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Inter- and intra-specific distribution of *Stowaway* transposable elements in AA-genome species of wild rice

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Abstract The presence or absence of miniature inverted-repeat transposable elements (MITEs) that belong to *Stowaway* family was analyzed at three loci, two of which are newly identified, in five wild rice species having the AA genome. The pattern of the presence or absence of MITEs was found to be highly associated with speciation in this plant group. In *Oryza rufipogon*, the pattern was also associated with differentiation into annual or perennial ecotypes. These results suggest either that gene flow has been highly restricted between different species, as well as between different ecotypes of *O. rufipogon* after they were differentiated, or that loci with or without MITEs have been selected in nature together with the linked genes that are responsible for adaptation to environments. In addition, a very low polymorphism with regard to the presence or absence of MITEs within each species or each ecotype suggests that the frequency of transposition of MITEs is very low, assuming that the loci that contain MITEs are free from selection pressure.

Key words Wild rice · Transposable element · Life-history traits · Geographic distribution · Phylogenetic relationship

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Introduction

Transposable elements have been found to be ubiquitous components in most living organisms. They comprise repetitive DNA sequences that constitute a significant part of the genomes of higher organisms (reviewed, for example, by Kidwell and Lisch 1997). An extreme example so far identified is maize, in which at least 50% of the nuclear genome was found to be made up of retrotransposons (SanMiguel et al. 1996). Transposable elements have been classified into two families: class-I and class-II elements. Class-I elements include retrotransposons and long and short interspersed elements (LINEs and SINEs, respectively), which transpose via an RNA intermediate. Class-II elements include transposable elements that transpose via DNA itself, such as the Ac-Ds family in maize, the P element in *Drosophila*, etc. In addition to these two classes, a new family of transposable elements, which are known as miniature inverted-repeat transposable elements (MITEs), has recently been discovered (Bureau et al. 1996). These share the characteristics of both class-I and class-II elements and their transposition mechanism is not yet known (reviewed by Kidwell and Lisch 1997). The MITEs, such as *Tourist* (Bureau and Wessler 1992; 1994a), and *Stowaway* elements (Bureau and Wessler 1994b) were both found to be located in the 5′ upstream regions, the 3′ downstream regions, or in introns of various genes of a wide variety of higher plants.

Cultivated rice (*Oryza sativa* L.), as well as its wild relatives of the genus *Oryza*, can be regarded as well-characterized higher organisms in terms of transposable elements. Various transposable elements such as Tnr1 (Umeda et al. 1991; Tenzen et al. 1994), Tnr2 (Mochizuki et al. 1992), Tnr3 (Motohashi et al. 1996), a Tnr1-like element (Nakamura et al. 1996) and *Stowaway* (Bureau and Wessler 1994b), as well as various retroelements such as p-SINE1 (Mochizuki et al. 1992; Hirano et al. 1994; Motohashi et al. 1997), RIREs (Nakajima et al. 1996; Noma et al. 1997; Uozu et al. 1997; Kumekawa et al. 1999) and Tos1-Tos20 (Hirochika et al.

1996), have been isolated from rice. Recently, Bureau et al. (1996) performed computer-based systematic searches to identify mobile elements in rice and *Arabidopsis*. They found 32 common sequences belonging to nine putative mobile-element families, among which were *Tourist*, *Stowaway* and four new families that had the characteristics of MITEs, while they found no mobile element in *Arabidopsis*. Distributions of p-SINE1 at seven loci, and those of Tnr1, Tnr2 and Ret1 each at one locus have been analyzed in rice strains with an AA genome (Mochizuki et al. 1993; Ohtsubo et al. 1993). Ohtsubo et al. (1993) suggested that the presence or absence of these elements is a useful character with which to infer the relationships of rice strains, and they classified 62 rice strains with an AA genome into ten groups. Iwamoto et al. (1999) recently analyzed the phylogenetic relationships of the genus *Oryza* and its related species based on the gain or loss of the p-SINE1-like intron in the catalase gene. Although the presence or absence of SINEs has been shown to be useful markers to reveal phylogenetic relationships in eukaryotes, the validity of MITEs, as such a marker, in plants has not yet been proven, mainly because the mechanism of transposition of these elements remains unclear.

In the present study, we examined whether the presence or absence of MITEs may reflect evolutionary events such as speciation, expansion of habitats, and differentiation into ecotypes in wild rice species which share the same AA genome with cultivated rice. These species are adapted to different kinds of ecological niches and differ in their breeding systems (reviewed by Morishima et al. 1992). A good example of this is *Oryza rufipogon* Griff. This species, which is a wild progenitor of *O. sativa*, is distributed in Asia, and exhibits a wide variation in life-history traits and mating systems. These variations are typically characterized by differences in perenniality as a result of natural selection in different habitats (Morishima et al. 1961; Oka and Morishima 1967; Sano and Morishima 1982; Morishima and Barbier 1990; Barbier et al. 1991). The distribution of transposable elements in natural populations has been well-analyzed in *Drosophila* (Charlesworth et al. 1992; Brunet et al. 1994; Kidwell 1994; Nuzhdin 1995). However, to our knowledge, no extensive study has been reported on the distribution of transposable elements in wild plants, especially in relation to ecological matters such as adaptation to environment or expansion of habitat, although the distribution of p-SINE1 at the *Waxy* locus was analyzed in the genus *Oryza* including accessions of *O. rufipogon* collected from several regions of Southeast Asia (Hirano et al. 1994). In the present study, we examined the presence or absence of MITEs in a number of wild rice accessions, which made it possible to find loci in which this character is associated with life-history traits in *O. rufipogon* as well as with speciation in AA-genome species. The locus that distinguishes between annual and perennial ecotypes of *O. rufipogon* is suggested as the first DNA marker for these traits.

Materials and methods

Plant materials

Wild rice plants belonging to five AA-genome species were used for the analyses. These were *Oryza rufipogon* Griff. (Asia and Oceania, the progenitor of *O. sativa*), *Oryza meridionalis* Ng (Australia), *Oryza glumaepatula* Steud. (America), *Oryza longistaminata* A. Chev. *et* Roehr. (Africa) and *Oryza barthii* A. Chev. (Africa, the progenitor of African rice *Oryza glaberrima*). In total, 144 accessions were analyzed. They were selected from genetic stocks maintained at the National Institute of Genetics (NIG), Mishima, Japan. These accessions were planted at experimental short-day plots at NIG, and various morphological and physiological characters were observed. Regarding longevity, *O. rufipogon* and *O. glumaepatula* showed continuous variation in perenniality within species. In this study, classification of these plants into different ecotypes was performed essentially as previously described (Morishima et al. 1961). First, quantitative traits of 22 characters were measured in each accession. The characters examined were as follows: 1, seed length/spikelet length; 2, spikelet length/spikelet width; 3, anther length/spikelet length; 4, stigma length/spikelet length; 5, flag leaf length/ flag leaf width; 6, apical hair length; 7, ligule length; 8, panicle length; 9, number of nodes on main culm; 10, length from panicle base to first node; 11, plant height; 12, culm elongation after heading; 13, number of days from sowing to heading; 14, reproductive effort; 15, seed fertility; 16, 100-seed weight; 17, 100-awn weight; 18, number of seeds per panicle; 19, frequency of effective tillers; 20, ratooning; 21, average regenerating ability; and 22, highest regenerating ability. Second, the data were analyzed by principal component analysis. As a result, 60% of the total multivariate variation was extracted by the first and second components, and the scatter diagram of the analysis showed two major distinct groups. One of these two groups has characters of high seed productivity, short (annual) life span, and low rate of outcrossing; while the other group has characters of low seed productivity, high regenerating ability (perennial), and relatively high rate of outcrossing. The plants belonging to the former and latter groups have been designated as annual and perennial types, respectively. The detailed results of the analyses with regard to various life-style traits of these plants have been described in Akimoto (1999). Based on the results, *O. rufipogon* was classified into perennial, intermediate and annual types, and *O. glumaepatula* was classified into perennial and intermediate types. *O. longistaminata* was strongly perennial, while *O. meridionalis* and *O. barthii* were exclusively annual.

Isolation of DNA and PCR-amplification

Total DNA was isolated from individual plants according to the method of Doyle and Doyle (1987). The presence or absence of MITEs at each locus was examined by PCR-amplification followed by agarose-gel electrophoresis of PCR products. The primers for PCR were chosen to anneal to the flanking sequences of inserted elements: 5′-GTGGTATTAAAGTGTATCAGAAGC-3′ and 5′-TAATTTCCATAACTTCCACCAGCT-3′ for the sequence in the 1st intron of the epsilon subunit of the mitochondrial F_1-ATPase gene; 5′-ATGTTAGTATGTTTCTGCCTTGGA-3′ and 5′-TCCTTCTCAGTGGTCTTCTCAG-3′ for the sequence in the 2nd intron of the heat-shock protein 82B gene; 5′-AAATTC AGGGTTACAAAGAGAAGC-3′ and 5′-TCGTGGACCTAAGTG AAACCTC-3′ for the sequence in the 4th intron of the homeobox gene 45. Each cycle of PCR consisted of denaturation for 30 s at 94°C, annealing for 30 s at 51°C, and extension for 30 s at 72°C. This cycle was repeated 25 times. PCR products were analyzed by electrophoresis on 1.5% agarose in TBE buffer. The expected sizes of PCR products using these primers were 516 bp or 364 bp when a MITE was present or absent, respectively, at the epsilon subunit of the mitochondrial F_1-ATPase gene locus, 567 bp or 415 bp when a MITE was present or absent, respectively, at the heat-shock protein 82 locus, and 432 bp or 280 bp when a MITE was present or absent, respectively, at the homeobox gene 45 locus.

Results

Amplification of MITEs from wild rice

In the present study, we examined the presence or absence of MITEs that belong to a family of *Stowaway* inverted repeat elements (Bureau and Wessler 1994b) inserted at three loci of rice: the first intron of a nucleus-encoded gene for the epsilon subunit of mitochondrial F_1-ATPase (abbreviated to "F_1-epsilon"), the second intron of the gene for heat-shock protein 82B ["HSP82"; Van Breusegem et al. (1994); "82B" is referred to as "82A" in the locus name of the data bases.], and the fourth intron of homeobox gene 45 ["OSH45"; Tamaoki et al. (1995)]. The *Stowaway* element in the F_1-epsilon locus has been detected in the course of a structural analysis of this gene in a cultivated rice variety, 'Nipponbare' (the details will be published elsewhere). The element in the HSP82 locus has previously been identified as a *Stowaway* element (Bureau and Wessler 1994b), while the element in the OSH45 locus was newly identified by a search of GenBank nucleic-acids databases by BLAST (Altschul et al. 1990) using the element found in the F_1-epsilon locus as a query sequence. These elements also showed sequence homology with Tnr1 (Umeda et al. 1991; Tenzen et al. 1994) with regard to a 13-bp sequence within the consensus sequence of the Tnr1 family located at both ends of the element.

Primers for PCR were made based on the nucleotide sequences of rice genomic DNA flanking each element in order to examine the presence or absence of the element. The PCR products were analyzed by agarose-gel electrophoresis (Fig. 1). This showed that the sizes of the PCR products were consistent with the sizes expected based on the presence or absence of the elements at each locus (for sizes of PCR products, see above). The

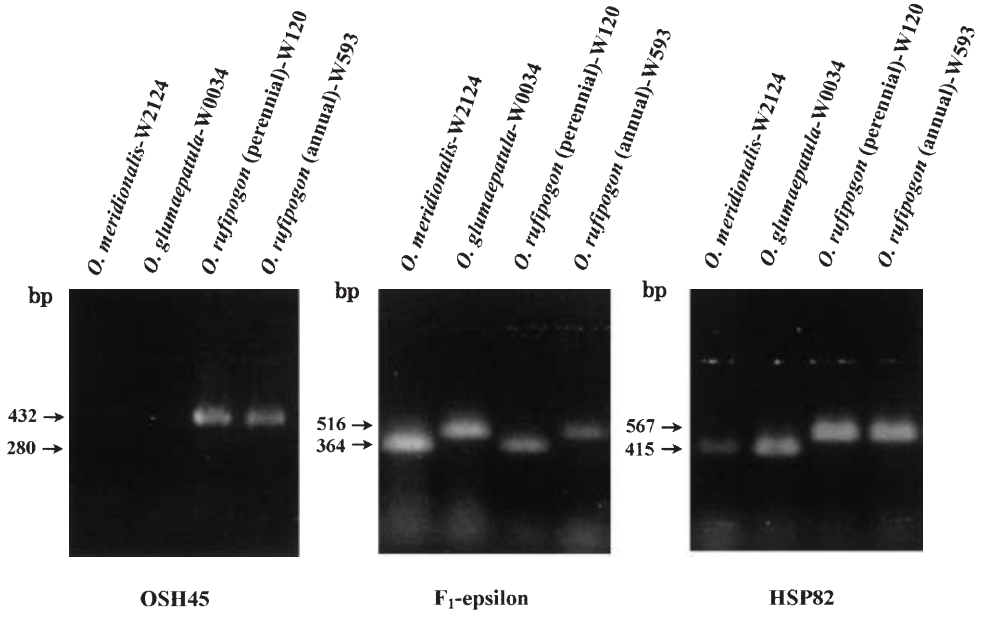

Fig. 1 Examples of the agarose-gel electrophoreses of PCR products that distinguish between the presence and absence of MITEs. The PCR products of three loci (OSH45, F_1-epsilon, and HSP82) from W2124 (*O. meridionalis*), W0034 (*O. glumaepatula*), W120 (*O. rufipogon*), and W593 (*O. rufipogon*) are shown

Table 1 Presence of MITEs at three loci in *O. barthii*

Accession	Origin	Ecotype	OSH45	F_1-epsilon	HSP82
W042	Unknown	Annual	+	+	+
W608	Unknown	Annual	+	+	+
W698	Guinea	Annual	+	+	+
W720	Mali	Annual	+	+	+
W747	Mali	Annual	+	+	+
W1063	Gambia	Annual	+	+	+
W1410	Siera Leonne	Annual	+	+	+
W1416	Siera Leonne	Annual	+	+	+
W1454A	Ivoly Coast	Annual	+	+	+
W1473	Chad	Annual	+	+	+
W1574	Nigeria	Annual	+	+	+
W1583	Chad	Annual	+	+	+
W1588	Cameroon	Annual	+	+	+
W1605	Nigeria	Annual	+	+	+
W1702	Mali	Annual	+	+	+
W1707	Chad	Annual	+	+	+
W1709	Cameroon	Annual	+	+	+
W1710	Cameroon	Annual	+	+	+

Table 2 Presence (+) or absence (–) or MITEs at three loci in *O. longistaminata*. N: no PCR product

Accession	Origin	Ecotype	OSH45	F_1-epsilon	HSP82
W1004	Ghana	Perennial	+	–	–
W1052	Gambia	Perennial	+	–	–
W1232	Tanzania	Perennial	N	–	–
W1429	Mali	Perennial	+	–	+/–
W1444	Cote-d'Ivoire	Perennial	+	–	–
W1460	Benin	Perennial	+	–	+/–
W1508	Madagascar	Perennial	+	–	–
W1540	Congo	Perennial	+	+	–
W1615	Nigeria	Perennial	+	–	–
W1624	Cameroon	Perennial	+	–	–
W649	Sierra Leone	Perennial	+	N	–
W708	Guinea	Perennial	+	+	+

sequences of PCR products from a rice cultivar were analyzed and were confirmed to contain the respective region (data not shown). In some cases, however, no PCR product was detected (see Tables 1–5). A similar phenomenon was observed in the analysis of Tnr1 (Tenzen et al. 1994). The failure of PCR-amplification may be due to the occurrence of mutations in the sequences of rice DNA to which primers should hybridize, as Tenzen et al. (1994) have already mentioned.

Variable occurrence of the element among the loci

Among the accessions examined in the present study, the F_1-epsilon and HSP82 loci showed polymorphisms with regard to the presence or absence of the element. The number of accessions from which no PCR product was amplified was two and four in the F_1-epsilon and HSP82 loci, respectively. The OSH45 locus showed either the presence of the element or a lack of amplification, and there was no accession that showed the absence of the element. The number of accessions from which no PCR product was amplified was higher (31 accessions) than in the cases of the F_1-epsilon (2 accessions) and HSP82 (4 accessions) loci. Remarkably, no PCR product was obtained from the OSH45 locus in any of the accessions of *O. meridionalis* (see Table 4). Thus, the sequence of this locus in *O. meridionalis* has probably changed.

Relationships between the presence or absence of the element and the geographic distribution, species and ecotypes of wild rice plants

The presence or absence of the inverted repeat element at the three loci was found to be highly correlated with the species and/or the geographic distribution of wild rice plants. All the accessions that belong to an African wild rice species, *O. barthii*, showed the pattern of OSH45 (+: presence), F_1-epsilon (+), HSP82 (+) (Table 1), and 6 out of 12 accessions of another African species *O. longistaminata* showed the pattern of OSH45 (+), F_1-epsilon (–: absence), HSP82 (–) (Table 2). Two accessions of this species (W1429 and W1460) showed a pattern that indicates both the presence and absence of the element (see Table 2). This probably indicates a heterozygotic state of this locus in these plants. All the accessions that belong to a Central and South American species, *O. glumaepatula*, showed the pattern of OSH45 (+), F_1-epsilon (+), HSP82 (–) except for four cases in which no PCR product was amplified from one or two loci (Table 3). Almost all the accessions of an Australian species, *O. meridionalis*, showed the pattern of OSH45 (N: no amplification), F_1-epsilon (–) and HSP82 (–) (Table 4). In the accessions of *O. rufipogon*, which are distributed in Asia and Oceania, the OSH45 locus showed no polymorphism with respect to the element, while the HSP82 locus showed a difference in only one accession (W1239), except for the cases of no amplification. On the other hand, the F_1-epsilon locus did show polymorphism in this species (Table 5). The ecotypes of wild rice have been classified in terms of their annual, perennial or intermediate types, as mentioned earlier. The polymorphism with regard to the F_1-epsilon locus was highly correlated with the ecotypes of *O. rufipogon*: the annual- and intermediate-type plants have the element, while the perennial-type plants have no element at this locus. Exceptions are three accessions of the annual ecotype (CB5c, W1683 and W630) that are F_1-epsilon (–), and an accession of the perennial ecotype (W2005) that is F_1-epsilon (+). The collection sites of the accessions analyzed suggest that the pattern of the presence or absence of MITEs appeared to be more correlated with species or ecotypes than with geographic distribution.

The extent of diversity at these loci was evaluated with Shannon and Weaver's index (*H′*, see Table 6). The lack of a PCR product was tentatively included as a character state assuming that it may indicate mutations that prevent the annealing of primers. This analysis showed that *O. longistaminata* and *O. rufipogon* have a relatively high diversity at these loci.

Discussion

Presence or absence of MITEs and phylogenetic relationships of wild rice species

The presence or absence of MITEs was highly conserved within each wild rice species except for *O. rufipogon*. In

Table 3 Presence (+) or absence (–) of MITEs at three in *O. glumaepatula*. N: no PCR product

Accession	Origin	Ectotype	OSH45	F_1-epsilon	HSP82
W0034	French Guiana	Perennial	N	+	–
W0036	Cuba	Perennial	+	+	–
W1167	Cuba	Perennial	+	+	–
W1169	Cuba	Perennial	+	+	–
W1183	Guiana	Perennial	+	+	–
W1185	Suriname	Perennial	+	+	–
W1186	Suriname	Perennial	+	+	–
W1187	Brazil (Amazon)	Perennial	+	+	–
W1189	Brazil (Amazon)	Intermediate	+	+	–
W1191	Brazil (Amazon)	Intermediate	+	+	–
W1192	Brazil (Amazon)	Intermediate	+	+	–
W1196	Colombia	Intermediate	+	+	–
W1246	Brazil (Amazon)	Intermediate	+	+	–
W1481	Brazil (Amazon)	Intermediate	+	+	–
W1482	Brazil (Amazon)	Intermediate	+	+	–
W1833	Brazil (Amazon)	Intermediate	+	+	–
W2135	Brazil (Amazon)	Intermediate	+	+	–
W2136	Brazil (Amazon)	Intermediate	+	+	–
W2140	Brazil (Amazon)	Intermediate	+	+	–
W2141	Brazil (Amazon)	Intermediate	+	+	–
W2143	Brazil (Amazon)	Intermediate	+	+	–
W2145	Brazil (Amazon)	Intermediate	+	+	–
W2147	Brazil (Amazon)	Intermediate	N	+	–
W2152	Brazil (Amazon)	Intermediate	+	+	–
W2160	Brazil (Amazon)	Intermediate	+	+	–
W2161	Brazil (Amazon)	Intermediate	+	+	–
W2162	Brazil (Amazon)	Intermediate	+	+	–
W2163	Brazil (Amazon)	Intermediate	+	+	–
W2165	Brazil (Amazon)	Intermediate	N	+	N
W2166	Brazil (Amazon)	Intermediate	+	+	–
W2168	Brazil (Amazon)	Intermediate	+	+	–
W2169	Brazil (Amazon)	Intermediate	+	+	–
W2173	Brazil (Amazon)	Intermediate	+	+	–
W2179	Brazil (Amazon)	Intermediate	+	+	–
W2184	Brazil (Amazon)	Intermediate	+	+	–
W2186	Brazil (Amazon)	Intermediate	+	+	–
W2187	Brazil (Amazon)	Intermediate	+	+	–
W2189	Brazil (Amazon)	Intermediate	+	+	–
W2191	Brazil (Amazon)	Intermediate	+	+	–
W2192	Brazil (Amazon)	Intermediate	+	+	–
W2199-1	Brazil (Pantanal)	Perennial	+	+	–
W2201-2	Brazil (Pantanal)	Perennial	+	+	–
W2202-1	Brazil (Pantanal)	Perennial	+	+	–
W2203-2	Brazil (Pantanal)	Perennial	+	N	N
W2203-3	Brazil (Pantanal)	Perennial	+	+	–

Table 4 Presence (+) or absence (–) of MITEs at three loci in *O. meridionalis*. N: no PCR product

Accession	Origin	Ecotype	OSH45	F_1-epsilon	HSP82
W1627	Australia (Adelaide River)	Annual	N	–	–
W1631	Australia (Kununurra)	Annual	N	–	–
W1635	Australia (Toritilla Flats)	Annual	N	–	–
W2069	Australia (Kununurra)	Annual	N	–	–
W2077	Australia (Darwin)	Annual	N	–	–
W2079	Australia (Darwin)	Annual	N	–	–
W2080	Australia (Darwin)	Annual	N	–	–
W2081	Australia (Katherine)	Annual	N	–	–
W2100	Australia (Ingham)	Annual	N	+	+
W2103	Australia (Cairns)	Annual	N	–	–
W2105	Australia (Ingham)	Annual	N	–	–
W2112	Australia (Cook Town)	Annual	N	–	–
W2114-2	Australia (Cook Town)	Annual	N	–	–
W2116	Australia (Weipa)	Annual	N	–	–
W2117	Australia (Weipa)	Annual	N	–	–
W2123	Australia (Brisbane)	Annual	N	–	N
W2124	Australia (Brisbane)	Annual	N	–	–

Table 5 Presence (+) or absence (–) of MITEs at three loci in *O. rufipogon*. N: no PCR product

Accession	Origin	Ecotype	OSH45	F_1-epsilon	HSP82
CB10b	Cambodia	Intermediate	+	–	+
CB5c	Cambodia	Annual	+	–	+
CB6c	Cambodia	Annual	+	+	+
CB8	Cambodia	Intermediate	N	+	+
CHW3	China	Perennial	+	–	+
CP52 11-1	Thailand	Perennial	+	–	+
CT3a	Vietnam	Perennial	+	–	+
LP10	Laos	Intermediate	+	+	+
LP5-1	Laos	Annual	+	+	+
LT7	Vietnam	Perennial	N	–	+
LV26	Laos	Perennial	+	–	+
LV27	Laos	Perennial	+	–	+
LV5	Laos	Intermediate	+	+	+
W106	India	Annual	+	+	+
W107	India	Annual	+	+	+
W108	India	Perennial	+	–	+
W120	India	Perennial	+	–	+
W1238	New Guinea	Perennial	+	–	+
W1239	New Guinea	Perennial	N	–	–
W1551	Thailand	Annual	+	+	+
W1666	India	Perennial	+	–	+
W1669	India	Perennial	N	–	+
W1681	India	Annual	+	+	+
W1683	India	Annual	+	–	+
W1685	India	Annual	+	+	+
W1690	Thailand	Annual	+	+	+
W1822	Bangladesh	Intermediate	+	+	+
W1852	Thailand	Annual	+	+	+
W1866	Thailand	Annual	+	+	+
W1922	Thailand	Annual	+	+	+
W1927	Thailand	Perennial	+	–	+
W1939	Thailand	Perennial	+	–	+
W1943	China	Perennial	+	–	+
W1945	China	Perennial	N	–	+
W1981	Indonesia	Perennial	+	–	+
W1994	India	Annual	+	+	+
W2003	India	Annual	N	+	+
W2005	India	Perennial	N	+	+
W2007	India	Intermediate	N	+	+
W2011	India	Annual	N	+	N
W2014	India	Annual	N	+	+
W2060	Bangladesh	Intermediate	+	+	+
W2078	Australia	Perennial	+	–	+
W2099	Australia	Perennial	+	–	+
W2109	Australia	Perennial	+	–	+
W2119	Australia	Perennial	+	–	+
W593	Maraya	Annual	+	+	+
W610	Burma	Annual	+	+	+
W629	Burma	Annual	+	+	+
W630	Burma	Annual	+	–	+
W636	Burma	Perennial	+	–	+
YZWR-146	China	Perennial	+	–	+

O. rufipogon, different patterns were detected in different ecotypes and the pattern was conserved within each ecotype. Conserved patterns were observed within each species even when different species showed overlapping distributions, such as occurs with *O. meridionalis* and *O. rufipogon* in Australia, and *O. barthii* and *O. longistaminata* in Africa.

Differences in the occurrence of MITEs at the three loci among the species indicated that gain (or loss) of a MITE at each locus occurred at temporally distinct phases during the differentiation of wild rice species. The possible timings of the gain or loss of MITEs that generated the observed polymorphisms in the three loci were located on a phylogenetic tree (Fig. 2), whose branching order was taken from a tree based on restriction fragment length polymorphism (RFLP) data of nuclear DNA (Wang et al. 1992). The three most-parsimonious possibilities are shown in Fig. 2. In panel A, only a gain of MITEs was assumed to occur, while in panels B and C, both gain and loss were assumed to occur. Because we could not obtain a PCR product with regard to the OSH45 locus in *O. meridionalis*, it may also be possible that the gain of a MITE in the OSH45 locus occurred before the branching of *O. meridionalis* from other species.

Table 6 Frequency distribution of MITE pattern at three loci found in five wildrice species

Species	Distribution pattern of MITEs at three loci[a]												Number of accessions	H'[b]
	N––	+––	N–N	+N–	N–+	N+–	++–	+–+	N+N	+NN	N++	+++		
O. longistaminata	1	7		1			1	1				1	12	1.35
O. rufipogon	1				3			23	1		5	19	52	1.27
Perennial	1				3			19			1		24	0.70
Annual and intermediate								4	1		4	19	28	0.94
O. meridionalis	15		1								1		17	0.44
O. glumaepatula						2	41		1	1			45	0.38
O. barthii												18	18	0

[a] Presence or absence of MITEs at three loci, namely, OSH45, F_1-epsilon, and HSP82; +: presence, –: absence, N: no PCR product; heterozygotic state observed in *O. longistaminata* was counted as 1/2

[b] $H'=-\Sigma p_i \ln p_i$ (Shannon and Weaver's diversity index), in which p_i is the frequency of each MITE pattern

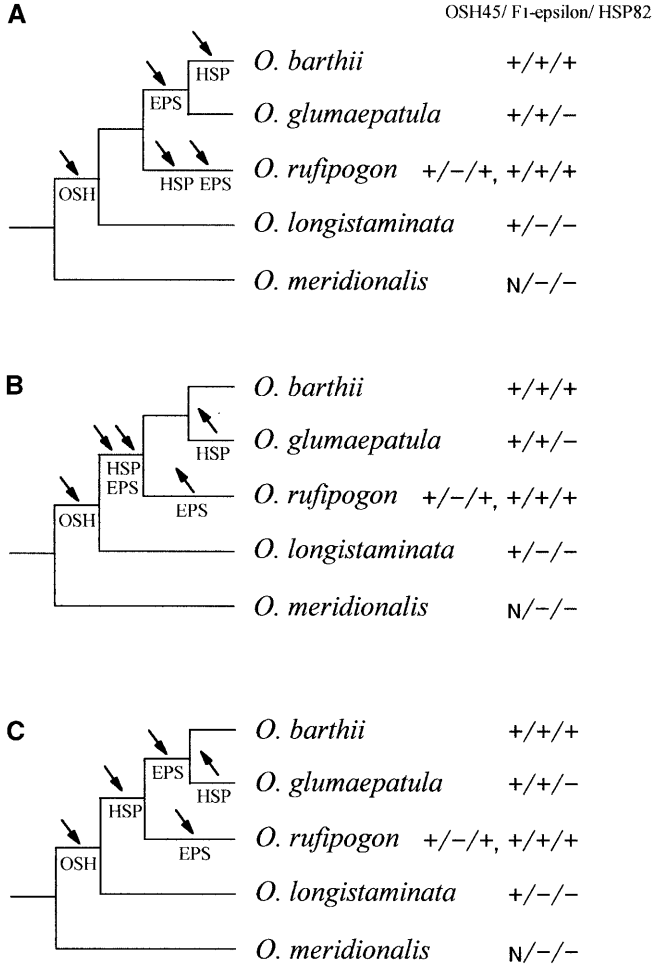

Fig. 2A–C Schematic representation of the timing of the occurrence of the presence or absence of MITEs at three loci during evolution of AA-genome species of the genus *Oryza*. The location of evolutionary events on the phylogenetic tree assumes only gain (panel **A**) or both gain and loss (panels **B and C**) of MITEs. *Downward and upward arrows* indicate the occurrence of gain and loss, respectively, at each locus. The branching order of wild rice species is taken from a phylogenetic tree that was constructed based on RFLP data of nuclear DNA (Wang et al. 1992). + and – indicate the presence and absence of a MITE at each locus, respectively, which were examined by PCR analysis. *N* indicates that no product was obtained by PCR amplification

In these three cases, the observed status of the three loci in the wild rice species can be explained by five evolutionary events that are shown by arrows in Fig. 2. The result shown in panel B assumes the generation of a perennial type of *O. rufipogon* from an annual type of *O. rufipogon* accompanying a loss of a MITE from the F_1-epsilon locus, whose evolutionary order is contradictory to the previous finding that the annual type evolved from the perennial type in *O. rufipogon* (reviewed by Morishima et al. 1992). If we accept the phylogenetic relationships of wild rice species shown in Fig. 2, it appeared that gain of MITEs in one, two, or all three of these loci occurred in these AA genome species after their divergence from the other species of the genus *Oryza*. Dally and Second (1990) obtained a different branching order of these wild rice species using RFLP data of chloroplast DNA. In this case, nine or more steps were required to explain the results (data not shown).

Mechanisms of the generation of the presence or absence of MITEs that is associated with speciation and differentiation into ecotypes

We propose two models that explain the mechanism responsible for the generation of the presence or absence of MITEs that are associated with speciation, as well as ecotype differentiation, in *O. rufipogon*.

One is that the presence or absence of the elements at each locus was established in the ancestral plants of each species or each ecotype of *O. rufipogon* before the expansion of their habitats. Subsequently, the state of each locus was conserved in each species or ecotype because of reproductive isolation that strictly prevented gene flow between different species as well as between different ecotypes. In this case, the frequency of transposition of MITEs would be expected to be very low. A similar possibility has been suggested with respect to Tnr1: Mochizuki et al. (1993) have mentioned that Tnr1 is not often excised since this element was present at the *Waxy* locus of all the rice strains they examined. Various levels of hybrid sterility have been observed in interspecific

crossings among species having the AA genome (Chu et al. 1969; Morishima 1969; Chu and Oka 1970; Naredo et al. 1998), which suggests that AA-genome species, in general, have developed reproductive isolation mechanisms. The notion of such a reproductive isolation between these related species has also been supported by analyses of other molecular markers such as rDNA spacer-length variation (Sano and Sano 1990). With regard to *O. rufipogon*, crossing between annual and perennial types has been shown to be possible under experimental conditions (Chu et al. 1969) and the possibility of gene flow has been suggested for natural populations in Thailand (Barbier 1989). However, two ecotypes tend to inhabit different niches and have slightly different flowering times, both of which could restrict gene flow between these ecotypes (Morishima et al. 1992). In fact, natural populations of *O. rufipogon* in which annual and perennial types of plants are growing sympatrically are rare.

The alternative model is that the presence or absence of the elements was independently selected in each species or ecotype during adaptation to its environment or habitat. This model can particularly be implicated in the result obtained for the ecotypes of *O. rufipogon*, since a continuum between typical annual and perennial types has been observed in natural populations, which indicates that the process of differentiation is still under way (Morishima et al. 1984). In the present study we found that the presence or absence of MITEs at the F_1-epsilon locus distinguished between these two ecotypes with a very high probability (with only 4 exceptions out of 52; see above), although six out of seven accessions of an intermediate type showed the same pattern as the annual type. In this model the F_1-epsilon locus may be closely linked with one or more genes that are involved in adapting to a particular environment. Subsequently, the F_1-epsilon locus with or without a MITE is transmitted together with surrounding genes that are mutually coadapted during ecotypic differentiation. Such a mechanism is very similar to that suggested by Morishima (1991) based on an analysis of *Pox-1* allozyme variations, which were reported to be linked with some characters of these ecotypes such as reproductive allocation, flowering time and anther length. Up to now *Pox-1* variation is the only molecular marker reported that can distinguish the two different ecotypes of *O. rufipogon*, the annual and perennial types. In addition, given the occurrence of outcrossing, this model can explain the gain of a MITE at the same site in the genome in different lineages (see Fig. 2A, C), which can hardly be explained by repetitive insertion, since a specific target for insertion of the *Stowaway* elements, i. e., a sequence consisting of more than just a few nucleotides, has not been detected (Bureau and Wessler 1994b).

In any case, the results of the present study showed that the presence or absence of MITEs is highly correlated with ecotype variation in *O. rufipogon*, as well as speciation in various AA-genome species of wild rice. This suggests that the presence or absence of these MITEs can be used as a marker when we examine inter- or intra-population relationships of wild rice. The presence or absence of the MITE in the F_1-epsilon locus would be the first DNA marker that distinguishes between annual and perennial types in *O. rufipogon*, which would be confirmed by genetic analysis using the plants of these ecotypes for crossing.

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