairwise genetic distances following Nei and Li (1979). Clusters were constructed following the UPGMA (unweighted pair group method using arithmetic average) method using the software package POPGENE version 1.21 (Yeh and Boyle 1997). The robustness of the dendrogram was tested by bootstrap analysis using the software package WINBOOT developed at IRRI (Yap and Nelson 1996). The RAPD and geographic distances were compared pairwise between populations by means of Pearson's correlation coefficient.

Gel elution and Southern hybridization: The PCR-amplified products were fractionated on agarose gel and vacuum blotted (vacuum blotter, Bio-Rad) onto nylon membrane (Hybond N^+, Amersham-Pharmacia) according to manufacturer's instructions. The selected PCR product was eluted from the gel using GFX^{TM} PCR DNA and Gel Band Purification kit (Amersham-Pharmacia), and was radiolabelled using the Prime-a-Gene labelling system (Promega). Southern hybridization was carried out as reported earlier (Thomas *et al*. 2000).

Table 2. Distances between the collection sites of *O. malampuzhaensis* populations.

	Distance (km)			
Population	Vi	Pi	Ny	Pm
Nr	76	83	100	110
Vi		8	37	50
Pi			32	45
Ny				14

Nr, Nilambur; Vi, Vellani; Pi, Peechi; Ny, Nelliampathy; Pm, Parambikulam.

Results

DNA fingerprinting

A total of 33 decamer primers randomly selected from C, E, F, J and AE kits were tested for their efficiency in generating reproducible amplification products in *O. malampuzhaensis* and *O. officinalis*. Of the 33 primers, 14 yielded clearly resolved reproducible amplification products among the accessions of *O. malampuzhaensis* with at least one polymorphic band. Individuals belonging to the same population exhibited practically no RAPD polymorphism among themselves. The subsequent analysis was therefore limited to only one representative individual from each population. The banding profiles yielded by the 14 selected primers were used for genetic analysis of *O. malampuzhaensis*. A representative RAPD pattern generated by OPC 14 is shown in figure 1. The 14 primers together yielded 87 fragments, of which 25 were polymorphic (table 3). Eight amplification products (32% of total polymorphism) were specific to the Nilambur population whereas no polymorphism was observed among the three populations of Parambikulam (codes 13, 14, 15).

Genetic distance

The pairwise genetic distance matrix was prepared on the basis of RAPD data (table 4). The genetic distance varied

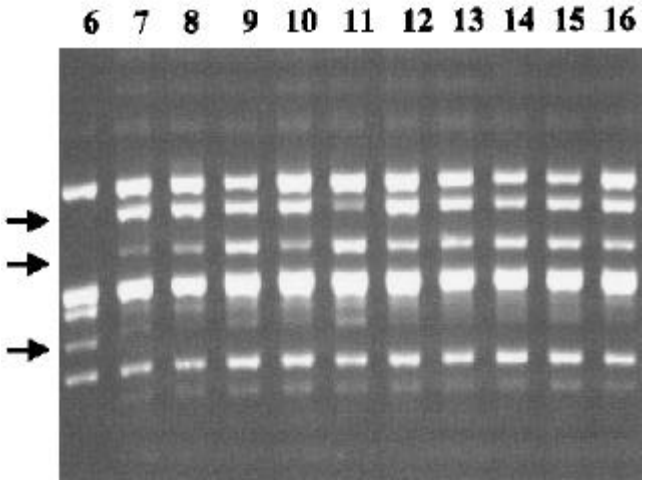

Figure 1. An example of RAPD pattern of *O. malampuzhaensis* produced using the random 10-mer primer OPC 14. Lane number corresponds to the population code given in table 1. The arrows indicate the polymorphic bands.

from 0 to 0.124 with a mean value of 0.046. The Nilambur population was clearly distinct from other populations. The four populations from Vellani and Peechi showed relatively higher distances among themselves and from other populations. Except for population 12, the populations from Nelliampathy and Parambikulam displayed comparatively lower distances among themselves. The Pearson test revealed significant ($P < 0.01$) correlation between RAPD and geographic distances ($r = 0.668$).

Cluster analysis

Cluster analysis was performed on RAPD data using UPGMA to generate a dendrogram showing overall genetic relationships among the populations of *O. malampuzhaensis* (figure 2). Three distinct clusters can be identified. The first cluster comprises four populations from Parambikulam (codes 13–16), one of the populations from Vellani (code 8) and the Nelliampathy population (code 11). The next cluster is formed by the populations from Peechi (codes 9, 10), and one population each from Vellani (code 7) and Parambikulam (code 12). The population from Nilambur (code 6) did not cluster with any other population and remains separate by itself.

Except for populations 8 and 12, populations within a cluster were geographically closer (table 2) than populations in different clusters. The geographically closer populations that clustered together were from similar altitudinal habitats. Population 6 is from a low altitude (110 m) whereas the Vellani (codes 7, 8) and Peechi (codes 9, 10) populations as a group are from a middle elevation with an average altitude of 330 m (derived from table 1). The Nelliampathy and Parambikulam populations are from a high altitude with an average altitude of 1050 m (derived from table 1). Since the populations showed a tendency to cluster according to their geographical proximity and altitudinal habitat, we examined the relationship between the means of the genetic distances of populations belonging to similar altitudinal levels. For this, the mean of the pairwise genetic distances of each population was first calculated; these means were then used to compute the mean genetic distance of populations belonging to similar altitudinal levels. The values obtained for low, middle and high altitudes are 0.115 ± 0.006, 0.046 ± 0.004 and 0.035 ± 0.005 respectively. These values differ significantly ($P < 0.05$). The mean pairwise distance of populations shows a decrease with increasing altitude (figure 3).

Table 3. List of primers used for the RAPD analysis of *O. malampuzhaensis* and the total and polymorphic amplification products yielded by each primer.

	Amplification products	
Primer	Total	Polymorphic
OPE 1	5	1
OPE 2	7	3
OPE 3	8	2
OPE 11	6	1
OPF 9	6	1
OPF 10	5	1
OPF 13	6	1
OPF 15	5	1
OPC 14	9	4
OPC 15	9	3
OPC 16	3	1
OPJ 9	8	3
OPJ 19	5	1
OPAE 8	5	2
Total	87	25

Development of O. malampuzhaensis-specific RAPDs

To identify RAPDs diagnostic for *O. malampuzhaensis*, the amplification products generated by the 33 random primers were scrutinized for the amplicons that are present in all the accessions of *O. malampuzhaensis* but absent in all the accessions of *O. officinalis*. Several such fragments were identified (table 5). However, we selected only the eight most intense fragments.

Table 4. Pairwise genetic distances among 11 populations of *O. malampuzhaensis* based on RAPD data. Population codes correspond to the codes given in table 1.

	7	8	9	10	11	12	13	14	15	16
6	0.113	0.124	0.114	0.121	0.108	0.113	0.124	0.105	0.114	0.116
7		0.039	0.021	0.030	0.041	0.029	0.034	0.040	0.034	0.041
8			0.085	0.039	0.021	0.042	0.015	0.031	0.015	0.015
9				0.055	0.044	0.009	0.038	0.033	0.041	0.041
10					0.086	0.011	0.035	0.052	0.035	0.035
11						0.044	0.007	0.018	0.007	0.007
12							0.038	0.045	0.041	0.041
13								0.017	0	0
14									0.017	0.017
15										0

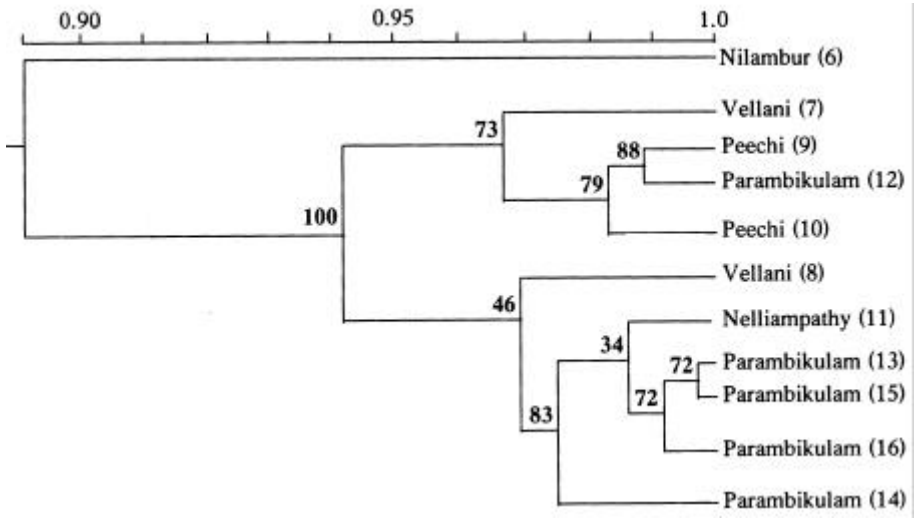

Figure 2. A UPGMA dendrogram of 11 populations of *O. malampuzhaensis* based on RAPD markers. Numbers shown at nodes represent percentage confidence limits obtained in the bootstrap analysis. The scale shows genetic similarity estimates according to Nei and Li (1979). Numbers in parenthesis are population code according to table 1.

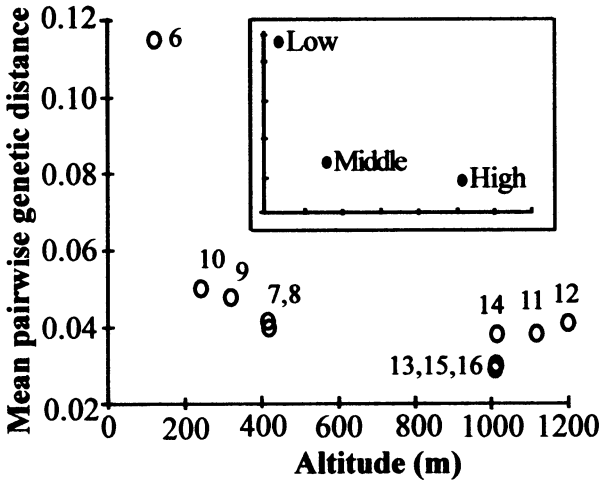

Figure 3. Scatterplot of the mean pairwise genetic distance of *O. malampuzhaensis* populations versus the altitude of their natural habitats. Numbers correspond to the population codes given in table 1. Relationship between mean pairwise genetic distance of populations belonging to similar altitudinal levels and mean altitude at such levels is shown in inset.

Table 5. List of the electrophoretically selected distinct *O. malampuzhaensis*-specific RAPDs.

Primer	*O. malampuzhaensis*-specific RAPDs (bp)
OPE 01	750, 1000
OPE 11	1500
OPF 09	1200
OPF 12	650, 1400
OPC 14	1400
OPJ 18	1000, 1500
OPAC 01	450, 800
OPAC 17	550
OPAE 04	500, 1200

Each of the *O. malampuzhaensis*-specific RAPDs was eluted from the gel and its specificity was further examined by Southern hybridization to the profile consisting of the corresponding fragment. A 1200-bp fragment generated by OPAE 4 and a 1500-bp fragment generated by OPE 11 demonstrated clear species specificity as they hybridized to the corresponding amplicon in all accessions of *O. malampuzhaensis*. However, these fragments hybridized to some other fragments also in both *O. officinalis* and *O. malampuzhaensis* but the sizes of such fragments were different. The results obtained with the primer OPAE 4 are shown in figure 4. The remaining 12 fragments did not demonstrate clear genomic specificity as they hybridized to fragments of corresponding size in *O. officinalis* also. Apparently, these fragments hybridized to some bands in *O. officinalis*, similar in size to that of the selected fragment, which were not visible on the ethidium bromide-stained agarose gel.

Discussion

A recent amplified fragment length polymorphism (AFLP) analysis of *Oryza* species that included two accessions of *O. malampuzhaensis* revealed a mean genetic distance of 0.0375 within *O. malampuzhaensis* (Aggarwal *et al*. 1999). Similar levels of RAPD distance observed in the present study (0.046), in spite of sampling more collections, reflect relatively low genetic diversity within

O. malampuzhaensis. From the low diversity and the scattered nature of relatively fewer natural populations, one would tend to treat *O. malampuzhaensis* as an ancestral 'declining species' presently represented by a few relic populations. The clustering of *O. malampuzhaensis* at a lower level in the dendrograms of *Oryza* species reported earlier (Second 1985; Wang *et al.* 1992; Aggarwal *et al.* 1999) however indicates recent origin because a recently originated species cladistically occupies a lower position in dendrograms (Wendel *et al.* 1994). Most likely, *O. malampuzhaensis* did not spread much from its centre of origin. Highly recalcitrant seed and the potential limitations associated with the clonal mode of propagation would have together contributed to limited natural spread of the species.

The pattern of genetic polymorphism within and among the populations of *O. malampuzhaensis* shows certain features of the spatial distribution of its genetic diversity. The populations are almost homogeneous, as evident from practically no RAPD polymorphism among individuals belonging to the same population. Instead, the genetic diversity within *O. malampuzhaensis* is seen between the populations, especially between the three geographical/

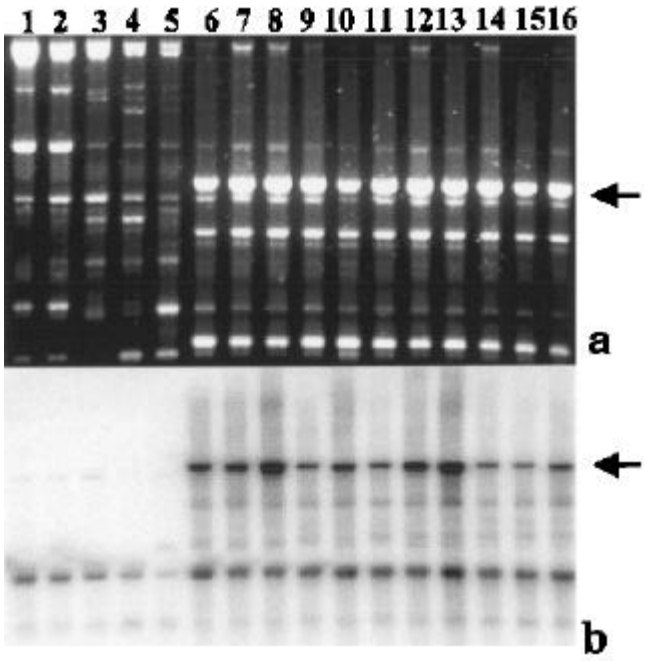

Figure 4. An *O. malampuzhaensis*-specific RAPD fragment generated by primer OPAE 4. (a) Ethidium bromide-stained agarose gel showing the RAPD profile of *O. officinalis* (1 to 5) and *O. malampuzhaensis* (6 to 16) accessions. Arrow indicates the 1200-bp *O. malampuzhaensis*-specific band. (b) Autoradiogram obtained following Southern blot hybridization of the gel in (a) with the DNA eluted from the 1200-bp *O. malampuzhaensis*-specific band. Arrow indicates the signal given by the 1200 bp band, demonstrating its specificity to *O. malampuzhaensis*.

altitudinal groups, as seen from the significantly different genetic distances of the three groups. The similar genetic distances within geographical groups and significant differences between the three geographical groups of populations demonstrate a distinct geography-dependent population differentiation in *O. malampuzhaensis*. This means the populations have differentiated locally as a result of natural selection under the micro-ecosystem prevailing at their natural habitat. The reason for the different behaviour of populations 8 and 12 is not clear. Species with restricted gene flow generally show a greater tendency to differentiate among populations (Govindaraju 1989; Hamrick *et al.* 1991). The limited scope of gene flow associated with clonal multiplication may be the primary reason for local differentiation in *O. malampuzhaensis*. The three geographical regions are not too far apart to have striking differences in components of ecological factors. It is possible that the marked difference in elevation at the three geographic locations may have caused substantial difference in certain components of environmental factors and such factors might have played a larger role in geography-dependent differentiation in *O. malampuzhaensis* as reported in the case of *Clarkia unguiculata* (Vasek 1964; Vasek and Sauer 1971; Jonas and Geber 1999).

Several features of *O. malampuzhaensis*, such as restricted distribution in a small geographical area, scattered smaller populations, and no variation within the populations, have strong correspondence with those of a typical 'island endemic' like *Gossypium mustelinum* (Wendel *et al.* 1994). Species that occur as isolated small patches are vulnerable to genetic drift and exhibit high levels of population divergence (Godt and Hamrick 1993; Diaz *et al.* 1999). The sharp local differentiation of populations from the Vellani–Peechi area over a short distance may be an indication of genetic drift. Hence it is possible that, in addition to natural selection, genetic drift also may be a factor responsible for population divergence in *O. malampuzhaensis*. However, it is exceedingly difficult to separate the effects of selection and genetic drift in population divergence of a species (Briggs and Walters 1997). A number of studies have reported low genetic diversity in rare and endangered plants (Waller *et al.* 1987; Gustafssson and Gustafsson 1994; Swensen *et al.* 1995). Genetic variation is the raw material of evolution and its magnitude is therefore of vital interest in governing the potential of a species to evolve and adapt. It is likely that the genetically isolated smaller populations of *O. malampuzhaensis* experience severe genetic drift. Over the long term that may result in further genetic depauperization and inability to respond to further changes in the habitat. Thus, there is a strong possibility of *O. malampuzhaensis* being driven to extinction.

Recent studies on wild barley (Owour *et al.* 1997; Nevo *et al.* 1998) and wild emmer wheat (Fahima *et al.*

1999; Li *et al*. 1999) have clearly demonstrated the value of RAPDs in assessing the level of DNA differentiation among their populations from different ecogeographic habitats. Among the populations of *O. malampuzhaensis*, genetic distance increases as the geographic distance from the Nelliampathy–Parambikulam area increases. This in turn shows that the populations from the Vellani–Peechi area and Nilambur experienced a higher evolutionary sorting during their acclimatization to the respective places. Higher levels of phenotypic variation (Wilken 1977) and allozyme polymorphism (Nevo 1995) have been reported earlier in populations from more stressful environments than in those from less stressful ones. It is possible that the ecological conditions prevailing at the Peechi–Vellani and Nilambur sites impart varying degrees of stress, which causes the induction of relatively high genetic variation among the populations in these sites. The induction of genetic variation in plants when they experience stress has been interpreted as a mechanism for increase in the pool of variability to enable evolutionary forces to select a variant with increased survival fitness (Cullis and Creissen 1987). In that sense, the relatively larger number of populations seen in Parambikulam as well as the lower divergence among themselves (three populations were indistinguishable) indicate that ecological conditions at high altitude may be the most suitable ones for the survival of *O. malampuzhaensis*.

O. malampuzhaensis is often confused with *O. officinalis* owing to their morphological similarities (Vaughan 1989b, 1994). The two RAPDs identified in the present study are valuable for the unambiguous identification of *O. malampuzhaensis* in collecting missions and for the management of its germplasm in germplasm banks. The specificity of these markers is well grounded as we used a larger sample of *O. malampuzhaensis* comprising collections from different geographical sites. These markers can either be directly used as species indicators or can be converted into more reliable and powerful sequence-characterized amplified regions (SCAR).

The results of the present study on population-genetic structure of *O. malampuzhaensis* would help in designing methods for its collection and conservation. Collecting missions for *O. malampuzhaensis* can be directed more to low elevations of the Western Ghats, as this area shows higher levels of genetic diversity of the species. Since high degree of genetic diversity was found between the populations, as many populations as possible should be sampled, but one or two individuals from each population would be enough to conserve the genetic diversity of this species. The forest ranges possessing genetic resources of *O. malampuzhaensis* are often disturbed for development programmes such as construction of hydroelectric projects. In addition, a gradual decline of natural populations of *O. malampuzhaensis* has been observed. Above all, the results of our genetic structure analysis reveal that *O. malampuzhaensis* is under the threat of extinction. Therefore, there is urgent need for preservation of *O. malampuzhaensis* by suitable conservation methods.

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