VI.5 Molecular Markers in Rice Systematics and the Evaluation of Genetic Resources

G. SECOND[1]

1 Introduction

Cultivated varieties of rice are the result of several thousands of years of human selection from the available genetic diversity, in various natural environments and human cultures. The process of gathering varieties from different geographic origins, planting them in the same field, and selecting particular genotypes among the offsprings of natural hybridization can still be observed in traditional societies and has no doubt been going on since early days. Modern breeding in the last century has done little more than to control the process of hybridization and selection in a more efficient way, and the result has been to adapt varieties to better controlled and fertilized environments. The basic approach consists of choosing parents with complementary characters in order to combine them. Unexpected (transgressive) characters are also obtained in the process. Evaluation of germplasm is generally a matter of screening for useful characters although the understanding of the genetic structure of genetic resources can give a clue to obtain transgressive characters (Second and Charrier 1989).

Ways to further improve efficiency of modern breeding may be seen in two philosophically different, though complementary, approaches:

- direct alteration of the genotype by *in vitro* engineering, regardless of the natural processes (molecular biology),
- making use of the natural processes but taking fuller advantage of available genetic diversity to create new genotypes.

We are concerned here with the second approach, which is directly dependent upon the ability to obtain, exchange freely, and assess the vast array of germplasm available in nature. The rapid worldwide devastation of natural environments constantly erodes the scope of genetic diversity that has evolved over millions of years and underscores the importance of attempts to preserve those genetic resources in whatever ways we can. Collections of genetic resources of rice are already very large, although more collection is still necessary (see Chang and Vaughan, Chap. VII.1, this Vol.). A guide line is needed to evaluate, utilize, and further complement these invaluable collections. Such a guideline may be found in recognizing that the genetic diversity is not distributed at random (there exists a

[1] Plant Breeding, International Rice Research Institute, P.O. Box 933, 1099 Manila, Philippines

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genetic "structure" at infra-specific level) and that the degree of similarity (genetic distance) allows us to group individuals, varieties, or accessions into a limited number of entities. As similar genotypes are more likely to share common characteristics, a limited number of individuals can then be chosen to efficiently represent a much larger group.

Further, the genetic structure can be interpreted in terms of phylogeny and an evolutionary scenario or domestication process can be drawn from the available information and tested with new data. This approach is actually nothing more than the traditional goal of taxonomy which is to produce a classification scheme that reflects phylogeny. In the past, the study of systematics has relied upon phenotypic evaluation as the basis for postulating evolutionary relationships. The advent of molecular DNA technology has enabled us to observe the genotype directly. The use of molecular markers in the study of taxonomy enhances our ability to make inferences about phylogeny and provides information which allows us to suggest possible genetic mechanisms that account for observed evolutionary patterns.

In this chapter, the impact of molecular markers in the systematics of cultivated varieties of rice and their wild relatives is assessed. We review the recent developments in the mapping of the genome that will not only allow us to reach unprecedented refinements in systematics but also provide tools with which to approach plant breeding as a science of experimental evolution.

Molecular markers represent locus-specific DNA variation detected either at the level of the protein product of a gene, as in the case of isozymes, or directly at the level of DNA. Variation of the DNA is detected directly either as restriction fragment length polymorphism (RFLP) or by determining the exact nucleotide sequence of specific areas of the genome. These locus-specific polymorphims mark the chromosome segments on which they are located and thus all genetic factors located on the same segment, within the law of genetic linkage and recombination. Further, it is important to note that they are codominant (heterozygotes can be distinguished from homozygotes), their expression is independent of the environment, and they can be scored at the seedling stage.

Molecular maps, composed of hundreds of molecular markers distributed throughout the entire genome, are of invaluable assistance in both taxonomic studies and plant breeding. These maps order the array of available markers, providing information about chromosomal location and linkage distance relative to one another. This knowledge enables a researcher to select markers efficiently, in order to scan the entire genome or to focus on a particular chromosomal area. Based on such studies, comparative maps between related species can be constructed, and structural differences due to chromosomal rearrangements or major insertions or deletions can be detected (Bonierbale et al. 1988; Tanksley et al. 1988a).

So far, in rice, isozymes have been used mostly for studying the genetic structure of cultivated varieties and their wild relatives (Endo and Morishima 1983; Second 1982, 1984, 1985a,b; Glaszmann 1987, 1988; de Kochko 1987a,b). Their contribution to mapping will remain limited because few isozyme markers are available. On the contrary, RFLP's provide an unlimited number of markers. RFLP's have been applied in rice:

— to obtain a linkage map of the chromosomes. This map is already denser than

the map previously available and the project is heading toward a saturated map (McCouch et al. 1988).
- at the level of chloroplast DNA to mark the plastome, and thus the cytoplasm, generally maternally inherited as opposed to the nucleus, which is inherited biparentally (Dally 1988; Dally and Second 1989).

Rice is a good material for using molecular markers:

- there is large amount of variation, including within cultivated varieties (Second 1982; Wang and Tanksley 1989),
- it is a diploid with a relatively small genome (C = 0.6 pg, Bennett and Smith 1976, that is in the order of 6×10^8 base pairs. The genome size however, varies, considerably among species of rice (Iyengar and Sen 1978), and also among cultivated varieties (Nagato et al. 1981). Its genome contains accordingly less repeated sequences than in wheat – even diploid – or in corn, for example. It shows some remarkable features of sequence organization (Deshpande and Ranjekar 1980; Gupta et al. 1981).
- important germplasm collections are available.

Further reasons to make fundamental studies in rice genetics and systematics include:

- rice is a staple crop in the world,
- breeding efforts look more and more to introgression of agronomically useful genes from wild species,
- the distribution of the cultivated varieties and the wild species, including the *Oryza* genus and the Oryzeae tribe, represents a very interesting model for evolutionary biogeography studies. Some of the wild species are still found in fairly undisturbed habitats,
- rice can be studied in a large variety of natural environments.

2 Methods

2.1 Isozymes

Starch, agar, and acrylamide gels have all been used. However, starch gel has been the most popular (see Endo and Morishima 1983 for a review). Typically, small pieces of leaves or a young germination shoot are ground with a small quantity of distilled water. Small pieces of paper are saturated with the homogenate and placed into a block of starch gel in various buffers and subjected to electrophoresis. The block of starch gel is subsequently cut in several slices that can each be stained for a different enzyme activity. More than 17 enzyme systems have been detected in starch gels.

A method to study the differential thermostability of the isozymes separated electrophoretically was also developed (Second and Trouslot 1980; Second 1982).

RFLP's offer the possibility of detecting many more markers than isozymes. However, the latter are easier and less expensive to detect. They consequently

remain the method of choice for preliminary surveys on a few markers. RFLP's offer, however, a greater degree of resolution because more markers are available and can be used wherever laboratories are set up to take advantage of the technology.

2.2 Chloroplast DNA Variation

The direct observation of the restriction patterns of purified chloroplast DNA (cpDNA) under fluorescence was mostly used. Although probing of cpDNA sequences in Southern blot of restricted total plant DNA was also adopted (Ichikawa et al. 1986), direct observation allowed a better resolution for small addition/deletion commonly encountered.

Purification of cpDNA in rice proved to be relatively difficult and was accomplished only recently. An aqueous method allowed the publication of the first restriction map of rice chloroplast DNA (Hirai et al. 1985); the total cpDNA sequence was also determined (see Hirai, Chap. VI.4, this Vol.); survey of the restriction polymorphism among cultivated varieties and A-genome species was published (Ishii et al. 1986, 1988).

A modification of a nonaqueous method allows one to obtain sufficient amounts of cpDNA from a single plant and the survey of many different genotypes, including sterile plants. The method utilizes freeze dried leaves and thus allows for the conservation and the transportation of plant material (Dally and Second 1989a). A survey of the restriction polymorphism in *Oryza*, section *Oryza*, including cultivars and male sterile lines was made (Dally 1988; Dally and Second 1989).

2.3 Nuclear DNA RFLP

Nuclear DNA RFLP techniques involve cloning unique (or low copy number) sequences of DNA from the nuclear genome. These clones are then radioactively labeled and used as probes to detect homologous sequences in total plant DNA which has been cut with various restriction enzymes, fragment separated on agarose gel and blotted onto nylon membranes. Alleles are identified by differences in the size of the restriction fragments to which the probe hybridizes. The segregation of RFLP markers can be monitored in progenies from controlled genetic crosses and, by using standard genetic analysis, linkage groups can be constructed (McCouch et al. 1988). Once the library of clones is established, it can be stored indefinitely and dispatched to different laboratories.

The use of mapped markers involves the extraction of total DNA from individual plants according to a simple procedure, the screening of the library for polymorphic markers in the specific rice parents to be studied, and the scoring of the markers in a segregating population. Standardized techniques are used for the DNA-DNA hybridization. Progress is being made in the use of nonradioactive labeling of the probes.

3 The Genetic Structure and Phylogenetic Relationships in *Oryza* section *Oryza*

3.1 Taxonomy

The *Oryza*, section *Oryza*, corresponds exactly to the section *Sativa* in Roschevicz (1931) later named *Eu-Oryza* in Roshevits (1937) after Baillon (1894). It corresponds also exactly to the section *Sativae* in Tateoka (1964). As retained here, its name follows the international code for botanical nomenclature. The following treatment of this section is based on our study of its genetic structure and phylogenetic relationships (Second 1984, 1985a,b). The isozyme diversity was interpreted in view of the geographic distribution and ecological situation as well as from previous studies on chromosome pairing in hybrids and population biology. For the sake of simplicity, we present it as an introduction to the summary of molecular data while it should be understood as its conclusion. Table 1 summarizes the taxonomy of the *Oryza*, section *Oryza*, as described below.

Two groups of species among which natural hybrids have never been found but artificial hybrids were made in various combinations (see Nayar 1973; Jena and Khush 1986, 1990) constitute the section *Oryza*. They both have a pan-tropical distribution. Their genome characterization based on chromosome pairing in a few F_1 hybrids (see Nayar 1973 and Katayama 1982) was generalized in view of the corresponding isozyme polymorphism. They are:

— The *Sativa* group, which comprises all forms with the A genome. It includes only diploid forms and all cultivated varieties. In the wild, it is found generally in flood plains or temporary pools, in full light but sometimes in partial shade. It is also often found as a weed in cultivated fields. Differences in life history forms represent the main axis of its morphological diversity (Morishima 1969) with typical annual and autogamous vs. perennial and allogamous life forms and intermediates. Two endemic wild African species represent the two extreme annual and perennial life forms: *O. breviligulata* (= *O. barthii*) and *O. longistaminata*, respectively, with no intermediate. A continuum between annual-autogamous and perennial-allogamous forms is found in Asia, Australasia, and inter-tropical America with geographic forms corresponding to each continent as well as a variety of intermediates. This variety of forms with intermediates defies any clearcut subclassification and we propose to understand it as a "complex species" called *O. rufipogon*, with various geographic and life history varieties. The various reproductive barriers, mainly F_1 hybrid pollen sterility, often found within populations of the same geographic and life history form, as well as between them (see Oka 1988), do not allow the application of the biological species concept to further subdivide *O. rufipogon*. The practical impossibility to define so far clearcut morphological types within *O. rufipogon* also does not justify any further species recognition, although several authors have attempted it (see Chang 1988). On the contrary, *O. breviligulata* and *O. longistaminata* both correspond to the biological species concept (see Ghesquiere 1988 for *O. longistaminata*) and are easy to typify morphologically.

Table 1. List of species in *Oryza* section *Oryza* (revised from Second 1985b)

	Geographic dispersion	Life form[a]	Rep. syst.[b]	Gen. symb.[c]
The *Sativa* group				
Cultivated species:				
O. sativa L. (*japonica* and *indica* subsp. Kato)	Asian origin, Worldwide	I	S, I	A
O. glaberrima Steud	Africa (and S. America)	A	S	A
Wild species:				
O rufipogon Griff[d] (Complex species)	Tropical Asia, Australia and America	A, I, P	S, I	A
O. longistaminata A. Chev. & Roehr.	Africa	P	O	A
O. breviligulata A. Chev. & Roehr. (= *O. barthii* A. Chev)	Africa	A	S	A
The *Latifolia* group				
O. officinalis[e] Wall. ex Watt (Complex species)	Asia, New Guinea	P	S	C
O. eichingeri A. Peter	Africa	P	S	C
O. alta Swallen *O. grandiglumis* (Doll.) Prod *O. latifolia* Desv.	Tropical America	P	S	CD
O. punctata Kotschy (2x and 4x species)	Africa	A(2x) P(4x)	S S	B BC
O. minuta J.S. Presl.	Africa	P	S	BC
O. australiensis Domin	Tropical arid Australia	A	S	E

[a] Life forms: A, annual; P, perennial; I, intermediate.
[b] Reproductive system: S, selfing largely predominant; O, self-incompatible; I, intermediate.
[c] Cytogenetic genome symbols (Nayar 1973), A, B, C and E are diploid 2x = 24), BC and CD are allotetraploid (2x = 48).
[d] *O. glumaepatula* Steud is sometimes used for (all?) American forms *O. meridionalis* Ng et al. was proposed for (all?) Australian forms (see Chang 1988). Many other names were also given (see Oka 1988).
[e] *O. collina* (Trimen) S.D. Sharma & Shastry was proposed for forms originating in Sri-Lanka. A tetraploid form found in India and similar to tetraploid *O. punctata* is called *O. malampuzahensis* Krishnaw. & Chandra. This name was not retained by Tateoka (1963).

The cultivated varieties of rice belong to two different species. The common rice, of Asian origin but with a present worldwide distribution, belongs to *O. sativa* with two subspecies, *indica* and *japonica*, and a variety of geo-agro-ecotypes. Some of the African varieties belong to a different species, *O. glaberrima*. From the standpoint of a biological species concept, *O. sativa* and *O. glaberrima* include respectively their wild ancestors, *O. rufipogon* and *O. breviligulata*.

— The *Latifolia* group comprises diploid forms with the B, C, and E diploid genome and tetraploid forms with the BC and CD genomes. While the species with BC genome are clearly allotetraploids, the D genome most probably does not exist at diploid level (Second 1989). Based on greenhouse observations, these species seem

to be all self-pollinated. Fewer experimental data were obtained on this group on which to base a sound taxonomy. However, the present status may be presented as follows:

O. punctata is an African species with diploid-annual (genome B) and tetraploid-perennial (genome BC) forms. The distinction of the two can be reliably done by counting the chromosomes in root tips or, as shown in Fig. 1, by analyzing the isozyme patterns such as phosphoglucose-isomerase or malate dehydrogenase (Second and Trouslot 1980). They show a partly disjunct distribution. The diploid form is found in temporary pools of the savanna zone (also sometimes as a weed in Southern Africa) and the tetraploid form in shaded disturbed environments in the forest zone (Sano 1980 and Second unpubl.).

O. officinalis is an Asian species with a large distribution and a variety of forms, all perennial. It is found in humid forest as well as in disturbed areas – sometimes as a weed in cultivated fields – in the forest climatic zone. Although it often presents F_1 pollen hybrid sterility between accessions (Hu and Chang 1967) and a large isozyme polymorphism, it is considered to share a single genome C. An allotetraploid form BC has been described (*O. malampuzahensis* which can be found in

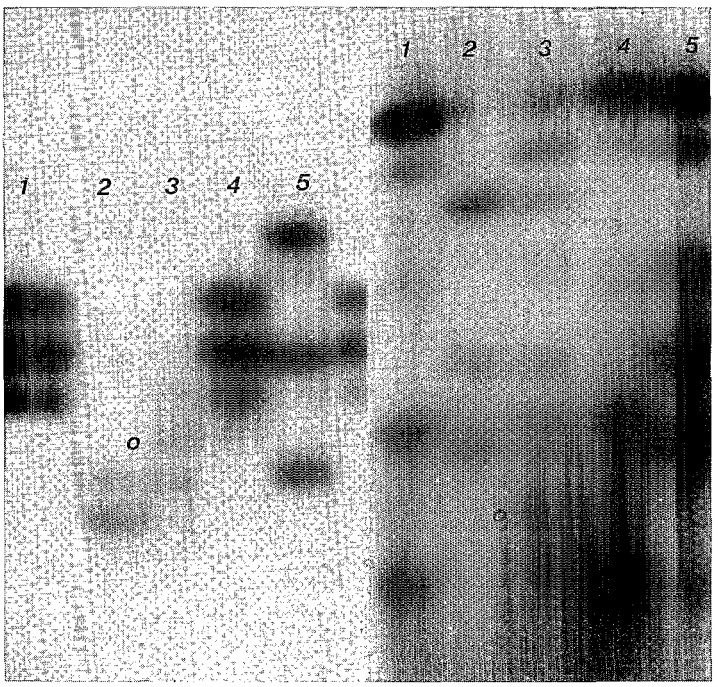


Fig. 1. Isozyme patterns of phosphoglucose isomerase (*left*) and malate dehydrogenase (*right*) stained after electrophoresis on starch gel of leaf extracts of the following species. *1*: *O. punctata* diploid; *2*: *O. officinalis*; *3*: *O. punctata* tetraploid; *4*: *O. punctata* diploid; *5*: *O. sativa*. The isozyme patterns are clearly distinct for the three diploid species. The allotetraploid species combines the bands of the parental species and shows additional bands (indicated by *O*) corresponding to heterodimer isozymes. (Second and Trouslot 1980)

Coimbatore area, India-Tateoka 1963, D. Vaughan, pers. commun.). A different form (= *O. collina*?) is found in Sri-Lanka.

O. eichingeri, an African species with a genome C found rarely in forest.

O. minuta, a species distributed only in the Philippines with a tetraploid BC genome.

O. latifolia, *O. alta*, and *O. grandiglumis* share a tetraploid CD genome and are found only in inter-tropical America. The distinction into three species is based on herbarium studies and was considered suspect by Tateoka (1963). It is not confirmed by either the distribution of reproductive barriers (see Nayar 1973) nor the isozyme and cpDNA polymorphisms. It thus seems more appropriate to consider a single complex species, *O. latifolia*.

O. australiensis with a genome E is the only representative of the group in Australia where it is endemic. It is an annual, found always in full light and in arid environment. Although a clearly isolated and distinguishable species, its inclusion in the *Latifolia* group is consistent with its morphological relationship as well as isozyme and cpDNA evidence.

3.2 The Genetic Structure of the Section *Oryza* as Seen at Isozyme Level

3.2.1 The Wild Species

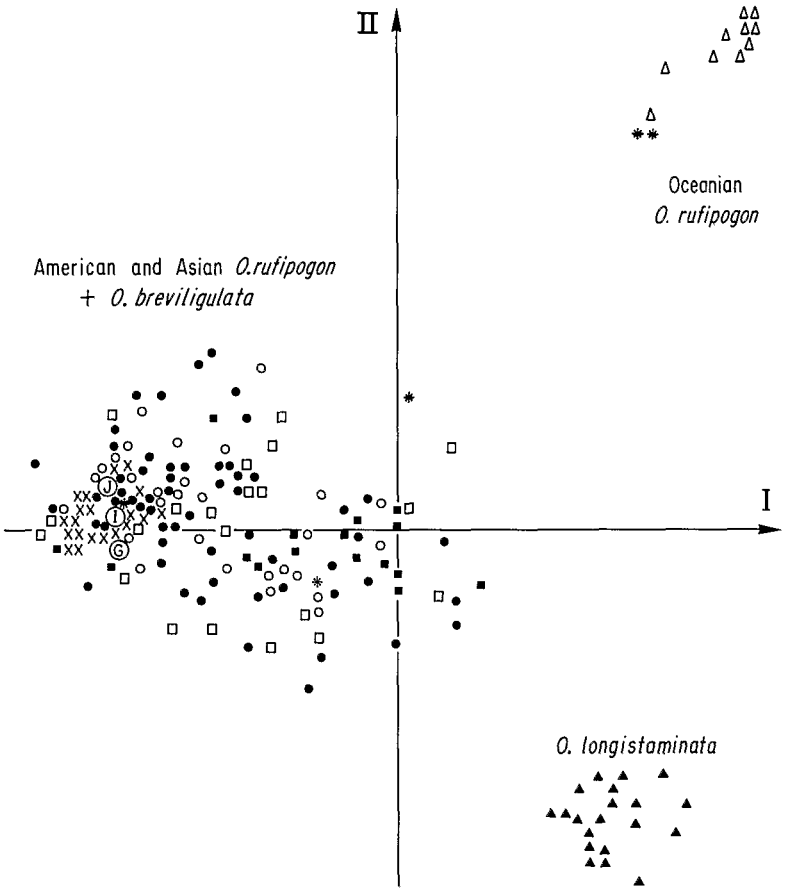
3.2.1.1 The *Sativa* Group

A fairly complete collection of the *Sativa* group with Australian, Chinese, South and Southeast Asian, African, and American origins was studied at 24 isozyme loci (Second 1985). A striking result is apparent in Fig. 2. Only *O. longistaminata* and the Australian form of *O. rufipogon* can be unambiguously distinguished from the remaining taxa of the group. Intermediates between the Asian and Australasian forms of *O. rufipogon* are found, however, in New Guinea (other intermediates were recently collected in Australia (Second 1987) but await further characterization. As judged from herbarium vouchers, other peculiar phenotypes occur in New Guinea).

The maximum genetic distance was found between the Australian taxa and others with common alleles differing at 10 out of 24 loci. On the other hand, the three basic isozyme patterns in cultivated rice, *glaberrima*, "ancestral" *indica* and *japonica* (see Sect. 3.2.2.1) were all found to be relatively similar to each other and to *O. breviligulata*, compared with the diversity found within the Asian form of *O. rufipogon* itself.

When the Asian *O. rufipogon* was considered alone, some Chinese strains appeared divergent from any strain originating outside China, while being very close to the *japonica* isozyme set. They originated in Guangxi province and showed no trace of introgression from cultivars. On the other hand, the *indica* isozyme set was closer to many strains found in South and Southeast Asia.

In view of the large polymorphism in the *Sativa* group, another striking result was that the American form of *O. rufipogon*, although morphologically slightly divergent, shares all its common alleles with the Asian form of *O. rufipogon*. This fact



Δ : Australia ; * : New Guinea ; ▲ : *O. longistaminata*, Africa ;
X : *O. breviligulata*, Africa ; ● : India, Sri-Lanka, Nepal, Bengladesh,
Burma, Thaïland, Cambodia ; ○ : China ; □ : Malaya, Indonesia,
Philippines ; ■ : South-America, Cuba .
Ⓖ : Glaberrima ; Ⓘ : Indica ; Ⓙ : Japonica .

Fig. 2. The genetic structure of the spontaneous forms of the *Sativa* group of species as seen at isozyme level. One hundred and eighty one strains are plotted in the first plane of a principal coordinate analysis of the genetic distances in strain comparison at 24 loci. The geographic origins or taxonomic classifications are indicated by a conventional sign. The positioning of cultivated rice is indicated by the three basic patterns of *indica*, *japonica*, and *glaberrima* types of cultivars. (Second 1985a)

definitively proves that the American strains did not diverge independently from their Asian relatives for a very long time. They have rather been recently introduced, hence probably by man, and naturalized in America.

3.2.1.2 The *Latifolia* Group

Strains representing most of the various forms and species described in this group (except from China) were studied (Second 1984). Figure 3 shows the relationships among them as analyzed at 17 loci.

Representatives of each diploid genome were found at extremes of the distribution, while allotetraploids, and also some diploids with the CC genome, were found intermediate between them; intermediate diploids include *O. eichingeri* and a group of *O. officinalis* from Indonesia and the Philippines. Accordingly, two groups were formed within *O. officinalis*: one presumed ancestral (CC1) and one presumed introgressed (CC2) with genes from BB (differentiation is supposed to take place monotonously with geological time but intermediate derivatives can arise through hybridization). The genome D is not known; a putative isozyme set D was deduced from the comparison between C1 and CD genomes.

Compared to conventional taxonomy, the classification was made easy within such complexes as *O. eichingeri*, diploid and tetraploid *O. punctata*, or between *O. officinalis* and tetraploid *O. minuta* or *O. malampuzhaensis*.

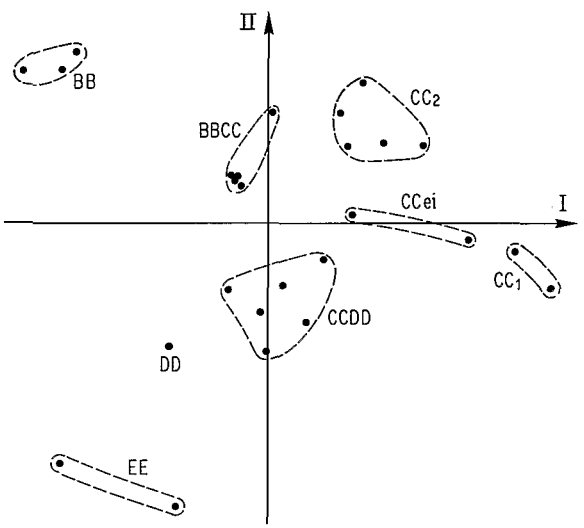


Fig. 3. The genetic structure of the *Latifolia* group of species as seen at isozyme level. Twenty six strains are plotted in the first plane of a principal coordinate analysis of the genetic distances in strain comparison at 17 loci. The groups are symbolized according to the genome or subgenome they represent and correspond to the following taxonomical classification. *BB* = *O. punctata* (diploid); *CC1* = *O. officinalis* "ancestral"; *CC2* = *O. officinalis* introgressed with genes from genome BB: *CCei* = *O. eichingeri*; *DD* = a putative isozyme set corresponding to the unknown DD genome; *EE* = *O. australiensis*; *BBCC* = *O. punctata* (tetraploid), *O. minuta*, and *O. malampuzahensis*; *CCDD* = *O. latifolia*, *O. alta* and *O. grandiglumis*. (Second 1984)

A remarkable observation was that the maximum distances found between the B, C, and E genomes [Standard Nei's distance, (Nei 1975) of the order of 1] was not greater than those found between the Australian and other strains of the *Sativa* group with the single genome A. This observation was best accounted for by a parallel evolution of both the *Latifolia* and the *Sativa* groups under the same forces of migration or isolation between continents (see Sect. 3.4).

3.2.2 The Cultivated Species

Isozymes were particularly useful to unravel the genetic structure of cultivated rice. The results may be summarized as follows:

3.2.2.1 On Two Species of Cultivated Rice

While no one would dispute an Asian origin for *O. sativa*, Portères (1950), after A. Chevalier (see Roschewicz 1931), suggested an independent domestication of *O. glaberrima* from *O. breviligulata* in Africa. This hypothesis was challenged, however, particularly by Nayar (1973), who proposed that the origin of *O. glaberrima* was also in India.

The relationship between *O. sativa*, *O. glaberrima*, and the latter's wild and weedy relative *O. breviligulata* were studied in detail at 40 loci (Second 1982). The following conclusions were reached:

- As shown in the multivariate treatment of the data in Fig. 4, the cultivated, wild and weedy African species form a group clearly distinct from *O. sativa*. The diversity of *O. breviligulata* is greater than that of *O. glaberrima*: domestication has reduced the gene diversity but has not selected new alleles.
- Two groups are clearly distinguished among *O. sativa* which correspond to the subspecies *indica* and *japonica* Kato (1930) or to the traditional distinction (among others) of the Hsien and Keng types by the Chinese.
- Intermediate types between the *indica* and *japonica* subsp. exist that show particular alleles and/or particular characters such as adaptability to upland or to counter season cultivation, short cycle and high quality grains (slenderness, high cooking elongation, fragrance, etc.) which probably represent secondary acquired characters in the course of the domestication process.
- The distribution of F_1 pollen sterility relationships among *O. sativa* is related to some extent to its isozyme polymorphism as it leads to the extraction of two small groups of varieties with a complementary set of isozymes, assumed for that reason to represent the "ancestral" *indica* and *japonica* isozyme sets, respectively.
- An approximately equal genetic distance was found between *O. glaberrima* and the "ancestral" *indica* and *japonica* in the three combinations as shown in Fig. 4: about 15 allelic discordances over 40 loci scored.

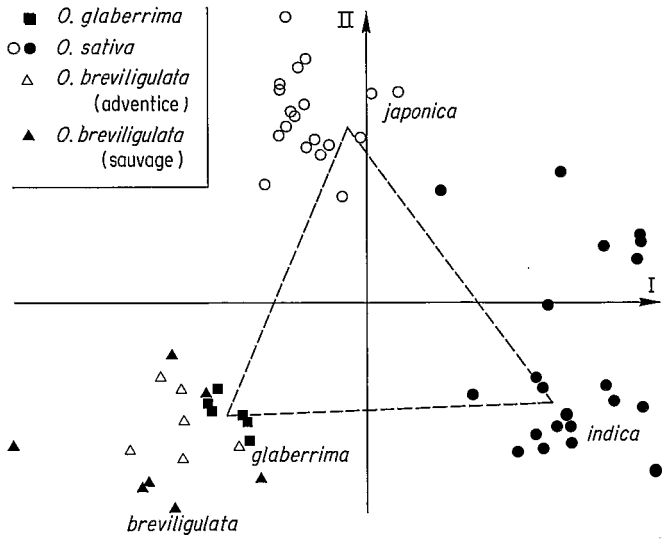


Fig. 4. The genetic structure of Asian cultivated rice (*O. sativa*), its relationship with African annual rice, cultivated (*O. glaberrima*) and wild or weedy (*O. breviligulata*), as seen at the isozyme level (Second 1985b). Sixty strains are plotted in the first plane of a principal coordinate analysis of the genetic distances in strain comparison at 40 loci. Their classification is indicated by conventional signs. Among *O. sativa*, an *open symbol* indicates a negative phenol reaction and allows the distinction of an *indica* and a *japonica* group. The *dotted triangle* shows that approximately equal genetic distances are found among the three groups of cultivated rice (about 15 allelic discordances over 40 loci scored)

3.2.2.2 On Two Main Subspecies Among *O. sativa*

Several other studies were reported on the isozymic differentiation within *O. sativa* (see Endo and Morishima 1983 for a review). The most complete and up to date are, however, those of Glaszmann (1987, 1988) for the Asian varieties and de Kochko (1987a,b, 1988) for the African varieties, including Madagascar. The former includes a study of 1688 traditional varieties from Asia on 15 polymorphic data. From a multivariate analysis shown on Fig. 5, six groups of varieties could be identified. As many intermediates are found, the definition of "groups" is based on the relative abundance of varieties with closely related genotypes more than on any discontinuity in the relatedness of the various genotypes found. Only two groups of varieties (groups 3 and 4) appear, however, well distinguishable. They represent a small number of deep water varieties originated in Bangladesh. While group 1 and 6 clearly corresponded to the subspecies *indica* and *japonica* Kato, groups 2,3,4 and 5 were little represented in the studies conducted previously and their distribution is largely restricted to South and West Asia, mostly in the Himalayan foothills. They are genetically partly intermediate in the *indica/japonica* differentiation, but also show originalities in their allelic composition which seem to be generally related to the diversity of *O. rufipogon* in South Asia (Glaszmann 1988).

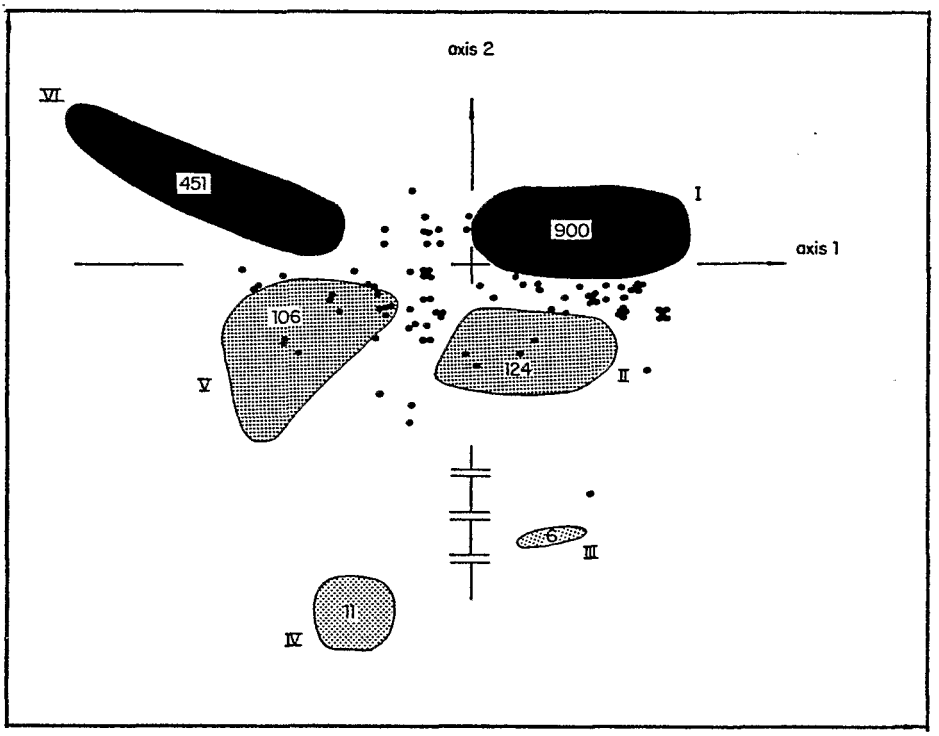


Fig. 5. Isozyme classification of the varieties of *O. sativa*. The positioning of six varietal groups on planes (*1,2*) of a factorial analysis of correspondences of isozyme variation at 15 loci among 1688 varieties. *Isolated dots* represent 90 varieties with intermediate positions or unstable classification. (Glaszmann 1987)

However, that the *indica/japonica* trend of variation was related to the diversity of *O. sativa* as a whole in spite of the existence of other trends was corroborated by the congruence found in an independent classification of the same varieties made on a morpho-physiological basis by Cheng (1985) and their isozyme classification (Glaszmann 1987; Zhou et al. 1988): the varieties were found in the same order on the *japonica/indica* axis of differentiation from the morphological and isozyme standpoint, respectively. A similar congruence had also been found earlier between the morphological classification by Oka (1958) and the isozyme analysis of the same varieties (Second 1982 and unpubl.). This implies that the genetic structure unraveled in two main subspecies has a biological foundation. It further signifies that a common classification can be shared between those who utilize only morphological markers and those who include molecular markers (RFLP's allow a similar classification as isozymes, Wang and Tanksley 1989 and unpubl.). This is an important issue because unless species, subspecies, varieties, or individuals are correctly characterized and named, knowledge about them cannot be communicated universally. It should not be overlooked, however, that many of the varieties called "*indica*" in the IRRI-IBPGR classification of cultivated varieties of rice, in

particular many upland varieties, also belong to the subspecies *japonica* Kato, as judged from their molecular marker imprint (Glaszmann 1987). They should be better referred to as "tropical *japonica*". Actually, many discrepancies between classifications could be indicative of the large genetic flow which has taken place between the *indica* and *japonica* subspecies (Second 1982) and which is likely to increase in the future. It would then seem useful to recognize that many varieties fall in the intermediates between the *indica* and *japonica* subspecies.

On the other hand, the commonly accepted belief that a third main subspecies can be recognized in *O. sativa*, namely "*javanica*" simply does not correspond to any data (see Oka 1988). "*Javanica*" varieties represent a tropical form of *japonica* subsp. and thus one of the many agro-ecotypes that can be recognized among cultivated rice (Glaszmann and Arraudeau 1986).

Rice appears as a crop in which spontaneous hybridization is possible among all cultivated varieties, subspecies, or species and with their direct wild ancestors in spite of several crossing barriers with variable intensities (see Oka 1988). Detailed studies underway by various workers with molecular markers should distinguish the relative contribution in the build-up of the wide polymorphism of cultivated rice varieties, of (1) hybridization between the *indica* and *japonica* subsp., (2) hybridization between cultivars and wild species in the range of their distribution and (3) the *de novo* mutation since domestication. In Asia, it is difficult to distinguish the contribution of wild rice through hybridization since domestication, from its contribution as a direct ancestor. However, the study of Asian rice in Africa provides in this regard an interesting situation.

3.2.2.3 The Case of Asian Rice in Africa and Madagascar

Both *indica* and *japonica* subsp. are grown in Africa, sometimes in close proximity and in traditional conditions which favor the selection of new varieties from natural hybridization events. Weedy forms of rice belonging to the A genome (*O. longistaminata*, *O. breviligulata*) are also often mixed in the fields. Their hybridization with Asian cultivated rice can be unambiguously followed from their specific molecular markers.

The study by de Kochko (1987a,b, 1988) of 831 traditional varieties recently collected in Africa and Madagascar at 37 presumed isozyme loci shows that the introduction of Asian rice in Africa has been on a large genetic basis. The degree of isozyme diversity is nearly equal in the African vs. Asian varieties of *O. sativa*. The *indica* subsp. is associated with the lowland culture (irrigated or submersed) in low or medium altitudes while the *japonica* subsp. is associated with the upland culture (rainfed) or the high altitude lowlands. However, fewer extreme *indica* or *japonica* and more intermediate genotypes are found in Africa and Madagascar as compared with Asia. Five new rare alleles which have never been found, either in the wild species or in the Asian cultivated varieties, including three null alleles, were observed. One possible explanation may be found in the mutagenic effect of hybridization between species or subspecies. This latter explanation received strong support from the observatoin of several new alleles (including revertant from null alleles with slightly different migration velocity and reduced activity) in natural hybrid derivatives between *O. sativa* and *O. longistaminata* (Ghesquiere 1988).

New morpho-physiological types of plants are found in Africa and particularly in Madagascar (Ahmadi et al. 1988) associated with a reduced divergence between *indica* and *japonica* subsp. There is little room for doubt that, along with the introduction of numerous varieties from Asia, hybridization between the *indica* and *japonica* subsp. has played a large role in the evolution of new varieties in Africa and Madagascar. This hybridization not only resulted in new recombined genotypes, but is likely also to promote higher mutation rates thus providing the necessary genetic diversification for selection of new cultivars (Second 1986). Hybridization with wild species has probably played a still larger role in Asia than in Africa, where the reproductive barriers separating *O. longistaminata* are particularly developed (Ghesquiere 1988).

3.3 The Genetic Structure as Seen at Chloroplast DNA RFLP Level

As an example of the observed variation, Fig. 6 shows various restriction patterns of an EcoR1 digestion of the chloroplast DNA isolated from 19 individual plants taken in the *Sativa* group of species and varieties. Figure 7 depicts a cladistic analysis of 112 mutations distinguishing 32 different restriction patterns found in a study of the cpDNA diversity of 320 plants representing the section *Oryza*. A maximum of ten different restriction enzymes were used (Dally 1988; Dally and Second 1989).

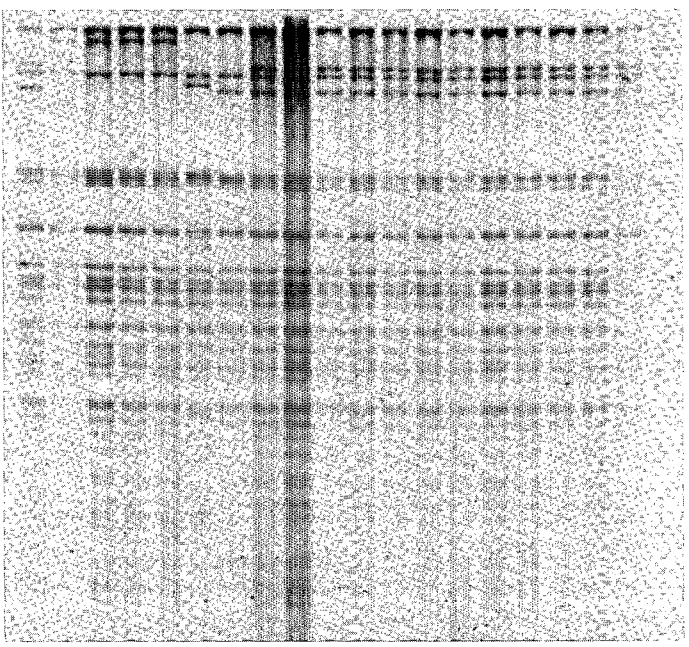


Fig. 6. Chloroplast DNA variation. Nineteen restriction patterns from EcoR1 digestion of cpDNA isolated from various wild and cultivated species and varieties of the *Sativa* group of *Oryza*. On the *extreme right*, a DNA molecular size marker. (Dally 1988)

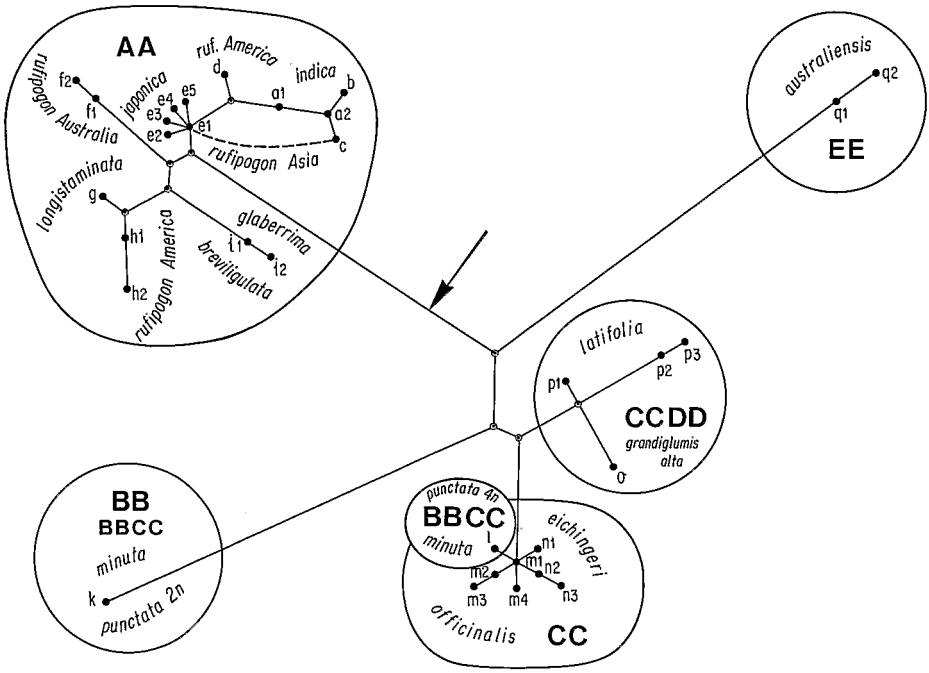


Fig. 7. The genetic structure of the section *Oryza* as seen at the level of the polymorphism of chloroplast DNA: Cladogram showing the relationship between 32 cpDNA restriction patterns distinguished by 112 mutations. Branch lengths are proportional to the number of branch specific mutations. The *arrow* represents the root of the cladogram and confirms the distinction of a *Sativa* and a *Latifolia* group of species. (Dally 1988)

The agreement with the genetic structure deduced at the chromosome level ("genomes") and with more detail at the isozyme level is striking. A new type of information appears, however, particularly on the differential similarity of the nucleus and the cytoplasm in presumed hybrid derivatives, compared with the parents. For example, certain American forms of *O. rufipogon*, with clear affinity to Asian *O. rufipogon* at the isozyme level, have close similarity with the African *O. longistaminata* at the cpDNA level. Several other cases appear. They can be explained by a nucleo-cytoplasmic substitution following an introgressive hybridization event: as the cytoplasm is predominantly inherited in a uniparental fashion from the mother, the nucleus of the recurrent male parent may largely substitute the female parent nucleus in a backcrossing process (F_1 hybrids are often male sterile but partly female fertile).

As suggested from the study of cytoplasmic male sterile lines, cpDNA could, however, also rarely be transmitted through the male parent (Second et al. 1989). The possibility of intermolecular recombination and subsequent selection has thus to be considered but requires further studies.

Ten plastotypes were distinguished among cultivated varieties. One corresponded to *O. glaberrima*. The nine others were found in *O. sativa*. They clustered

in the cladistic analysis in two groups which corresponded largely to the *indica/japonica* differentiation with one dominant type each. Intermediate *indica/japonica* varieties (Glaszmann's isozyme groups 2, 3, 4, 5) shared the *japonica* plastotype more often than the *indica* plastotype. The rare plastotypes corresponded to certain of the cytoplasms which induce male sterility and to unique varieties with particular features distributed in various parts of the world (Dally and Second 1988, 1989).

3.4 Evolutionary Interpretation

An evolutionary scenario based on the geographic (different continents and subcontinents) and ecological (undisturbed vs. man-disturbed) structure of the genetic diversity found in both the *Latifolia* and the *Sativa* groups appear to be possible assuming the postulate that migration of wild rice in nature occurs only on land and over short distances at a time, but that man has promoted the migration of wild and cultivated rice (directly and indirectly) across oceans and high mountains. The history of the paleoenvironment since the Tertiary era indicates the possible routes (in time and space) of migration between continents to account for the pan-tropical distribution of the section *Oryza*. The application of the molecular clock concept to molecular divergence between taxa presumably isolated over geological time provides an additional guideline. The consistency of the obtained scenario is self-supporting (Second 1984, 1985a,b).

A common ancestor of the genus *Oryza* in Eurasia during the Tertiary era is a reasonable assumption, since only in Asia are the forest-adapted species such as *O. meyeriana*, *O. ridleyi*, and *O. officinalis* found (Based on their relationships with Asian species, *O. eichingeri* in Africa and the American species are assumed to be recently naturalized). Both the *Sativa* and *Latifolia* groups migrated to Australia and Africa during the Tertiary era by land and remained subsequently isolated by geographic and/or climatic barriers. A much more recent exchange between Asia and Australasia has, however, occurred during the glaciations when the sea level was low. The recent uplift of the Himalaya range (Liu and Ding 1984) can explain the divergence between Chinese and non-Chinese Asian populations of wild rice. As indicated in Table 2, a remarkably good agreement was found between electrophoretic dating and the sequence of events in the paleoenvironment that should have created barriers to the migration of wild rice within the Old World.

A remarkable feature of that scenario is that it explains the differentiation of both the *Sativa* and the *Latifolia* groups of species through the rise of the same geographical or climatic barriers between continents or subcontinents. In this scheme, man intervened by carrying seeds, both of wild species and cultivars, across the natural barriers: (1) between China and South/Southeast Asia; (2) between Asia and Africa; and (3) to America.

New forms of *Oryza* arose in the process of introgression and allotetraploidization, in a way that is suggestive of what could be done to breed new types of rice.

The origin of the CD genome, found only in America, is most puzzling and still not properly resolved. Recent information concerning that problem is found in the

Table 2. The correspondence between electrophoretic dating for the main genetic divergences within the *Sativa* and *Latifolia* groups and the tectonic or climatic events in the paleoenvironment that should have created a barrier to the migration of species of *Oryza* according to their environmental requirements (After Second 1985b)

Electrophoretic dating of genetic divergence	Tectonic or climatic events in the paleoenvironment
Australian vs. non-Oceanic strains of the *Sativa* or *Latifolia* group: 15 My[a]	Collision of the Australasian plate with Southeast Asia
Asian vs. African strains, *Latifolia* group (basically forest-adapted): 15 My	– Opening up of the Red Sea – Establishment of a climatic barrier between Asia and Africa according to the sequence:
O. longistaminata (adapted to humid savanna in Africa) vs. Asian *O. rufipogon*: 7 My	humid forest humid savanna dry savanna desert (for rice)
O. breviligulata (adapted to dry savanna in Africa) vs. Asian *O. rufipogon*: 2–3 My	
Chinese vs. South/Southeast Asian *O. rufipogon*: 2–3 My *indica* vs. *japonica* subspecies of *O. sativa*: 2–3 My	Emergence of the Himalayas as a barrier to land migration

[a] My = millions of years.

diversity of cpDNA. The CD genome species are significantly divergent from all others and are also polymorph (Fig. 8). However, all mutations specific to the CD species (mostly addition/deletion vs restriction site mutation) affect restriction bands which are also variable among other species and are likely to correspond to "hot spots" in the cpDNA molecule. In contrast, the differentiation of the cpDNA corresponding to the B and E genomes include site mutations on bands otherwise monomorphic in the section *Oryza*. It is thus plausible that the CD-genome species have rapidly acquired this divergence at the cpDNA level.

Due to their close relationship, in particular with the C-genome species at both the isozyme and RFLP levels, an independent evolution of the American species of *Oryza* for many millions of years appears inconsistent with the molecular data. As it may be doubted that seeds could naturally cross the large stretch of ocean from Asia to America, the only possibility left is their introduction by man, probably during the last three centuries. The divergence of the nuclear D genome would have been rapidly acquired at the tetraploid level.

When compared with the published mutation rate estimated in cpDNA of grasses, the accumulated number of site mutation in the cpDNA of the E and B genomes allowed an estimation of the date of divergence compatible with our earlier estimation based independently on isozyme divergence, i.e., 15–20 million years (Dally 1988).

Overall, the above hypothesis for the evolution of the section *Oryza* appears compatible and agrees well with all facts known to the author. In a previous attempt

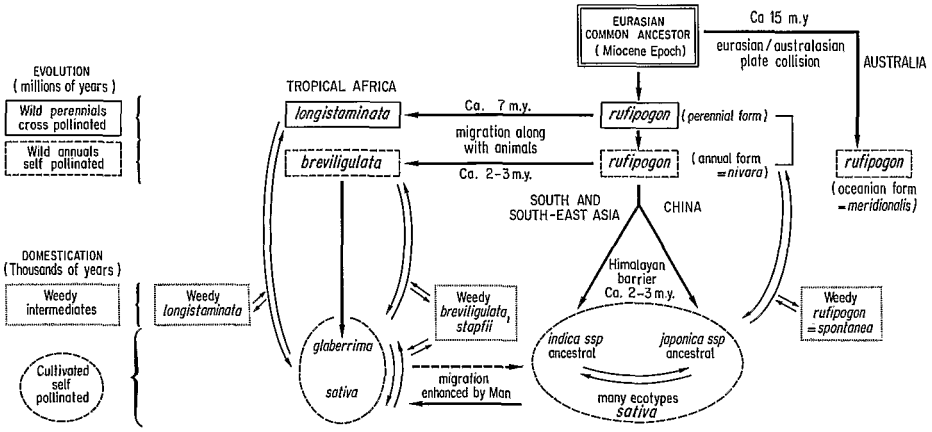


Fig. 8. Phylogenetic relationships of the two cultivated rice species in Asia and Africa. Taxa *boxed by solid lines* are wild perennials. Taxa *boxed by broken lines* are annuals. Taxa *boxed by dotted lines* are weedy types. *Arrows in heavy lines* indicate direct descent. *Double arrows* indicate introgressive hybridization, which seem to occur between all sympatric or parapatric forms except between *O. longistaminata* and *O. breviligulata* which are isolated by reproductive barriers particularly developed. The subspecies *indica* and *japonica* are meant to represent incipient cultivars in China and South/Southeast Asia respectively which later hybridized between them and with various forms of *O. rufipogon*. They correspond to a fundamental dichotomy within *O. sativa*. (Second 1985b)

to explain the pan-tropical distribution of wild species of rice, Chang (1976) claimed that continental drift was responsible for the distribution not only of the Oryzeae tribe but also of all the species of *Oryza*. The closest common ancestor of *O. glaberrima* and *O. sativa* would date back to the Cretaceous. Such claims to explain the separation of extant species or closely related species from continental movement that took place in the Mesozoic are particularly suspect (Briggs 1987). Our explanation appears, on the contrary, consistent with the present state of knowledge on the origin and evolution of the grasses (see Clayton and Renvoize 1986).

As more molecular data accumulate, this explanation of the present day distribution and genetic structure of the section *Oryza* will be further tested, but also may provide a framework model to pinpoint some features of the molecular evolution of specific markers.

The application of the molecular clock concept might be consistent only because:

1. Prior to a recent period, the species of rice were isolated during millions of years on different continents, without any possibility of genetic exchange. When this genetic exchange recently became possible, it was slowed down by various reproductive barriers and the coadaptation of the genomes. Besides, some of the species have remained strongly isolated.
2. Isozymes represent active products of genes, which are the same in most organisms and thus, in the long run, leave room for only nearly neutral evolution. Similarly, chloroplast DNA is saturated in genes and highly con-

served besides a few "hot spots". With the total rice chloroplast DNA sequence now available (Hirai, Chap. VI.4, this Vol.), more refined tests of the molecular clock in rices will become possible. Other types of sequences may then appear to be much less conservative. For example, according to our hypothesis, the American species of wild rice were introduced recently in America and yet show some extent differentiation. They may represent a good case of very rapid divergence. The cultivated varieties vs. their wild ancestors are another case of rapid evolution as pointed out by Darwin. They represent good material to study the "fluidity" of part of the genome vs. the conservatism of other parts, including genes.

Our explanation may also point to interesting studies of the evolution in ecological adaptation of various taxa. It is, for example, striking to note that, in both Africa and Australasia, the adaptation of the forest-loving *Latifolia* group to very arid environment was irreversible. The existence of counterparts of this group in the forests of Africa and New Guinea is due to recent (Pleistocene) introductions. A similar trend appears when comparing the perennial vs. the annual life form of *O. rufipogon* in Australasia.

Figure 8 diagrammatically depicts the proposed phylogenetic relationships of cultivated rices with their ancestors in the *Sativa* group. Emphasis is given to the role of the Himalaya mountain range as a geographic barrier which promoted in the past the differentiation of the wild progenitors of the *indica* and *japonica* subsp., respectively. The sequence of events that led to the present status of domestication is not yet clear. It involved hybridization between Chinese and South Asian origins of cultivated or/and wild rice. A valuable comparison of the genetic structure of *O. sativa* with what is observed in hybrid zones in natural environments can then be made, including the appearance of new genetic diversity (Second 1982, 1986; concerning hybrid zones see Govindaraju et al. 1989).

4 Mapping of the Rice Genome

Although it is possible to make taxonomic inferences from unmapped markers distributed throughout the genome, knowing the chromosomal position of the markers adds a new dimension to the study of molecular markers in systematics. In particular, using numerous mapped markers, it should be possible to detect "mosaic" genomes, that is genomes constituted of chromosome segments from different evolutionary lineages: when comparing the chromosome similarity in two individuals from two different varieties or species, different chromosome segments would have different degrees of similarity. Such situations are to be expected in the case of reticulate evolution that characterizes the rice evolutionary scenario as drawn above: through introgression or allotetraploidization, two divergent lineages genetically intermix. However, it is in breeding work that maps based on molecular markers will have the greater impact.

Tanksley et al. (1988b) have presented the promises of the integration of RFLP mapping in plant breeding programs: (1) to expedite the movement of desirable

genes among varieties, (2) to allow the transfer of novel genes from related species, (3) to make possible the analysis of complex polygenic characters as ensembles of single Mendelian factors, and (4) to establish genetic relationships between sexually incompatible crop plants.

The accomplishment of a saturated molecular map in rice is in rapid progress. We will only summarize here the present status of that map.

4.1 RFLP Map

As so far published (McCouch et al. 1988), the RFLP map in rice is composed of 135 loci corresponding to clones selected from a PstI genomic library. This molecular map covers 1389 cM of the rice genome and exceeds the current classical maps (Kinoshita 1986; Khush and Singh 1986) by more than 20%. As a by-product of this mapping project, the earlier proposed notion that rice contains a high proportion of single copy sequences (Deshpande and Ranjekar 1980) was documented, it was shown that rice contains a significant amount of RFLP variation and indirect evidence indicated that a large proportion of the RFLP's in rice are generated by insertions/deletions. One clone hybridized to sequences in both the *indica* and *japonica* genomes which has apparently transposed since the divergence of the two cultivars from their last common ancestor. Also, rice DNA appeared to be less C-methylated than many other crop plant DNA's.

The map was generated from F_2 segregation data (50 individuals, see Fig. 9 for an example) from a cross between an *indica* and a tropical *japonica* rice cultivar (isozyme groups 1 and 6 respectively, Glaszmann pers. commun.). Primary trisomics (Khush et al. 1984) were used to assign linkage groups to each of the 12 rice chromosomes as reproduced in Fig. 10. Twenty five of the loci analyzed in the F_2 population deviated significantly from the expected monogenic ratio, among which 23 were in favor of the *indica* allele. When considered in bulk, the remaining loci showed also an average significant deviation in favor of the *indica* alleles, in accordance with the general trend in *indica/japonica* hybrid derivatives so far evidenced (Oka 1988). Several possible mechanisms could account for that. At any rate, this map shows that there is a high rate of recombination in hybrids

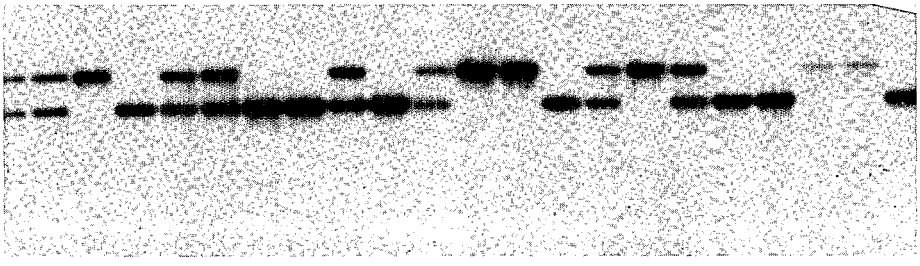


Fig. 9. Autoradiogram obtained from hybridizing a single copy clone onto DNA from the F_2 population used to derive the molecular map in Fig. 10. *Lower band* = *japonica* parent homozygote; *upper band* = *indica* parent homozygote; *both bands* = heterozygote

between the subspecies *indica* and *japonica*, contrary to the common belief of rice breeders.

As appears on the map in Fig. 10, several chromosomes are in pieces as if large sections of these chromosomes were not polymorphic in this cross. Several possible reasons could also account for this, including the random effect. Or, as hypothesized(see Second 1982), it could be that the genomes of all modern *O. sativa* cultivars are hybrid derivatives of two ancestral *indica* and *japonica* divergent lineages and thus have certain chromosome segments in common. Whether certain chromosome segments are shared by all *O. sativa* varieties or whether different varieties have different chromosome segments in common can now be answered through direct experimentation.

A solution to the problem of differential similarity between chromosome segments in the mapping of the rice genome could lie in the use of a backcross family from a hybrid between *O. sativa* and *O. longistaminata*. According to Ghesquière (1988), Mendelian segregations for isozyme markers are more regular in such backcross populations than in F_2s of *indica/japonica* hybrids. This cross shows a high degree of polymorphism for RFLP, thus speeding the screening of polymorphic markers. A map of the rice genome in 12 linkage groups is now being rapidly obtained from this cross (M. Causse et al. unpubl.).

4.2 Isozyme Map

Wu et al. (1988) provided the largest data set and the review of current status of the chromosomal locations of 19 isozyme loci through trisomic analysis. They are distributed on 8 of the 12 chromosomes of rice. Additional linkage data were obtained by Guiderdoni et al. (1989) among doubled haploid lines derived from a *japonica* × *indica* cross. These same lines are currently being mapped by RFLP's as well (S. McCouch pers. commun.) so that most of the isozyme markers should soon be included in the RFLP map.

5 Short-Term Perspectives

In spite of its importance as a dietary staple for a large part of the world's population, until recently relatively few researchers were involved in basic genetic and breeding research in rice as compared with other major cereals. This situation has changed due to a conjunction of factors including (1) the recent developments in molecular biology and in vitro manipulation methods which have encouraged interest in rice as a model, and (2) the availability of worldwide collections of rice and its wild relatives which make genetic, ecological, and evolutionary studies on this material very attractive.

In the field of molecular markers, progress is being made in the study of repeated sequences and ribosomal gene families with the particular aim of determining genome-specific sequences which can be used in *in situ* hybridization or dot blot experiments (Zhao et al. 1989; Cordesse et al. 1989, P, Gustafson, G. Kochert

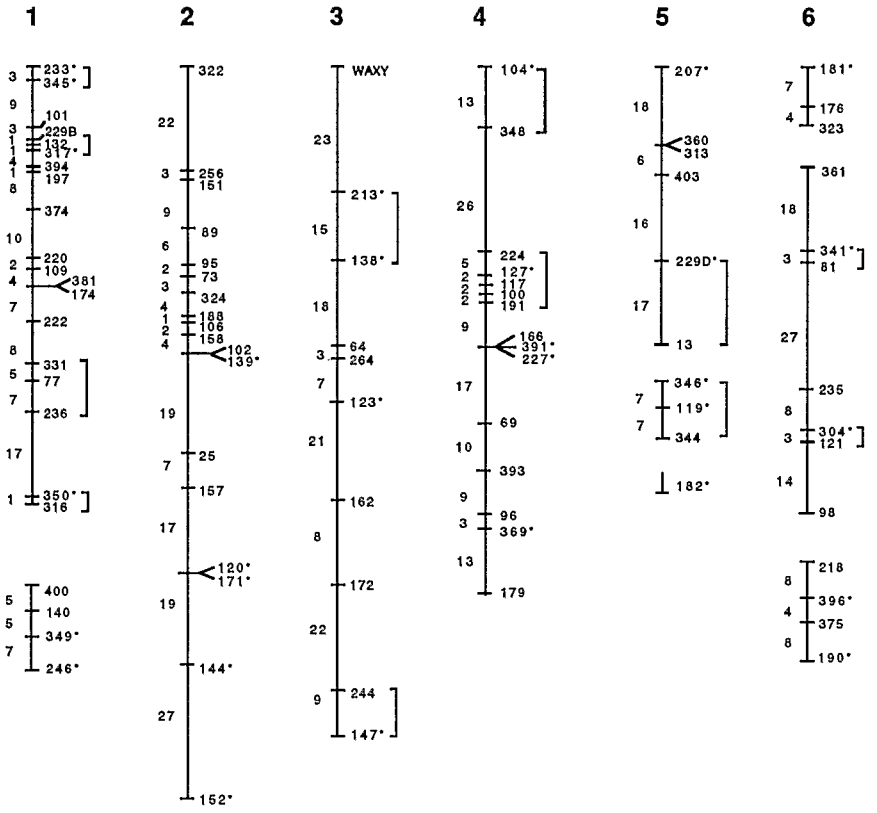


Fig. 10. RFLP molecular map of rice. *Numbers at top* indicate chromosomes. *Asterisks* indicate chromosomal location verified by trisomic dosage analysis. Orders where the confidence level is below 99% are bracketed. (McCouch et al. 1988)

pers. commun.). Encouraging results for high resolution "fingerprinting" of cultivars were obtained (Dallas 1988). The RFLP markers developed for the mapping project are being utilized in the same way as isozyme diversity for phylogenetic studies. Knowing their linkage position in the genome will allow the mapping of the variation between varieties and taxon, and hopefully allow researchers to distinguish between ancestral forms and likely hybrid derivatives, hence greatly improving our understanding of the intricate rice phylogeny. Besides, such a large number of cloned probes have become available that it is possible to choose among them for a reasonable degree of polymorphism in the set of taxa considered. It will soon be possible to quantify the divergence between any set of taxa.

The map itself will become more and more saturated to make feasible the application of other techniques to isolate genes of interest between markers located a few million bases apart (in rice, calculation based on the size of the map and the total DNA content show that 1 centimorgan of recombination equals approximately half million base pairs on average). The physical location of markers on the

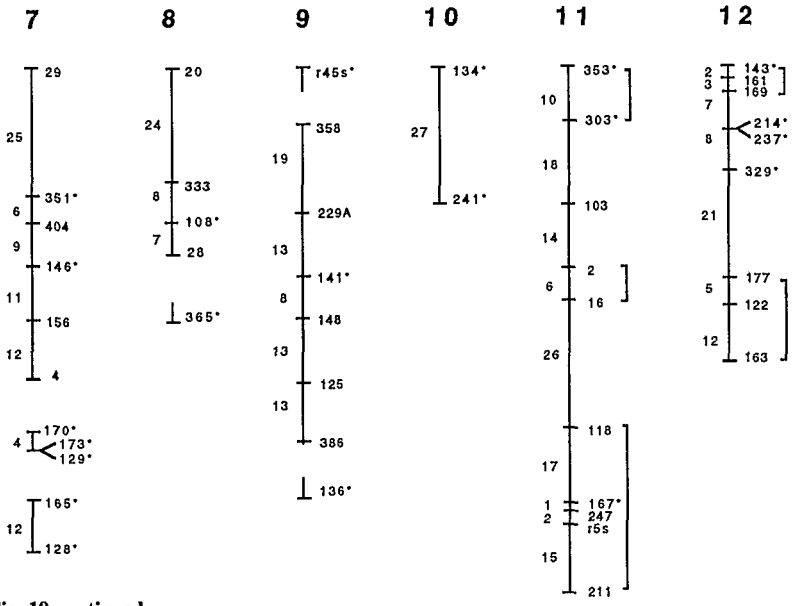


Fig. 10 continued

chromosomes is being pursued through *in situ* hybridization techniques and physical mapping of the chromosomes based on the pulsed field electrophoresis of large DNA restriction fragments and their hybridization with genetically mapped clones. The location of some major disease and stress resistance genes on the RFLP map is being pursued through the use of near isogenic lines (S. Tanksley pers. commun.).

The development of large projects such as the sequencing of the human and possibly the rice genomes will no doubt have a tremendous impact on the development of the technology. Automatized analyses of comparative sequencing and chromosome marking will become available. We are at a turning point in the uses to which this technology will be put, and rice may become a model plant for these accomplishments.

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