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Genetic Diversity of Myanmar Rice Cultivars Detected by DNA markers

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Genetic diversity of Myanmar cultivated rice accessions was evaluated by DNA markers. The materials included 110 accessions from six different regions of Myanmar and 17 accessions from other countries. Twelve RFLP markers, 6 STS markers and 28 CAPS markers were used. An UPGMA dendrogram was constructed to infer the phylogenetic relationships of the materials. The stability of the nodes in the tree was tested by the bootstrap analysis. All accessions except two could be distinguished from each other by at least one DNA marker. The dendrogram revealed 2 well–distinguished groups, namely, Group I and Ⅱ. Group I corresponded Japonica. It was further divided into two subgroups Ia and Ib. The subgroup Ia contained Japanese Japonica accessions and all the accessions in the subgroup Ib were Myanmar accessions clearly differentiated from the subgroup Ia. Indica accessions were contained in Group Ⅱ. It comprised small clusters. Bootstrap analysis was performed to determine the confidence levels of the forks in the dendrogram. In the resulting consensus tree, only 6 forks had bootstrap values above 80%. The grouping of the UPGMA tree and the majority–rule consensus tree were comparable in the subgroups Ia, Ib and Ⅱe. However, the Indica subgroups, Ⅱa, Ⅱb, Ⅱc and Ⅱd were not significantly differentiated.

INTRODUCTION

Rice, *Oryza sativa* L., is one of the agronomically and nutritionally important cereal crops and is the principal staple food for more than half of the world population. A main part of the genetic improvement of a crop resides in the creation of new combinations of genes from the available germplasm. In this perspective, the first concern of breeders is to know the genetic structure of the existing germplasm.

A large number of crop species, including rice, originated from the Hindustan center, which contained India, Assam, and Myanmar (Vavilov *et al.*, 1926). The origin sites of cultivated rice are located in a wide region extending from the Ganges plains, northern Myanmar, northern Thailand, Laos, northern Vietnam and southwest and southern China. (Matsuo, 1997; Chang, 1976). Nakagahra (1978) estimated the center of the diversity of Asian rice might be located at mountainous regions on the northern side of Southeast Asia, such as Myanmar, Thailand, and Yunnan in China.

As an origin of rice cultivation, Myanmar is one of the countries which have been found high in genetic diversity of rice and a part of the crop diversity area, and it also have heterogeneous geographical and ecological conditions such as hills and mountains. Geographically, Myanmar has six distinct regions: central dry zone, coastal strip region, delta region of Ayerwady and Sittaung rivers, eastern mountain region, northern mountain region, and western mountain region. As a staple food and crop with potential for export, rice is grown extensively in all of the six regions under all agro–ecological conditions.

There are 5770 accessions of rice germplasm collected and conserved in Myanmar Seed Bank Project in 2000 (Oka, 2000). The accurate identification of plant material in a gene bank is essential for effective germplasm characterization because breeders have no means to select appropriate materials for breeding programs without such information. The structure of genetic diversity is also important when considering the development of 'core collections' (Jackson *et al.*, 1998). Further, without determining diversity reliably it would not be possible to identify molecular marker/quantitative trait association which have been shown to be useful in the process of germplasm evaluation (Virk *et al.*, 1996).

The genetic variation of Asian cultivated rice has been studied by many researchers to classify landrace groups, to investigate phylogenetic differentiation, and to provide basic information for breeding. Oka (1953a and 1953b) systematically surveyed variations in several physiological, biochemical, and morphological characters and performed genetic analyses of hybrid sterility. He classified rice varieties into continental and insular types, subdividing the latter into tropical insular and temperate insular. The three groups of rice are generally recognized as Indica, Javanica, and Japonica. Variation of isozyme loci was intensively surveyed to make precise classification (Glaszmann, 1987; Nakagahra, 1978).

Although these classical methods were central, they are restricted in their resolving power mainly because of the small number of variables available. Nowadays,

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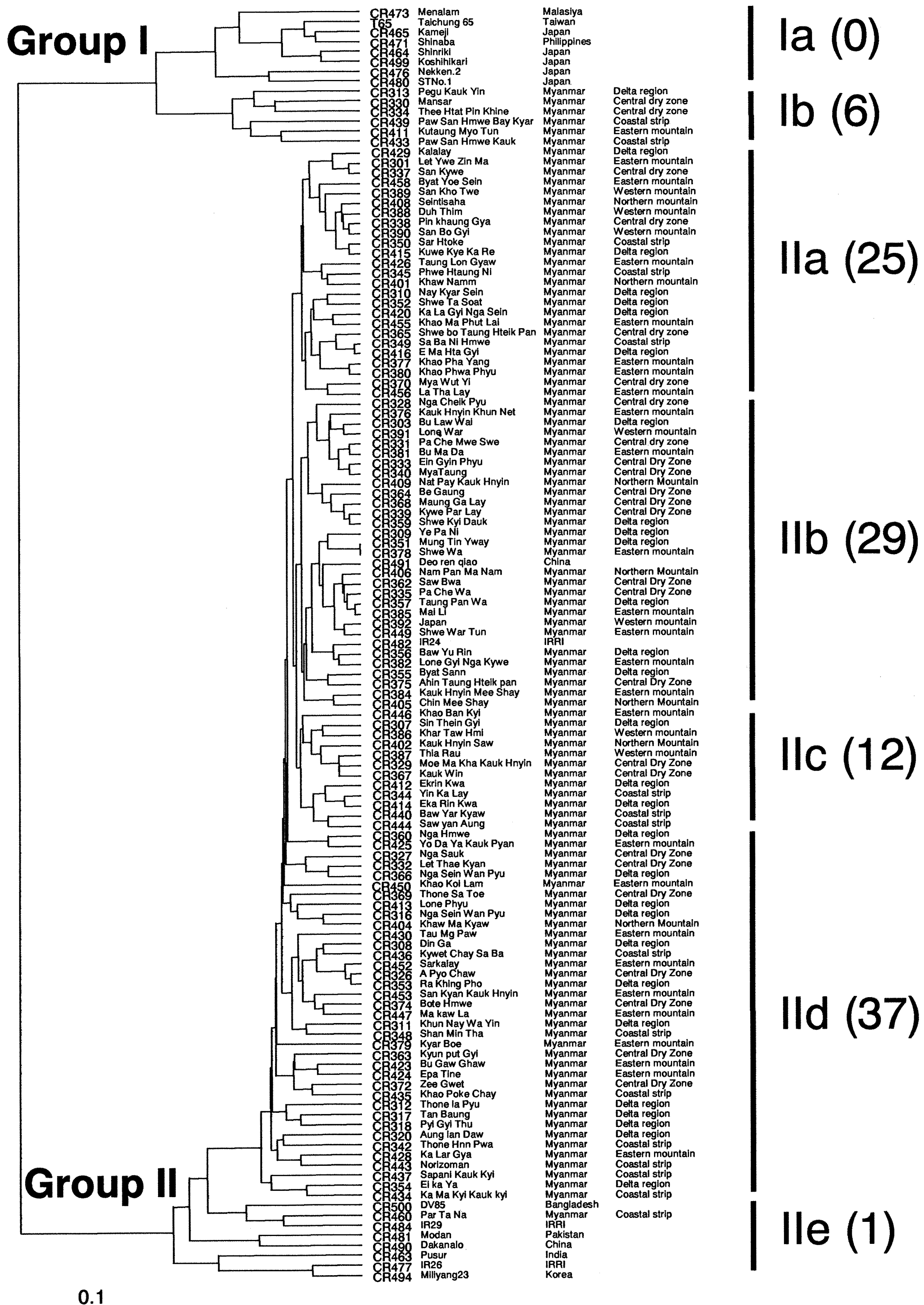

Fig. 1. Dendrogram generated by UPGMA method. Accessions from Myanmar are designated as CR301 to CR460. Numbers of Myanmar accessions contained in each group are shown in parentheses.

there are several different DNA based methods for the identification of cultivars. Restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) have been widely used. RFLP are differences in the lengths of DNA fragments following digestion with restriction endonucleases. These polymorphisms are generally detected using single or low copy number nuclear DNA sequences as hybridization probes on Southern blots of restriction–digested genomic DNA. The development of RFLP marker has provided powerful tools in assessing the genetic differentiation and detecting the phylogenetic relationship between cultivated rice and wild rice (Wang and Tanksley, 1989; Zhang *et al.*, 1992; Wang *et al.*, 1992; Nakano *et al.*, 1992; Doi *et al.*, 2000; Sun *et al.*, 2001). This approach, however, requires appreciable amounts of relatively pure DNA, is time consuming, costly, and is technically demanding.

On the other hand, PCR based markers such as random amplified polymorphic DNA (RAPD, Williams *et al.*, 1990) and amplified fragment length polymorphism (AFLP, Vos *et al.*, 1995) have the merit of revealing diversity without prior knowledge of genome sequence information but have the drawbacks of generating mainly dominant and anonymous markers (Skroch and Nienhuis, 1995; Shim and Jørgensen, 2000). Simple sequence repeat (SSR) markers have also been extensively used to generate multi–allelic and very variable markers (Ghebru *et al.*, 2002; Enoki *et al.*, 2002). Cleaved amplified polymorphic sequence (CAPS) methodology generates another type of molecular markers combining both PCR and RFLP techniques (Konieczny and Ausubel, 1993). This requires minute amounts of genomic DNA and simple electrophoresis systems to reveal polymorphism. CAPS markers have been successfully applied to a number of crop and forest species for which extensive nucleotide information is available (Chen *et al.*, 1994; Ghreyazie *et al.*, 1995; Tsumura *et al.*, 1997; Perry *et al.*, 1999).

The main objective of this research is to identify the genetic variation of Myanmar rice cultivars by using DNA markers and to get the information that allow to select appropriate materials within Myanmar rice cultivars for further breeding program.

MATERIALS AND METHODS

Plant materials

The numbers of the accessions and names of collection regions were shown in Table 1. The materials included 110 accessions from 6 different regions of Myanmar and 17 accessions previously analyzed (Doi *et al.*, 2000). The 17 accessions including typical Japonica varieties and Inidica varieties were used as controls (Fig. 1).

DNA extraction

Frozen leaves of a single plant in each accession were collected and ground in liquid nitrogen. DNA was extracted from the ground tissues by the CTAB (Cetyltrimethyl ammonium bromide) method (Murray and Thompson, 1980).

Detection of RFLP

Probes used in this study were selected from clones located on the RFLP map constructed by Saito *et al.* (1991). RFLPs were detected from the combination of total DNA digested with *Dra* I, *Hind* III, or *Eco* RV and 12 probes (Table 2). DNA samples were digested with restriction endonuclease, and were electrophoresed on 0.8% agrose gels using TAE (40 mM Tris–HCl pH 8.0, 40 mM acetic acid, 1 mM EDTA) as the running buffer. The gels were blotted onto a positively charged nylon membranes by capillary transfer in 0.4 N NaOH for 12 h. The membranes were washed in 2×SSC, dried, and baked at 120°C for 20 min. Hybridization signals were detected according to the protocol of the ECL direct nucleic acid labeling and detection system (Amersham Biosciences, Tokyo, Japan) on X–ray films (FUJIFILM, Tokyo, Japan) for 1–3 h.

Genotyping of PCR marker loci

Thirty–four PCR–based polymorphic markers including 6 sequence tagged site (STS) and 28 CAPS markers covering 12 chromosomes of rice (Rice Genome Research Program) were used. The names of the markers and their located chromosomes are shown in Table 3. PCR amplification was performed on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using the following amplification condition: 30 cycles of 94°C (30 sec), 60°C (1 min), and 72°C (1 min). The amplified products were digested with restriction enzymes as necessity. The DNA fragments

Table 1. Number of accessions in 6 regions of Myanmar and other countries

Region	Number of accessions
Central dry zone	25
Coastal strip region	18
Delta region	26
Eastern mountain region	27
Northern mountain region	7
Western mountain region	7
Other countries	17
Total	127

Table 2. RFLP markers used in this study

Probe (Npb)	Chromosome	Enzyme	Number of detected alleles
67	2	*Dra* I	3
132	2	*Eco* RV	3
15	3	*Eco* RV	2
129	3	*Hind* III	2
49	4	*Hind* III	2
81	5	*Eco* RV	2
27	6	*Eco* RV	3
33	7	*Dra* I	3
117	7	*Hind* III	3
126	8	*Eco* RV	3
13	9	*Eco* RV	2
32	10	*Hind* III	2

Table 3. PCR markers used in this study

Marker	Type	Chromosome	Restriction Enzyme	Number of detected alleles
S13048	CAPS	1	*Bam* HI	3
C955	CAPS	1	*Afa* I	2
S13994	CAPS	1	*Eco* RI	2
C178	CAPS	1	*Hae* Ⅲ	2
C137	CAPS	1	*Hind* Ⅲ	2
C777	STS	2	–	2
S10844	CAPS	2	*Apa* I	3
C932	CAPS	2	*Hha* I	2
S1792	CAPS	3	*Hind* Ⅲ	2
R2247	CAPS	3	*Hae* Ⅲ	2
C1329	CAPS	3	*Eco* T22I	3
S1322	CAPS	4	*Hae* Ⅲ	2
R00746	STS	4	–	3
R2232	CAPS	5	*Hha* I	5
R1436	CAPS	5	*Eco* T14I	3
C903	STS	5	–	2
S974	CAPS	5	*Mbo* I	2
S1520	CAPS	6	*Hind* Ⅲ	2
R3879	STS	6	–	2
S11633	CAPS	7	*Xho* I	2
R1382	CAPS	7	*Xho* I	2
R1789	CAPS	7	*Mva* I	2
R1943	CAPS	8	*Hind* Ⅲ	2
C166	CAPS	8	*Dra* I	2
R79	CAPS	9	*Hinf* I	2
S1974	CAPS	9	*Hinf* I	2
S1456	CAPS	9	*Mbo* I	2
C51124	STS	10	–	2
C63320	STS	10	–	2
C961	CAPS	10	*Dra* I	2
S11148	CAPS	10	*Eco* T22I	3
C562	CAPS	11	*Hind* Ⅲ	2
C950	CAPS	11	*Eco* RI	2
S1436	CAPS	12	*Hind* Ⅲ	2

were electrophoresed on 2.0% agarose gels in 0.5×TBE buffer at 120 V for 2 h, and stained with ethidium bromide.

Data analysis

The character was scored as 1 and 0 for the presence and absence of the fragment, respectively. The 1/0 matrix was used to calculate dissimilarity coefficients following Nei (1979). The resulting distance matrix was used to construct an unweighted pair–group method with arithmetic means (UPGMA, Sokal and Michener, 1958) dendrogram using software package PHYLIP (Felsenstein, 1993) to infer phylogenetic relationships. The stability of the nodes in the tree was tested by bootstrap analysis (Felsenstein, 1985). The data matrix was used to generate 1000 alternate data sets using SEQBOOT program. The alternate trees produced were used to construct a single majority–rule consensus tree using the CONSENSE program in PHYLIP package.

RESULTS

DNA polymorphisms in Myanmar rice cultivars

A total of 106 alleles were detected with an average of 2.4 alleles per locus using both 12 RFLP markers and 34 PCR markers. The total number of observed alleles per markers ranged from 2 to 5. The CAPS marker R2232 showed the highest number of alleles, 5 (Table 3).

Phylogenetic trees

All accessions except CR351 and CR378 could be distinguished from each other by at least one DNA marker. The dendrogram revealed 2 well distinguished groups, named as Group I and Group Ⅱ (Fig. 1). Group I seemed to correspond Japonica because it contained the accessions from Japan. It was further divided into subgroups Ia and Ib. Most accessions in the subgroup Ia were Japonica varieties originated in Japan, and all accessions contained in the subgroup Ib were Myanmar accessions. Although Myanmar accessions were divided from other Japonica varieties, the distance between the two subgroups was close and clearly differentiated from Group Ⅱ.

Group Ⅱ contained Indica accessions. It comprised subgroups (Ⅱa, Ⅱb and Ⅱc) plus accessions which formed no cluster (Ⅱd and Ⅱe in Fig.1). The accessions of the subgroup Ⅱe are found to be somehow distant from the subgroups Ⅱa, Ⅱb, Ⅱc and Ⅱd. Only one Myanmar accession (CR460) was included in the subgroup Ⅱe.

Bootstrap analysis was performed to determine the confidence levels of the forks in the dendrogram. In the resulting consensus tree, only 6 forks had bootstrap values above 80% (Fig. 2). The grouping of the UPGMA tree and majority–rule consensus tree were in general comparable except the subgroups Ⅱa, Ⅱb, Ⅱc and Ⅱd. The composition of Group I (subgroups Ia and Ib) and the subgroup Ⅱe were identical in both dendrograms. However, most of the forks in the subgroups Ⅱa, Ⅱb, Ⅱc and Ⅱd showed very low bootstrap values. The values of bootstrap analysis suggested that constituents of the subgroups Ⅱa, Ⅱb, Ⅱc and Ⅱd were not significantly differentiated.

Regional distribution

Regional distribution of all groups classified in the UPGMA tree was shown in Fig. 3. The accessions classified in Group I were found only in central dry zone, coastal strip region and delta region. The accession CR460 belonging to the subgroup Ⅱe was originated in coastal strip region.

DISCUSSION

A range of studies on the genetic diversity of natural populations of cultivated rice and wild rice species have been reported, including morphological, isozyme, and DNA levels (Oka, 1953a; Glazmann, 1987; Ishii *et al.*, 1996; Fuentes *et al.*, 1999; Joshi *et al.*, 2000; Virk *et al.*, 2000). However, studies on DNA level of Myanmar local rice varieties were very few. In the present study, 12 RFLP markers and 34 PCR markers were used to analyze 110 Myanmar landraces from core collection of Myanmar Seed Bank Project (Oka, 2000).

An average of 2.4 unique fragments per marker loci (Tables 2 and 3) is rather small compared with previous

Group II

Group I

Fig. 2. A majority–rule consensus tree based on the analysis of most parsimonious tree from 1000 bootstrapped data sets generated by SEQBOOT program. The underlines indicate the bootstrap values greater than 80% (800).

studies: average of 5.3 fragments observed in 192 accessions of A genome species (Doi *et al.*, 2000). 3.4 fragments in 70 accessions of *O. sativa* (Wang and Tanksley, 1989) and 11.2 fragments in 93 accessions from whole genus *Oryza* (Wang *et al.*, 1992). This is reasonable because the germplasm analyzed in this study are only cultivated rice and traditional varieties originated in Myanmar. Each pair of accessions showed a difference for at least one marker except CR351 and CR378, these accessions shared the identical genotype. This means that DNA marker is a powerful tool to classify germplasm.

Six accessions (CR313, CR330, CR334, CR433, CR439, and CR411) were classified into Group I (Fig. 1) which contained Japonica varieties from Japan. In the study of Kawase *et al.* (1991), genetic differentiation based on RFLP in Asian cultivated rice were well classified into two groups, Indica and Japonica, and Myanmar rice cultivars were included in both groups. Our results indicated that Japonica–like Myanmar accessions (subgroup Ib) were clearly differentiated from Japanese varieties (subgroup Ia), suggesting that these Myanmar accessions might be Javanica or tropical Japonica accessions. Adding more control accessions belonging to tropical Japonica should be helpful to clarify this question. Climate conditions, geographical heterogeneity or the wide area of rice cultivation in central dry zone, coastal strip region and delta region might be the reason for that these Japonica–like accessions were found (Fig. 3).

The majority rule consensus tree generated from bootstrap analysis (Fig. 2) indicated that only six nodes showed the stability greater than 80%. The significance of these nodes enforced the pattern of clustering of the subgroups Ia, Ib and IIe (Figs. 1 and 2). However, most of the forks in the subgroups IIa, IIb, IIc and IId showed low bootstrap values and unstable (Fig. 2). This indicated that constituents of these subgroups were not significantly differentiated. A similar pattern of the difference between the methods of the dendrogram construction was reported in *Triticeae* tribe (Monte *et al.*, 1993).

Previous studies also suggested that Myanmar rice varieties are high in genetic variation. In analysis of esterase isozyme, the highest genetic diversity was found in the area covering Myanmar, Thailand, Laos, and Yunnan Province of China (Nakagahra *et al.*, 1984). The results presented in this study indicated that Myanmar rice genetic resources are diverse in molecular level. The knowledge on genetic variation is important for breeders to fully draw the potential of germplasm. It is required to know the genetic diversity as the basic information. This is the first study on the genetic variation of Myanmar rice in DNA levels and the information generated from this experiment would allow us to select appropriate parents within Myanmar rice cultivars for further breeding program.

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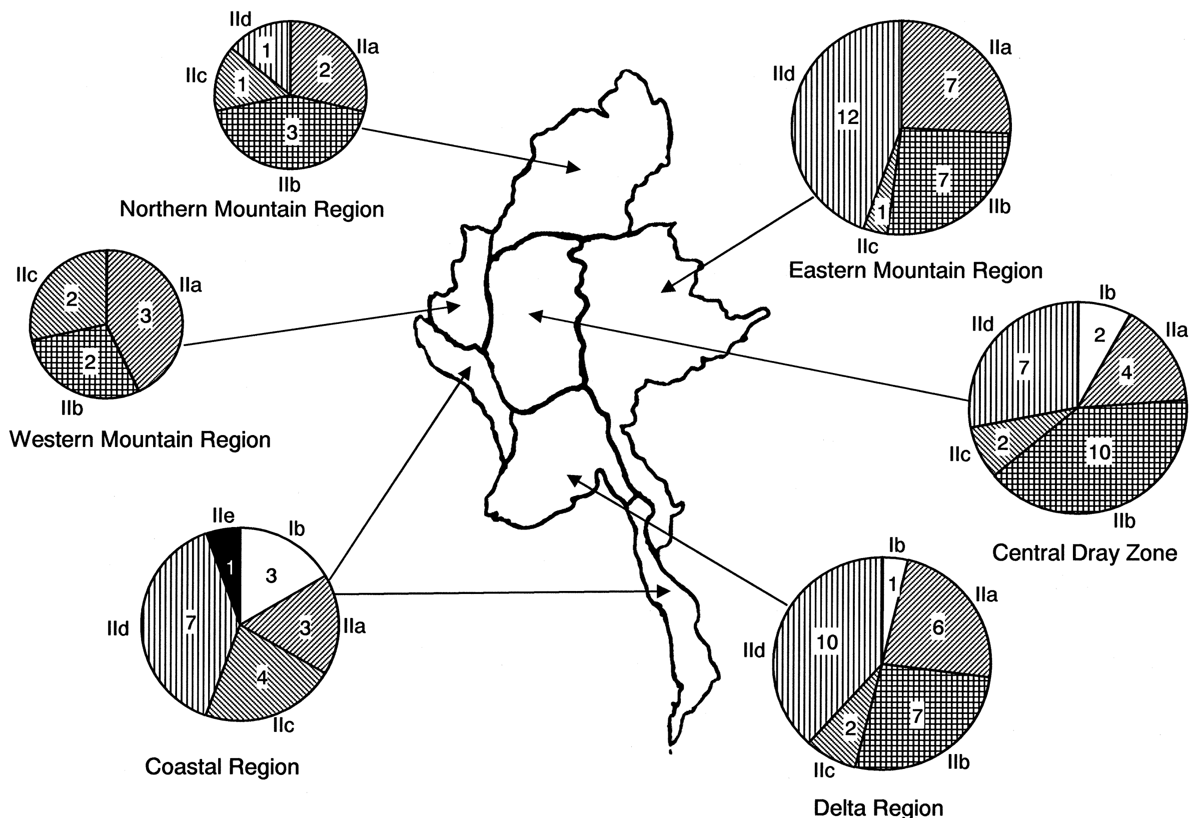

Fig. 3. Geographical distribution of the DNA marker–based groups of Myanmar accessions.

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