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ISOZYMES AND PHYLOGENETIC RELATIONSHIP IN *ORYZA*

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Isozymes have proven to be good markers for making systematic and phylogenetic inferences in many living organisms. A technique was developed to reveal isozymes at more than 40 loci in rice and was applied to the study of the genetic structure of the *Sativa* and *Latifolia* groups of species. This paper presents a summary of the results and their interpretation that were published elsewhere. The distinction of two cultivated species and of the *indica* and *japonica* subspecies of *O. sativa* was confirmed. No selection of particular alleles was observed in cultivated *O. glaberrima* compared with its direct wild ancestor. The interrelationships among wild species of both the *Sativa* and *Latifolia* groups can be interpreted as reflecting divergence due to genetic isolation. They suggest an evolutionary scenario coherent with tectonic and climatic modifications in the Old World since the mid-Tertiary era. Before domestication, the Himalaya range enhanced a differentiation of *O. rufipogon* in China and South/Southeast Asia, which is related to the *indica-japonica* genetic structure. Reciprocal introgression between the *indica* and *japonica* subspecies has probably been determinant in building the genetic diversity of *O. sativa*.

The term isozyme describes different molecular forms of enzymes with the same substrate specificity. They may represent products of different alleles at a given locus (allozymes) or products of different loci. Their study has gained popularity since the 1960s when the simple electrophoretic technique was developed and revealed an unexpectedly large amount of genetic polymorphism at the molecular level within populations and species of various organisms. Additional techniques coupled with electrophoresis, such as the study of isozyme thermostability, have unraveled still more variability, although it is now well established that these techniques are able to evidence only part of the total variability at the level of DNA. The polymorphism "hidden" by these techniques is large when individuals that are compared belong to more distantly related taxonomic units.

The isozyme approach has been widely applied to the study of plant genetics and breeding (20). Still, compared with animals, it has been applied very little to the study of phylogeny in plants. The reasons for this situation appear to be well exemplified in the case of *Oryza*.

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Research on rice isozymes started early (4) but was limited to general enzymes that are stained by artificial substrate and show complex zymograms (peroxydases, esterases, acid phosphatases). Considering the large genetic diversity present in a single species group like the Sativa group in *Oryza*, it is not possible to infer loci and allelic relationship from such complex zymograms. The earlier studies failed to use the basic power of isozymes compared with morphological characters.

Another factor seems to have refrained researchers from using isozymes for phylogenetic studies on rice and other plants. Earlier studies showed that isozymes are often under apparently strong direct selection. Their polymorphism can be supposed to be subjected to genetic convergence according to a selection pressure like domestication (5). On the contrary, it has become more and more apparent that enzyme electrophoresis provides data that differ fundamentally from morphological, macroscopic characters. They represent primary products of structural genes that appear to be well conserved among organisms compared to other classes of DNA. They are thus genetically determined in a simple way and are generally neutral to direct selection or subjected to only slight selection coefficients (although they may be associated with selected genes on the chromosome segments). Besides, they can be compared on the same ground between the cultivars and the weedy or wild forms of a domesticated species complex.

We developed a technique on starch gel to strain more than 13 rice enzymes encoded at more than 40 loci. Provisional inferences on genetic determinism were based on the comparison of polymorphism observed in self-pollinated and closely related outcrossed species, and on general knowledge of the secondary structure of the enzymes considered. At present, more than 20 of these loci have been checked for Mendelian segregation of various alleles among the Sativa group of species (19). So far the results have always confirmed previous assumptions and have enhanced confidence in an assumed genetic determinism.

This technique was used to determine genetic structure and genetic distances among cultivated rice and the Sativa and Latifolia groups of species constituting the section *Eu-Oryza* of the genus *Oryza*. Because of the large genetic diversity studied, and because of some technical constraints, not all loci were always surveyed; but at least 17 or 24 were analyzed.

Detailed results have been published elsewhere (14, 15, 16, 17). This paper gives a general overview of the main results and their interpretations from the phylogenetic viewpoint.

THE GENETIC STRUCTURE OF CULTIVATED RICE

While none would dispute an Asian origin for *O. sativa*, Portères (12) suggested an independent domestication of *O. glaberrima* in Africa. This hypothesis was challenged, however, particularly by Nayar (9), who proposed that the origin of *O. glaberrima* was also 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. The following conclusions were reached:

- As shown in the multivariate treatment of the data in Figure 1, 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* corresponding to the *indica* and *japonica* subspecies or to the traditional distinction (among others) of the Hsien and Keng types by the Chinese.
- Intermediate types between the *indica* and *japonica* subspecies exist that show particular alleles and/or particular characters such as inducing cytoplasmic male sterility or adaptability to upland or floating conditions. This point and the previous one are clearly corroborated by a study of a much larger collection of *O. sativa* (7).
- 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. All genotypes among *O. sativa* except rare alleles can be explained by hybridization between these varieties, which are assumed for that reason to represent the "ancestral" *indica* and *japonica* isozyme sets.
- An approximately equal genetic distance was found between *O. glaberrima* and the "ancestral" *indica* and *japonica* in the three combinations as shown in Figure 1.
- Some of the weedy strains (according to their occurrence in rice fields or recent fallows) of *O. breviligulata* show two alleles frequent in *O. sativa* but never found in wild strains of the same species, nor in *O. glaberrima*. In connection with field observations and artificial hybridization experiments, this strongly suggests that, according to Nayar's hypothesis, some of the weedy strains of *O. breviligulata* have their origin in the introgressive hybridization of *O. glaberrima* by *O. sativa*.

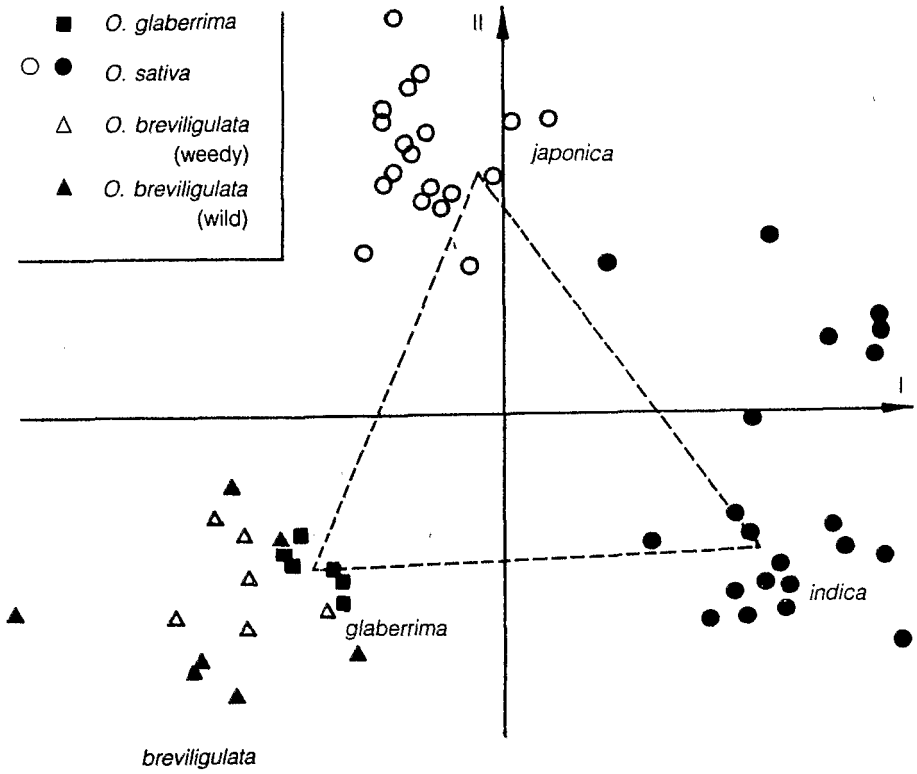


Fig. 1. Sixty strains of cultivated rice or wild and weedy *O. breviligulata* plotted in the first plane for a principal coordinate analysis of the genetic distances scored at 40 isozyme loci. Their classification is indicated by conventional symbols. Among *O. sativa*, an open symbol indicates a negative 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).

It should be stressed here that, most unfortunately, the distinction of the *indica* and *japonica* subspecies made above does not correspond to the morphological distinction of the *indica*, *japonica*, and *javanica* morphological types widely in use, at IRRI in particular. However, it very closely corresponds to the "Continental" and "Insular" groups of Oka (10) and doubtless to a basic subspecific differentiation of *O. sativa* in which the *javanica* type is part of the *japonica* subspecies. Illustrative of this fact is the observation that both the *indica* and *japonica* subspecies present all morphological types of spikelets often used to distinguish the *indica*,

japonica, and javanica morphological types.

The above results are interpreted as reflecting a differentiation of the direct ancestors of cultivated rice into three species or subspecies prior to domestication. The rich ecotypic and varietal diversity of present *O. sativa* would have arisen (a) from introgressive hybridization between the *indica* and *japonica* incipient cultivars themselves and between the cultivars and the local wild rice in the various areas of their dissemination and (b) from selection by man superimposed on this basic genetic structure.

It is well known that hybridization may be an evolutionary stimulus, particularly in areas disturbed by man and for the origin of domesticates, both plants and animals (1). We followed that model for the origin of rice. However, two main questions arise:

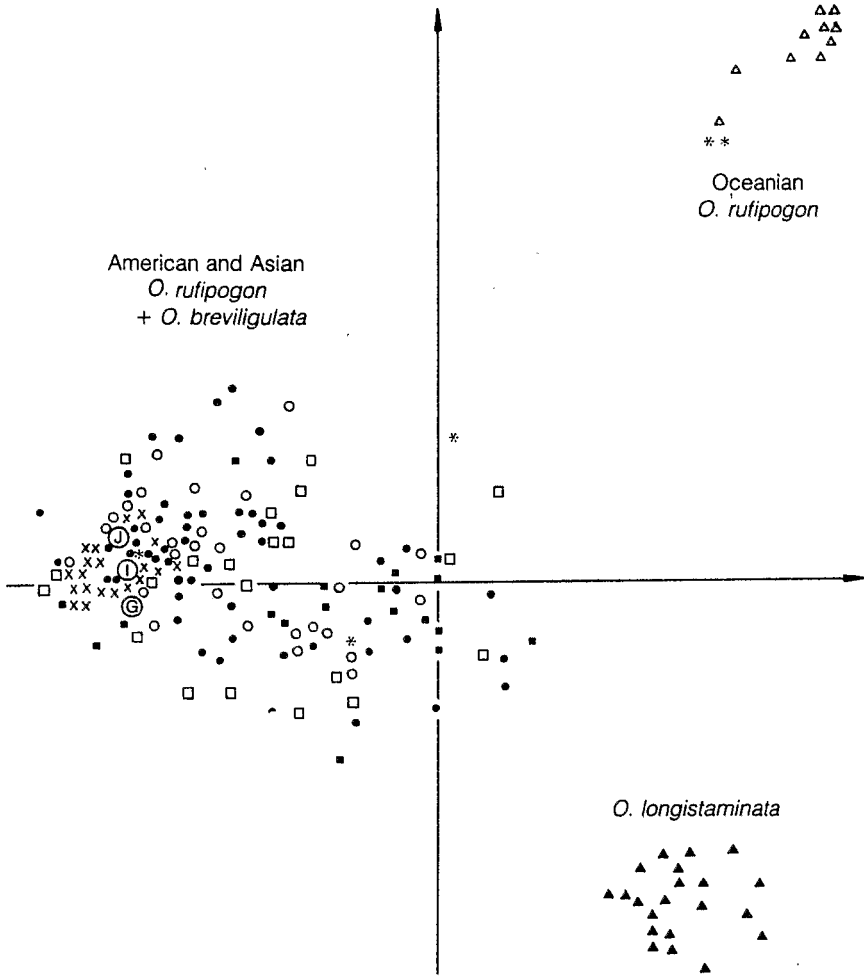
- What kind of evidence do we have to warrant the assumption of a long time of isolation of the various ancestors of cultivated rice in different environments prior to their domestication?
- Is the introgressive hybridization of *O. sativa*, which has probably built most of its diversity at the isozyme level, a mere artifact of its success in bringing it to various ecological and geographical environments or, on the contrary, does this phenomenon explain its success to a significant degree?

Elements of the answers to these questions should lie in the study of the wild relatives of cultivated rice.

THE GENETIC STRUCTURE OF THE SATIVA GROUP

A fairly complete collection of the Sativa group with Australian, Chinese, South and Southeast Asian, African, and American origins can be constituted and studied at 24 isozyme loci. A striking result is apparent in Figure 2. Only *O. longistaminata* and the Australian form of *O. rufipogon* can be unambiguously distinguished from the remaining taxa of the group. The maximum genetic distance was found between the Australian taxa and others with common alleles differing at 10 out of the 24 loci. On the other hand, the *glaberrima*, *indica* and *japonica* cultivated rice were all found to be relatively similar to each other and to *O. breviligulata* compared with the diversity 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 Kwangsi Province and showed no trace of introgression from cultivars.



△ : Australia; * : New Guinea; ▲ : *O. longistaminata*, Africa;
 × : *O. breviligulata*, Africa; ● : India, Sri Lanka, Nepal, Bangladesh,
 Burma, Thailand, Cambodia; ○ : China; □ : Malaya, Indonesia,
 Philippines; ■ : South America, Cuba
 ⊙ : Glaberrima; ⊠ : Indica; ⊡ : Japonica

Fig. 2 Strains of spontaneous forms of the Sativa species group plotted in the first plane of a principal coordinate analysis of genetic distances scored at 24 isozyme loci. The geographical origins and taxonomic classification are indicated by conventional symbols. The position of cultivated rice is indicated by the three "ancestral" isozyme patterns of *indica*, *japonica*, and *glaberrima* types of cultivars introduced as supernumerary individuals.

On the other hand, the *indica* isozyme set was closer to many strains found in South or 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 definitely proves that the American strains studied did not evolve independently from their Asian relatives for a very long time. They have rather been recently introduced, probably by man, and naturalized in America.

In contradiction to the above statement, Barnes and Pental (2) found a divergence at the DNA level of some American strains compared to a few Asian ones. However, from our study (17), those Asian strains most closely related to American strains are found in Malaysia and Indonesia. These were not included in the DNA study cited above.

THE GENETIC STRUCTURE OF THE LATIFOLIA GROUP

As understood here, the *Latifolia* group includes *O. australiensis*, because its affinity at the isozyme level confirms macroscopic observations. The *Latifolia* group thus comprises three diploid genomes (defined on cytogenetic grounds) — BB with maximal distribution in Africa, CC in Asia, and EE in Australia — and two tetraploid genomes — BBCC found in Africa and Asia and CCDD found in America.

Strains representing most of the various forms and species described in this group were studied, but only one was probably from China (it corresponded to the CCDD genome). Figure 3 shows the interrelationships 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 genome BB.

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

A remarkable observation was that the maximum distances found between the BB, CC1, and EE genomes (Nei's distance on the order of 1)

was not greater than those found between the Australian and other strains of the Sativa group with the single genome AA.

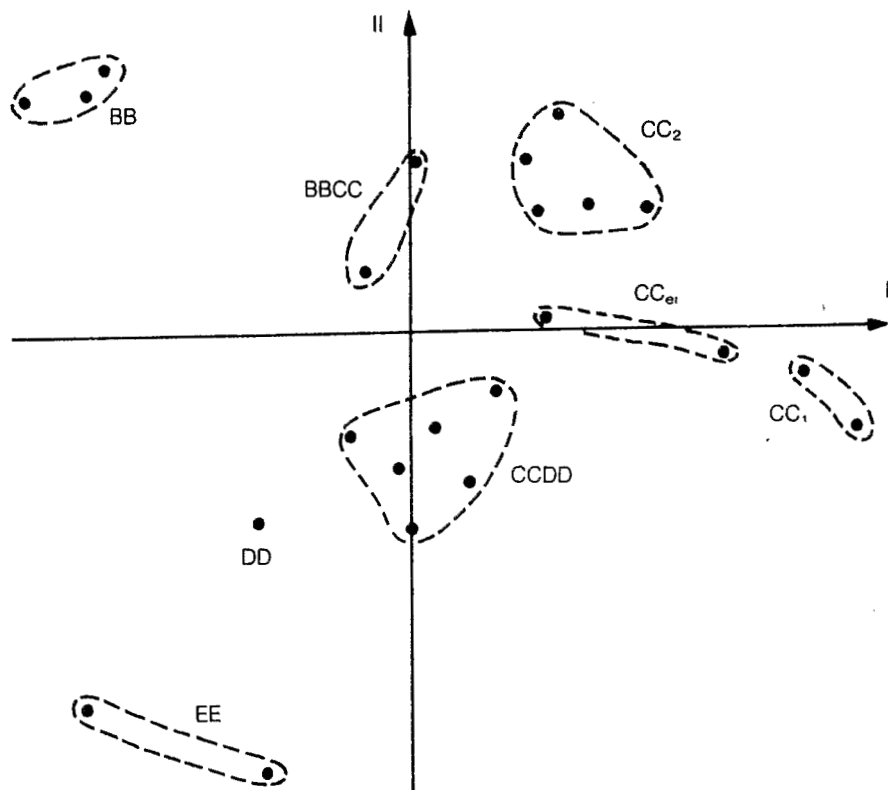


Fig. 3 Twenty-six strains representative of the various forms of the Latifolia species group plotted in the first plane of a principal coordinate analysis of the genetic distances scored at 17 isozyme loci. The groups are symbolized according to the genome or subgenome they represent and correspond to the following taxonomical classification. BB= *O. punctata* (diploid); CC1= *O. officinalis* "ancestral"; CC2= *O. officinalis* introgressed with the genes from genome BB; CCEi= *O. eichingeri*; DD= a putative isozyme set of genome DD; EE= *O. australiensis*; BBCC= *O. punctata* (tetraploid), *O. malampuzhaensis*, and *O. minuta*; CCDD= *O. latifolia*, *O. Alta*, and *O. grandiglumis*.

EVOLUTIONARY INTERPRETATION

A coherent evolutionary interpretation of the genetic structure found in both the Latifolia and the Sativa groups appears to be possible based on the two following postulates:

- In the case of genetic isolation, there exists a correlation between isozyme divergence and the time since divergence occurred (molecular clock).
- Migration of wild rice naturally occurs on land only and by short distances at a time, but man has promoted the migration of wild and cultivated rice (directly and indirectly) across oceans and high mountains.

A common ancestor of the genus *Oryza* in Eurasia seems to be a reasonable assumption, since only in Asia are found the forest-adapted species such as *O. meyeriana*, *O. ridleyi*, *O. officinalis*. (Based on their relationship with Asian species, *O. eichingeri* in Africa and the American species are assumed to be recently naturalized.) Both the Sativa and Latifolia groups may have migrated to Australia and Africa during the Tertiary era by land and remained subsequently isolated by geographical and/or climatic barriers. The recent uplift of the Himalaya range (8) can explain the divergence between Chinese and non-Chinese Asian populations of wild rice. As indicated in Table 1, 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.

Because of the relative affinity of the wild annual *O. breviligulata* with all cultivated forms and also because accumulating favorable genes should be easier within self-pollinated types, it is assumed that incipient domestication of rice started from annual forms. It might have begun independently in various areas in Asia and Africa, as it does not involve catastrophic changes in the plant architecture nor physiology. As a matter of fact, incipient domestication of annual forms is apparently still going on in Western India (18). However, a differentiation of the wild progenitors in Africa, South/Southeast Asia, and China existed prior to domestication as well as in the respective cultures of the different ethnic groups in these areas. It may be assumed that a combination of these factors has resulted in the genetic differentiation of cultivated rice into three main subspecies or species.

Subsequently, disturbance of primitive habitats and seed transportation promoted introgressive hybridization between gene pools geographically, ecologically, or genetically isolated. In that sense, no doubt, the various forms of Asian *O. rufipogon* have all contributed to the origin of *O. sativa*. In turn, many strains of the wild and weedy *O. rufipogon* have introgressed genes from cultivars. The situation, which can be studied in Africa, with introgression going on between *O. glaberrima* and *O. sativa* on one hand and between *O. sativa* and the wild perennial *O. longistaminata*

Table 1. 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.

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:
<i>O. longistaminata</i> (adapted to humid savanna in Africa) vs Asian <i>O. rufipogon</i> : 7 My	humid forest_____
	humid savanna_____
<i>O. breviligulata</i> (adapted to dry savanna in Africa) vs Asian <i>O. rufipogon</i> : 2-3My	dry savanna_____
	desert (for rice)
Chinese vs South/Southeast Asian <i>O. rufipogon</i> : 2-3 My <i>Indica</i> vs <i>japonica</i> subspecies of <i>O. sativa</i> : 2-3 My	Emergence of the Himalaya as a barrier to land migration

^a My=millions of years.

(6) on the other, could be illustrative of what has happened in Asia since prehistoric times.

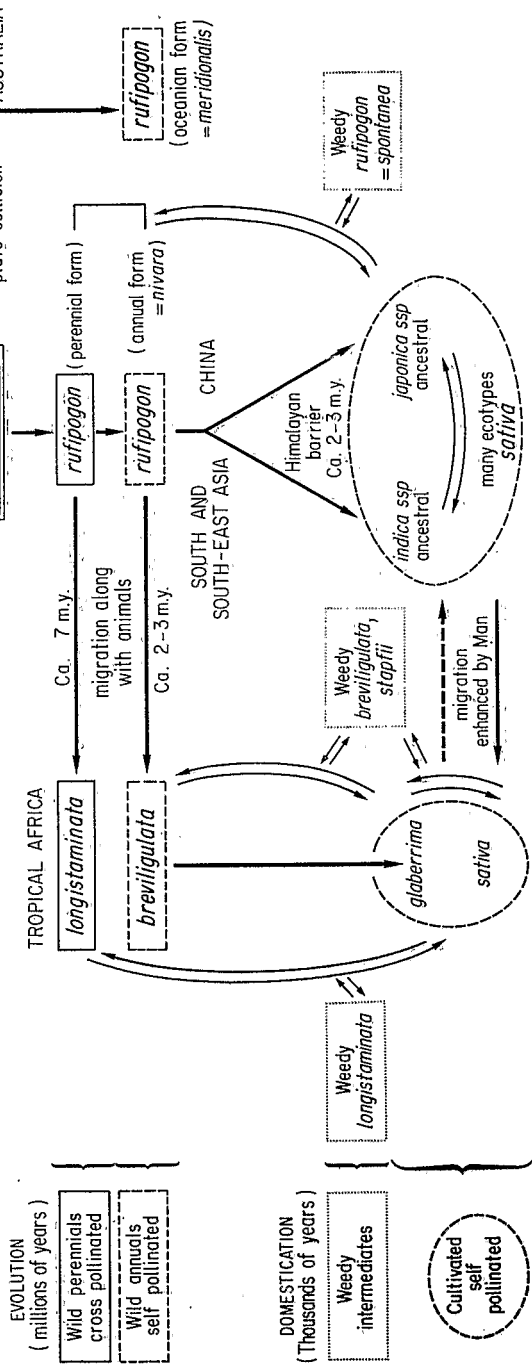
Figure 4 diagrammatically depicts the proposed phylogenetic relationships of cultivated rice with its ancestors in the Sativa group. It is deduced from the isozymic genetic structure but appears to be coherent with all facts known to the author.

This model of evolution for rice seems to be in accordance with published knowledge as a whole, even if it is contradictory with many interpretations previously put forward (3, 11) as discussed in Second (16).

We may briefly answer now the two questions raised earlier:

- The role of the Himalayan mountain range as a geographic barrier becomes even more probable when one considers that (a) the Sativa group is found in open habitats (humid or only locally humid savannas) and (b) conditions suitable for *O. rufipogon* probably

Fig. 4 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 seems to occur between all sympatric or parapatric forms. However, *O. longistaminata* and *O. breviligulata* are isolated in their natural habitat by reproductive barriers particularly developed. The subspecies *indica* and *japonica* are conceptual in the sense that they are meant to represent incipient cultivars in China and South/Southeast Asia. They correspond nevertheless to a fundamental dichotomy within *O. sativa*.



existed in Central Asia until its desertification during the Quaternary era (21) and in central China until historical times. Low levels of migration might have occurred among the areas of China and South Asia through the mountainous area of Indochina. However, there have never existed large, emerged plains between them, even when the sea was at its lowest and the islands of Southeast Asia were part of the continent.

- In the *Latifolia* group, allopolyploids seem to have developed by combination of genomes originating on two different continents — Africa and Asia for the BBCC forms in particular. They are adapted to environments newly opened due to the disturbance of man. By analogy we should assume that in the *Sativa* group introgression between cultivars native to China and South/Southeast Asia and South-South-East Asia have promoted the appearance of new cultivated types. The *Javanica* type of insular South-East Asia and the *Hsien* type of Southern China are good candidates as representatives of such an event from the point of view of their genetic affinities, their growing environment, as well as historical considerations.

Also, besides introgressive hybridization *per se*, there is the possibility that the hybridization of two genomes previously isolated for a long time has a mutagenic effect as evidenced or presumed in other organisms like the mouse, frog, and *Drosophila* in particular (13). This could account for the rare electromorphs found in *O. sativa*, particularly in those varieties that do not really fall in the *indica* nor the *japonica* subspecies (7, A. Ghesquiere and A. de Kachko, personal communication). Some of these alleles are not observed in the wild species. This hypothesis represents an appealing model for the evolution under domestication of species polyphyletic in origin such as *O. sativa*.

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