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Speciation in *Pyricularia* inferred from multilocus phylogenetic analysis

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ABSTRACT

Pyricularia isolates from various host plants were subjected to a multilocus phylogenetic analysis based on rDNA-ITS, actin, β-tubulin, and calmodulin loci. A combined gene tree resolved seven groups with 100% bootstrap support, suggesting that they are monophyletic groups supported concordantly by all four loci. By incorporating biological and morphological species criteria, each of the seven groups was considered to be a current species. However, phylogenetic relationships among these species were unresolved in the single-gene trees and in the combined tree. Furthermore, the transition from concordance to conflict occurred more than once in the combined gene tree. They were interpreted by assuming that *Pyricularia* has evolved through repeating species radiation. The transition point other than the current species limit was considered to be the limit of the former species.

Keywords: Genealogical concordance, *Magnaporthe*, Multilocus phylogenetic analysis, *Pyricularia*, Speciation

Introduction

One of the most striking developments in fungal biology during the last 10 years has been the establishment of a new principle of species recognition. Traditionally, fungal species have been diagnosed by morphological species recognition (MSR) or biological species recognition (BSR) (Taylor *et al.* 2000). However, these traditional methods of diagnosing species have some drawbacks. For example, species diagnosed by MSR are sometimes composed of more than one species when diagnosed by BSR (Taylor *et al.* 2000). BSR is widely applied to sexually reproducing organisms including animals and plants, but cannot be applied to fungi that lack a sexual stage. Recent advances of molecular techniques have provided a method for recognizing species on the basis of phylogenetic trees constructed from DNA data, that is, phylogenetic species recognition (PSR) (Taylor *et al.* 2000). In PSR individuals are grouped objectively, but the decision about where to place the limit of the species is subjective (Taylor *et al.* 2000).

To avoid the subjectiveness of determining the limits of a species, Taylor and his coworkers applied multilocus sequence typing (Taylor & Fisher 2003) to recognize fungal species (Koufopanou *et al.* 1997; Geiser *et al.* 1998) and established this principle as GCPSR (Genealogical Concordance Phylogenetic Species Recognition) (Taylor *et al.* 2000). In GCPSR phylogenetic trees are constructed from more than one gene and their topology is compared. Theoretically, different gene trees should be concordant between species due to fixation of polymorphisms through random genetic drift after genetic isolation, but should show conflict within species due to recombination among individuals. Therefore, the transition from concordance to conflict determines the limits of species (Taylor *et al.* 2000).

GCPSR has been successfully used to identify cryptic species in human or animal

pathogenic fungi (Koufopanou *et al.* 1997; Kasuga *et al.* 1999, 2003; Cruse *et al.* 2002), an insect pathogenic fungus (Bidochka *et al.* 2005), plant pathogenic fungi (O'Donnell 2000; O'Donnell *et al.* 2000; Steenkamp *et al.* 2002), a food-associated fungus (Geiser *et al.* 1998), a model fungus (Dettman *et al.* 2003), and a lichenized fungus (Kroken & Taylor 2001). New Latin names were assigned to some of those cryptic species (Fisher *et al.* 2002; Couch & Kohn 2002; O'Donnell *et al.* 2004). In some of these studies, however, relationships among species were not concordant among gene trees. For example, Dettman *et al.* (2003) found eight phylogenetic species in *Neurospora* using GCPSR, but the phylogenetic relationships among those species were difficult to resolve; the internal branches that united multiple species received nonsignificant bootstrap support.

Pyricularia is the causal agent of blast disease of various monocot species. This genus includes several morphological species such as *P. higginsii* pathogenic on *Cyperus* (Luttrell 1954; Hashioka 1973), *P. zingiberi* pathogenic on *Zingiber* (Kotani & Kurata 1992), *P. zizaniaecola* pathogenic on *Zizania* (Hashioka 1973), etc. The most familiar species is *P. grisea* (Rossman *et al.* 1990) (teleomorph, *Magnaporthe grisea* (Hebert) Barr.) which has caused destructive epidemics on staple gramineous crops. *P. grisea* is isolated from rice (*Oryza sativa*), foxtail millet (*Setaria italica*), common millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*), wheat (*Triticum aestivum*), perennial ryegrass (*Lolium perenne*), crabgrass (*Digitaria sanguinalis*), etc. Kato *et al.* (2000) examined pathogenicity, mating compatibility, and RFLPs of *Pyricularia* isolates from various hosts, and found that the isolates from *Oryza*, *Setaria*, *Panicum*, *Triticum*, and *Eleusine* form a genetically close, interfertile group (CC group; cf., Kato *et al.* 2000), and are distinct from the isolates from *Digitaria* to which the Latin name *P. grisea* was first assigned. This finding led them to conclude that the CC

group is a species distinct from *P. grisea* and should be designated as *P. oryzae* (Kato *et al.* 2000). Recently, Couch and Kohn (2002) recognized two cryptic species in *Magnaporthe grisea* by using a multi-locus phylogenetic analysis. They assigned a new species name, *M. oryzae*, to the isolates from staple crops while restricting the name *M. grisea* to the isolates from *Digitaria*. The species boundary of *M. oryzae* was perfectly congruous to that of the anamorphic species *P. oryzae* proposed by Kato *et al.* (2000). In the present study we applied GCPSR to *Pyricularia* isolates from various host plants to examine speciation in the genus *Pyricularia*.

Materials and methods

Fungal materials

Fungal material included 97 *Pyricularia* isolates from 28 host species collected in Brazil, India, Nepal, China, Indonesia and Japan (Table 1). *P. higginsii* was transferred to *Dactylaria* and designated *D. higginsii* by Ellis (1976). Recently, however, Bussaban *et al.* (2005) suggested that *P. higginsii* should be maintained in *Pyricularia* on the basis of rDNA-ITS sequences. In the present paper we designate an isolate from *Cyperus* as *P. higginsii* according to Bussaban *et al.* (2005).

For long-term storage in our laboratory, those isolates were grown on sterilized barley seeds in vials, dried thoroughly at 25°C, and maintained at 4°C in containers with silica gel. They were transferred to a potato dextrose agar slant just before use and grown at room temperature. A part of the voucher strains were deposited to the Microorganisms Section of the NIAS Genebank (MAFF), National Institute of Agrobiological Sciences, Tsukuba, Japan, and CABI Bioscience, UK Centre (formerly

the International Mycological Institute (IMI)), Egham, UK (Table 1).

DNA amplification and sequencing

Total DNA was extracted from mycelia as described previously (Nakayashiki *et al.* 1999). Four gene regions were chosen for the GCPSR analysis: a portion of the nuclear ribosomal RNA gene repeat (rDNA-ITS: ITS1, 5.8S and ITS2), a portion of the actin (ACT) gene including two introns, a portion of the beta-tubulin (BT) gene including one intron, and a portion of the calmodulin (CAL) gene including three introns. They were amplified with primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990), ACT-512F (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT-783R (5'-TACGAGTCCTTCTGGCCCAT-3') (Carbone & Kohn 1999), Bt1a (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and Bt1b (5'-GACGAGATCGTTCATGTTGAACTC-3') (Glass & Donaldson 1995), and CAL-228F (5'-GAGTTCAAGGAGGCCTTCTCCC-3') and CAL-737R (5'-CATCTTTCTGGCCATCATGG-3') (Carbone & Kohn 1999), respectively, in a 50 µl reaction containing 1.5 units of HotStarTaq DNA polymerase (Qiagen, Hiden, Germany), 1 x PCR buffer provided by the manufacturer, 200 µM of each dNTP, 0.2 µM of each primer and 10 ng of template DNA, using a mastercycler (Eppendorf, Hamburg, Germany) programmed for 8 min at 95˚C, 30 cycles of 30s at 95˚C, 20s at 55˚C, and 1 min at 72˚C, followed by 5 min at 72˚C. PCR products were purified by ethanol precipitation, and sequenced directly with the same primers as in the amplification using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) and the ABI 3100 Genetic Analyzer following the

manufacture's instructions. Both forward and reverse strands were sequenced and checked against each other. The resulting sequences were assembled with Sequencher (TAKARA, Otsu, Japan), aligned with CLUSTAL W 1.7 (Thompson *et al.* 1994), and adjusted visually with Genetix Mac X. The DNA sequences and alignments were deposited in GenBank (Table 1) and TreeBASE (submission ID number, SN3200; submission name, Motoaki Kusaba; P.I.N. code, 15130).

Phylogenetic analysis

Phylogenetic trees were constructed using the maximum-parsimony (MP) method with the heuristic search using PAUP* Version 4.0 (Swofford 2002). Alignment gaps were treated as missing data. Bootstrap values (Felsenstein 1985) were calculated from 1000 replicates using the heuristic search option. Phylogenetic trees were also constructed using the Neighbor-joining (NJ) method (Saitou & Nei 1987) and maximum-likelihood (ML) method (Felsenstein 1981) of the program NEIGHBOR and DNAML from PHYLIP v 3.4 (Felsenstein 1991). For the NJ method, the evolutionary distance was calculated from the Kimura two-parameter method (Kimura 1980). For the ML method, the transition/transversion ratio was set to 2:1. NJ and ML analyses were performed with data sets that excluded insertions and deletions. Partition homogeneity tests (PHT) were performed using a program implemented in PAUP* Version 4.0 to detect significant conflict of phylogenetic signals between loci.

Results and Discussion

Identification and designation of alleles and genotypes

DNA sequencing revealed 21 (rd1-rd21), 15 (ac1-ac15), 16 (be1-be16), and 16 (ca1-ca16) alleles (Table 1) at the rDNA-ITS, actin, β-tubulin, and calmodulin loci, respectively, among the *Pyricularia* isolates tested. By combining these alleles at the four gene loci, the 97 *Pyricularia* isolates were classified into 29 unique multilocus genotypes (Table 1). These genotypes were designated using abbreviations of their host plants. For example, four genotypes found in isolates from *Oryza sativa* were never found in the other isolates, and therefore, were designated as Os1, Os2, Os3, and Os4 after *Oryza sativa*. Two genotypes were found in isolates from *Eleusine* spp., but one of them was shared by isolates from *Triticum* and *Lolium* while the other was shared by an isolate from *Eragrostis*. Consequently, three genotypes were shared by isolates from *Triticum*, *Eleusine*, *Lolium*, and *Eragrostis*, and therefore, were designated as TELE1, TELE2, and TELE3 after *Triticum*, *Eleusine*, *Lolium*, and *Eragrostis.*

Identification of species

The combined analysis of the four gene regions produced a total of 1882 aligned sites, of which 467 (24.8%) were parsimony informative (Table 2). To reveal the most basal taxon among the *Pyricularia* isolates, parsimony analysis was performed using *Magnaporthe salvinii* MS-1 as an outgroup. In this analysis only exon sequences of ACT, BT, and CAL were used because their intron sequences were difficult to align with corresponding sequences of *M. salvinii* MS-1 (GenBank accession nos. AF395975, AF396004, AF396030). MP trees from the combined exon data showed that isolates from *Cyperaceae* (with genotypes Ci, Kb1, and Kb2) constitute the most basal taxon among the *Pyricularia* isolates tested (Fig 1). This is reasonable because the isolates

from *Cyperaceae* are morphologically distinct from the other *Pyricularia* isolates, so as to have been transferred to another genus, *Dactylaria*, by Ellis (1976).

To improve the resolution, *M. salvinii* was omitted from the alignment, and MP trees were reconstructed from the whole data (ITS1, 5.8S and ITS2 of rDNA, exons and introns of actin, β-tubulin, and calmodulin genes) using Ci, Kb1, and Kb2 as outgroup taxa. Parsimony analysis of the four individual loci resolved the 26 genotypes into the same seven exclusive groups with 100% bootstrap support (data not shown). A PHT showed that there is significant incongruence among the four data set ($P<0.01$, Fig 2A). Nevertheless, the combined data set again resolved the seven groups with 100% bootstrap support in the parsimony analysis (Fig 3A), suggesting that they are monophyletic groups that are concordantly supported by all the four loci. The group consisting of Zm1-4 corresponded to *P. zingiberi*, a morphological species that produces sclerotium-like structures (Kotani & Kurata 1992). The group consisting of Zl1-2 corresponded to *P. zizaniaecola*, a morphological species characterized by oval conidia (Hashioka 1973). The group consisting of Ss and Pb shared a characteristic conidial morphology, that is, a pigmented mid-cell, and therefore, was considered to be another morphological species. This species was tentatively designated as *Pyricularia* sp. (SsPb). The group consisting of Dssh1-3 and that consisting of Os, TELE, Bp, SP and As corresponded to *P. grisea* and *P. oryzae*, respectively, which are biological species recognized by Kato *et al.* (2000). These biological species (*P. grisea* and *P. oryzae*) are morphologically very similar, and had been collectively classified into a single morphological species, *P. grisea* (Rossman *et al.* 1990). This morphological species (*P. grisea sensu lato* defined by Rossman et al. 1990) will be tentatively designated as *P. grisea* (M) below. *P. grisea* (M) included two additional phylogenetic groups; one consisted of Lo and Sg, and the other consisted of CE1 and CE2 (Fig 3A). They

corresponded to the L&S group and the C&E group, respectively, which were found by Kato et al. (2000). The branches to these groups were as deep as those to *P. oryzae* and *P. grisea*. Therefore, they were considered to be phylogenetic species and were tentatively designated as *Pyricularia* sp. (LS) and *Pyricularia* sp. (CE). For convenience, we will call *P. oryzae*, *P. grisea*, *Pyricularia* sp. (LS), and *Pyricularia* sp. (CE) as cryptic species within *P. grisea* (M). Isolates from *Kyllinga brevifolia* (with genotype Kb1 or Kb2) were morphologically very similar to *P. higginsii* with genotype Ci. However, Kb1 and Kb2 were deeply separated from Ci within the phylogenetic tree (Fig 3A). Therefore, the isolates from *K. brevifolia* were tentatively designated as *Pyricularia* sp. (Kb).

The seven phylogenetic groups were also found in NJ (Fig 3B) and ML (data not shown) trees with 100% bootstrap support. These phylogenetic analyses integrated with morphological and biological species criteria led us to a conclusion that each of these seven phylogenetic groups is an evolutionary species. When we mention just "species" below, it indicates these seven groups.

Evolutionary relationships among the seven species

Branches within *P. oryzae* (shown in red in Fig 3) were in conflict among the four gene trees, and poorly supported by bootstrap analyses in the combined gene trees (Fig 3A, B) as expected. According to the principle of GCPSR, branches above (proximal to) the species should be concordant among the four gene trees and strongly supported by bootstrap analyses in the combined gene tree. In fact, the phylogenetic relationships among the four cryptic species within *P. grisea* (M) were difficult to resolve; the internal branches that united these cryptic species received nonsignificant bootstrap

support (Fig 3A, B). We reconstructed phylogenetic trees by eliminating intron sequences from the combined data sets or by treating alignment gaps as a fifth character (for MP analysis), but the bootstrap support was not improved (data not shown). A high bootstrap support (100%) appeared again at a node that united all the four cryptic species (Fig 3A, B). This unit corresponded to *P. grisea* (M) (shown in grey in Fig 3A, B). *P. grisea* (M) was then united with *P. zizaniaecola* (shown in blue in Fig 3) with very high bootstrap support (Fig 3A, B).

To reveal why the internal branches within *P. grisea* (M) were poorly supported, a genotype was arbitrary chosen from each cryptic species within *P. grisea* (M), and the resulting four representatives (TELE1, Lo, CE1, and Dssh1) were subjected to a PHT. If the incongruence detected in Fig 2A is only due to the conflict within the species, the extraction of one genotype from each of the four species should remove the conflict. As expected, the actual summed tree length was not significantly different from those from 10000 artificial data sets in the PHT with the four representatives (Fig 2B), suggesting that the four gene data sets are congruent with respect to the relationship among the four representatives. When MP trees of the four representatives were constructed from the four individual genes, however, the four gene trees were apparently incongruent (Fig 4). To reveal why the data producing such incongruent trees were judged to be congruent by the PHT, informative sites were analyzed in detail. Each of the actual data sets of the four genes included various types of informative sites like artificial data sets produced by shaffling (Table 3). From these results, we suggest that the four species radiated from a common ancestor.

The internodes of *P. zizaniaecola, P. zingiberi,* and *Pyricularia* sp. (SsPb) also received low bootstrap support (Fig 3A, B). Again, a genotype was arbitrary chosen from each morphological species. When MP trees of the three representatives (Zl1, Zm1,

and Pb) were constructed from the four individual gene data sets, the four gene trees were apparently incongruent (Fig 5). When they were subjected to a PHT, however, the actual summed tree length was not significantly different from those from 10000 artificial data sets (Fig 2C), suggesting that the four gene data sets are congruent with respect to the relationship among the three representatives. From these results, we suggest that the three morphological species radiated from a common ancestor. Taken together, we conclude that the genus *Pyricularia* has evolved through repeating species radiation.

The poor support of species internodes is often found in the literature (Chaverri *et al.* 2003; Kasuga *et al.* 1999, 2003; Kroken & Taylor 2001; O'Donnell *et al.* 2000). Fungi, especially plant pathogenic fungi, seem prone to radiation, because they are often surrounded by more than one "new" niche and may become adapted to them through simple genetic changes. For example, saprophytic *Alternaria alternata* share a common variation of rDNA-ITS and mitochondrial DNA polymorphisms with *A. kikuchiana* pathogenic on Japanese pear, *A. mali* pathogenic on apple, *A. longipes* pathogenic on tobacco, etc. (Kusaba & Tsuge 1994, 1995, 1997). This fact suggests that these pathogenic *Alternaria* species evolved directly from a common population of saprophytic *A. alternata*. Nishimura and his coworkers (Nishimura *et al.* 1978; Nishimura 1980) proposed that these *Alternaria* species should be designated as pathotypes of *A. alternata*. If these pathotypes evolve in the future through the fixation of polymorphisms, we may recognize them as species that have radiated from a common ancestor.

GCPSR in fungi that has evolved through species radiaton

In GCPSR, the transition from concordance to conflict should determine the limit of species (Taylor *et al.* 2000). In *Pyricularia*, the transition from concordance to conflict appeared more than once (Fig 3A, B). Then a question arises: which is the true species limit? We suggest that, in the case of fungi that involve radiation in the speciation process in the past, the limit of current species should be determined by incorporating morphological or biological species criteria into the phylogenetic species concept. We determined the current species in *Pyricularia* by incorporating morphological and biological information (Fig 3). Then another question arises: what does the other transition point mean? It may represent a former species. For example, *P. grisea* (M) may have been a species before the cryptic species differentiated. Actually, some isolates of *P. oryzae* produce perithecia when crossed with *P. grisea* although the perithecia never mature (Yaegashi 1981). This may be a remnant of the past in which *P. oryzae* and *P. grisea* were members of the same species.

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Figure legends

Fig 1 – Phylogenetic relationships of *Pyricularia* genotypes inferred from analysis of the combined actin, β-tubulin, and calmodulin exon sequences (TreeBASE submission ID number SN3200-13632). A strict consensus of 6 MP trees is shown. The tree was rooted using *Magnaporthe salvinii* MS-1 (accession No. AF395975, AF396004, AF396030) as an outgroup taxon based on the anamorph morphology. CI, consistency index; RI, retention index. See Table 1 for the genotypes.

Fig 2 - Partition homogeneity tests in data sets of all *Pyricularia* genotypes (A), the genotypes TELE1, Lo, CE1, and Dssh1 representing the four cryptic species in *P. grisea* (M) (B), and the genotypes Zl1, Zm1, and Pb representing the three morphological species (C) (cf. Table 1, Fig 3). The observed summed tree lengths were compared with the distribution of summed tree lengths calculated for 10000 randomized data sets.

Fig 3 - Phylogenetic relationships of *Pyricularia* genotypes inferred from combined rDNA (ITS1, 5.8S and ITS2), actin (exons and introns), β-tubulin (exons and an intron), and calmodulin (exons and introns) sequences (TreeBASE submission ID number SN3200-13633, SN3200-13634). One of 39 MP trees (A) and an NJ tree (B) are shown. The trees were rooted using Kb1, Kb2, and Ci as outgroup taxa. CI, consistency index; RI, retention index. Numbers at nodes represent bootstrap support >50% from 1000 replications. The seven evolutionary species are color-coded with their names in the right column. The gray box indicates the morphological species previously described as *P. grisea* (i.e., *P. grisea* (M)). See Table 1 for the genotypes.

Fig 4 - Phylogenetic relationships of the four cryptic species within *P. grisea* (M) (TreeBASE submission ID number SN3200-13635, SN3200-13636, SN3200-13639, SN3200-13638). TELE1, Lo, CE1, and Dssh1 were arbitrary chosen from *P. oryzae*, *Pyricularia* sp. (LS), *Pyricularia* sp. (CE), and *P. grisea*, respectively (cf. Fig 3). MP trees were constructed from rDNA (ITS1, 5.8S and ITS2) (A), actin (exons and introns) (B), β-tubulin (exons and an intron) (C), and calmodulin (exons and introns) (D) sequences. The trees were rooted using Zl1 as an outgroup taxon. CI, consistency index; RI, retention index. Numbers at nodes represent bootstrap support >50% from 1000 replications.

Fig 5 – Phylogenetic relationships of the three morphological species of *Pyricularia* (TreeBASE submission ID number SN3200-13640, SN3200-13641, SN3200-13642, SN3200-13644). Zl1, Zm1, and Pb were arbitrary chosen from *P. zizaniaecola*, *P. zingiberi*, and *Pyricularia* sp. (SsPb), respectively (cf. Fig 3). MP trees were constructed from rDNA (ITS1, 5.8S and ITS2) (A), actin (exons and introns) (B), β-tubulin (exons and an intron) (C), and calmodulin (exons and introns) (D) sequences. The trees were rooted using Ci as an outgroup taxon. CI, consistency index; RI, retention index. Numbers at nodes represent bootstrap support >50% from 1000 replications.

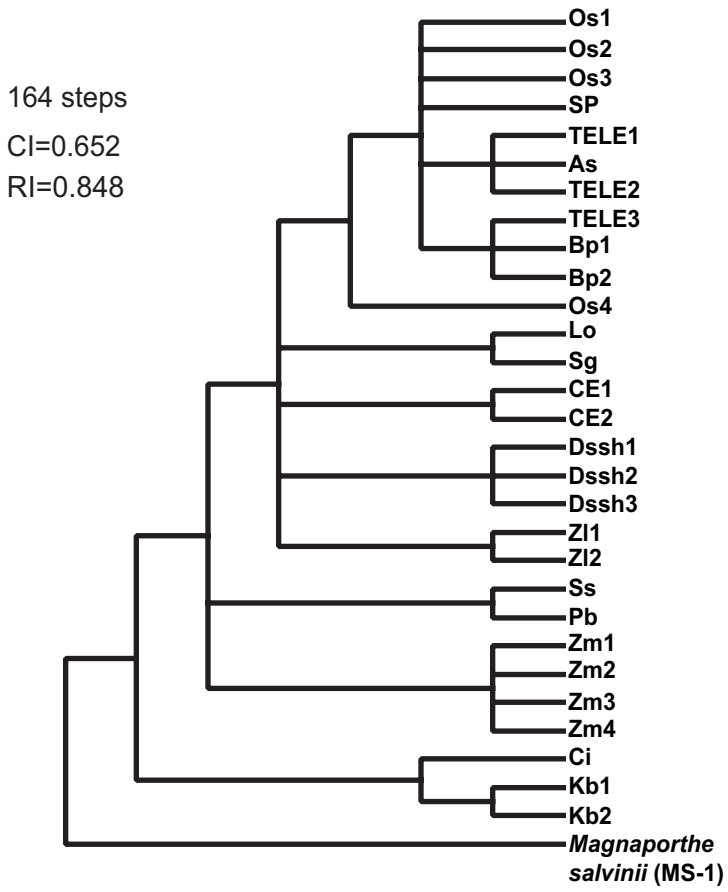
164 steps
CI=0.652
RI=0.848
Os1
Os2
Os3
SP
TELE1
As
TELE2
TELE3
Bp1
Bp2
Os4
Lo
Sg
CE1
CE2
Dssh1
Dssh2
Dssh3
Zl1
Zl2
Ss
Pb
Zm1
Zm2
Zm3
Zm4
Ci
Kb1
Kb2
Magnaporthe salvinii (MS-1)

Fig. 1.

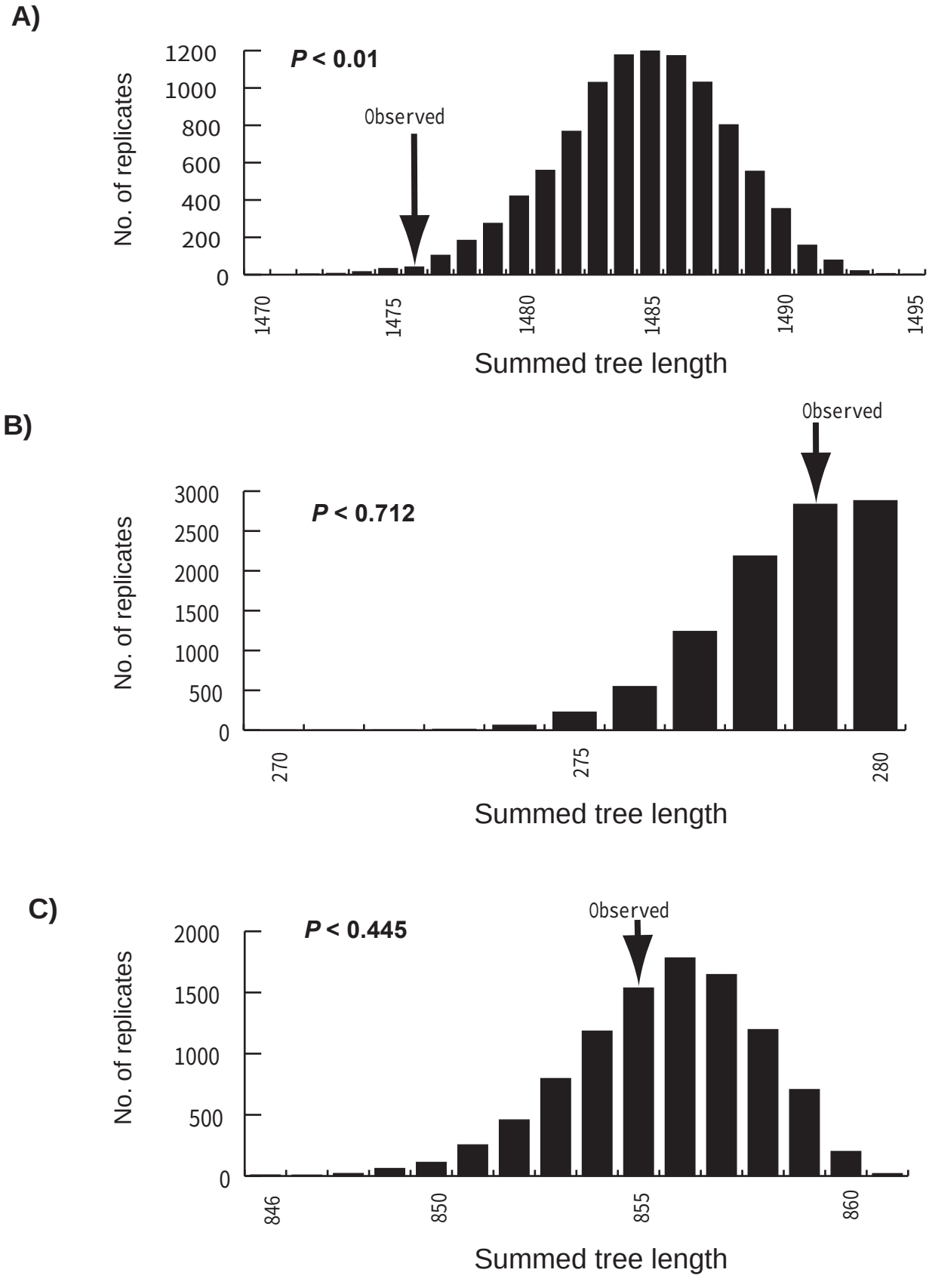
A)
P < 0.01
Observed
No. of replicates
1200
1000
800
600
400
200
0
1470
1475
1480
1485
1490
1495
Summed tree length
B)
Observed
P < 0.712
No. of replicates
3000
2500
2000
1500
1000
500
0
270
275
280
Summed tree length
C)
Observed
P < 0.445
No. of replicates
2000
1500
1000
500
0
846
850
855
860
Summed tree length

Fig. 2.

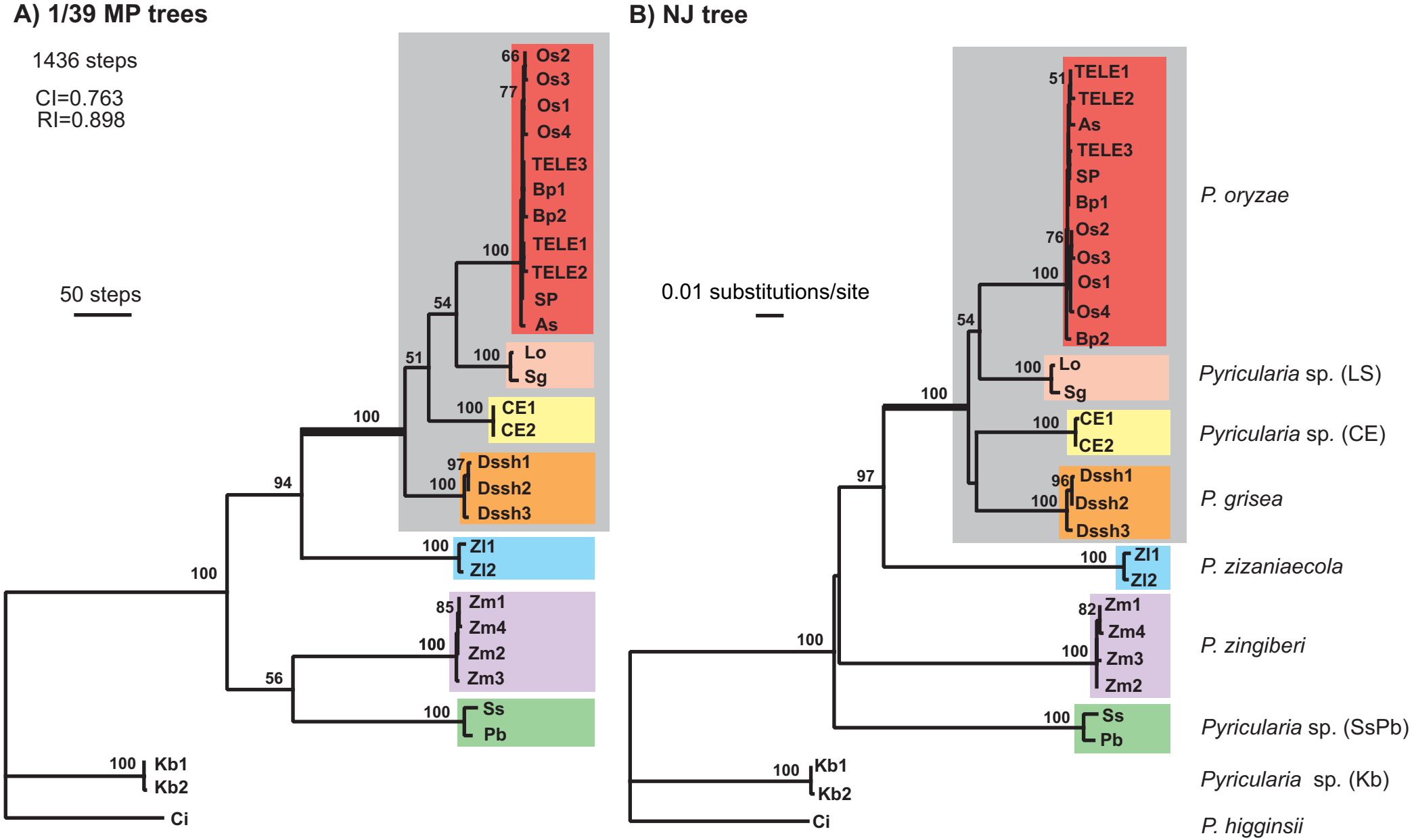
A) 1/39 MP trees
1436 steps
CI=0.763
RI=0.898
50 steps
B) NJ tree
0.01 substitutions/site
Os2
Os3
Os1
Os4
TELE3
Bp1
Bp2
TELE1
TELE2
SP
As
Lo
Sg
CE1
CE2
Dssh1
Dssh2
Dssh3
Zl1
Zl2
Zm1
Zm4
Zm2
Zm3
Ss
Pb
Kb1
Kb2
Ci
P. oryzae
Pyricularia sp. (LS)
Pyricularia sp. (CE)
P. grisea
P. zizaniaecola
P. zingiberi
Pyricularia sp. (SsPb)
Pyricularia sp. (Kb)
P. higginsii

Fig. 3.

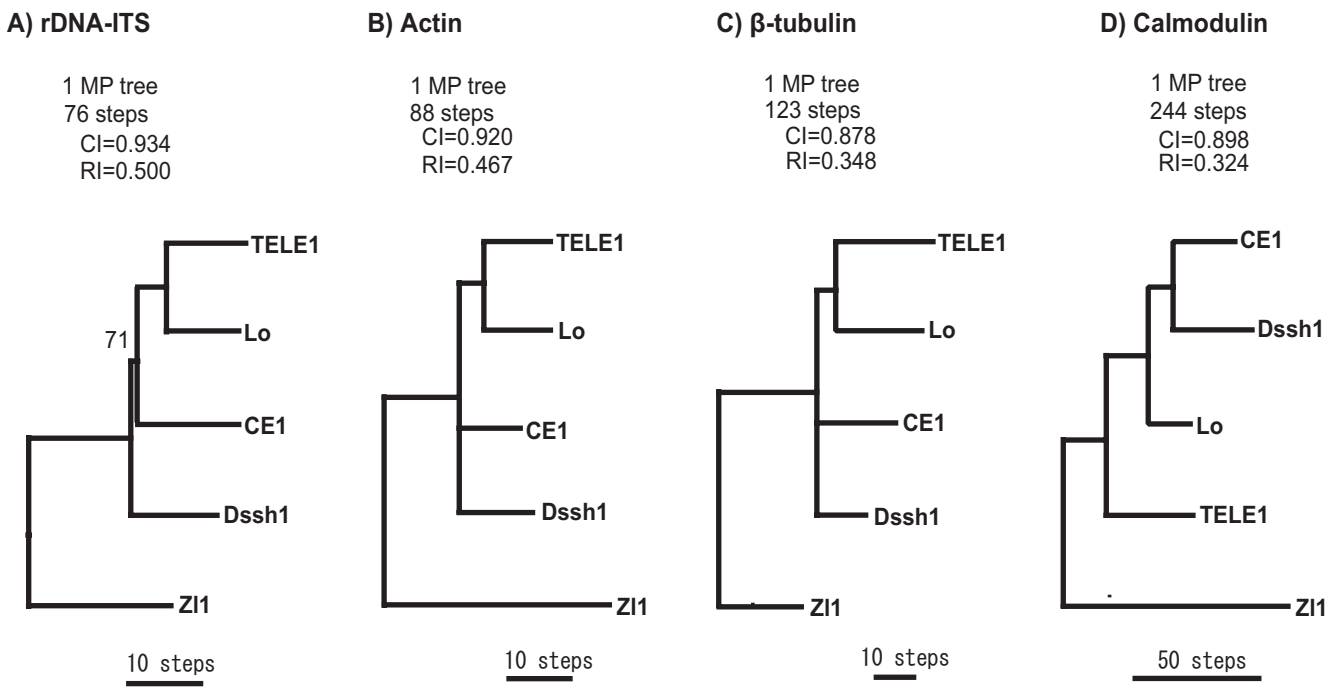
A) rDNA-ITS
1 MP tree
76 steps
CI=0.934
RI=0.500
TELE1
Lo
71
CE1
Dssh1
ZI1
10 steps
B) Actin
1 MP tree
88 steps
CI=0.920
RI=0.467
TELE1
Lo
CE1
Dssh1
ZI1
10 steps
C) β-tubulin
1 MP tree
123 steps
CI=0.878
RI=0.348
TELE1
Lo
CE1
Dssh1
ZI1
10 steps
D) Calmodulin
1 MP tree
244 steps
CI=0.898
RI=0.324
CE1
Dssh1
Lo
TELE1
ZI1
50 steps

Fig. 4.

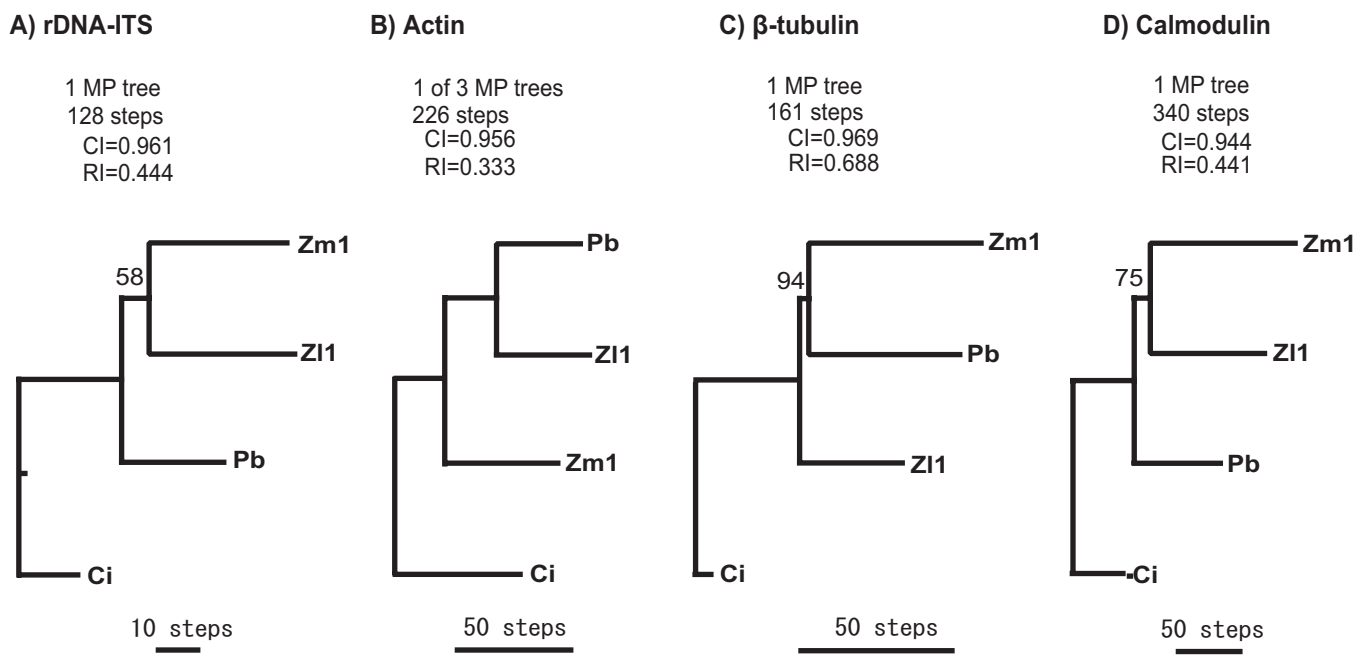
A) rDNA-ITS
1 MP tree
128 steps
CI=0.961
RI=0.444
58
Zm1
Zl1
Pb
Ci
10 steps
B) Actin
1 of 3 MP trees
226 steps
CI=0.956
RI=0.333
Pb
Zl1
Zm1
Ci
50 steps
C) β-tubulin
1 MP tree
161 steps
CI=0.969
RI=0.688
94
Zm1
Pb
Zl1
Ci
50 steps
D) Calmodulin
1 MP tree
340 steps
CI=0.944
RI=0.441
75
Zm1
Zl1
Pb
Ci
50 steps

Fig. 5.

Table 1 - *Pyricularia* isolates used and their allele types.

Isolate[a]	Host	Locality	Isolation year	Allele[b]				Genotype
				rDNA-ITS	Actin	β-Tubulin	Calmodulin	
Guy11	*Oryza sativa*	Guiana	1978	rd1	ac1	be1	ca1	Os1
Ken 54-04 (MAFF235006)	*Oryza sativa*	Japan	1954	rd2(AB274419)	ac1	be1	ca1	Os2
Ken 54-20 (MAFF235005)	*Oryza sativa*	Japan	1954	rd1(AB274418)	ac1(AB274439)	be1(AB274454)	ca1(AB274470)	Os1
Hoku 1	*Oryza sativa*	Japan	1948	rd1	ac1	be1	ca1	Os1
Ina 72 (MAFF235003)	*Oryza sativa*	Japan	1957	rd3(AB274420)	ac1	be1	ca1	Os3
Ina 168	*Oryza sativa*	Japan	1958	rd2	ac1	be1	ca1	Os2
Ken 53-33	*Oryza sativa*	Japan	1953	rd1	ac1	be1	ca1	Os1
P-2b	*Oryza sativa*	Japan	1948	rd1	ac1	be1	ca1	Os1
0903-4	*Oryza sativa*	Japan	1976	rd1	ac1	be1	ca1	Os1
2012-1	*Oryza sativa*	Japan	1976	rd1	ac1	be1	ca1	Os1
2403-1	*Oryza sativa*	Japan	1976	rd2	ac1	be1	ca1	Os2
1836-3	*Oryza sativa*	Japan	1976	rd2	ac1	be1	ca1	Os2
88A	*Oryza sativa*	Japan	1976	rd2	ac1	be1	ca1	Os2
CHNOS 59-6-1	*Oryza sativa*	China	1989	rd1	ac1	be1	ca1	Os1
CHNOS 60-8-1	*Oryza sativa*	China	1989	rd1	ac1	be1	ca1	Os1
Br10	*Oryza sativa*	Brazil	1990	rd2	ac1	be1	ca1	Os2
Br13	*Oryza sativa*	Brazil	1990	rd2	ac1	be1	ca1	Os2
Br15	*Oryza sativa*	Brazil	1990	rd1	ac1	be1	ca1	Os1
Br18	*Oryza sativa*	Brazil	1990	rd2	ac1	be1	ca1	Os2
PO-02-7306	*Oryza sativa*	Indonesia	1973	rd4(AB274421)	ac1	be2(AB274455)	ca1	Os4
PO-02-7501	*Oryza sativa*	Indonesia	1975	rd1	ac1	be1	ca1	Os1
PO-04-7501	*Oryza sativa*	Indonesia	1975	rd1	ac1	be1	ca1	Os1
PO-12-7301-2	*Oryza sativa*	Indonesia	1973	rd1	ac1	be1	ca1	Os1
PO-12-7301	*Oryza sativa*	Indonesia	1973	rd1	ac1	be1	ca1	Os1
VHT6.1	*Oryza sativa*	Vietnam	1998	rd1	ac1	be1	ca1	Os1
VTB6.1	*Oryza sativa*	Vietnam	1998	rd1	ac1	be1	ca1	Os1
VHG4.5	*Oryza sativa*	Vietnam	1996	rd1	ac1	be1	ca1	Os1
VHT3.3	*Oryza sativa*	Vietnam	1998	rd1	ac1	be1	ca1	Os1
GFSI1-7-2	*Setaria italica*	Japan	1977	rd5(AB274422)	ac1	be1	ca2(AB274471)	SP
NRSI2-2-2	*Setaria italica*	Japan	1977	rd5	ac1	be1	ca2	SP
NRSI3-1-1	*Setaria italica*	Japan	1977	rd5	ac1	be1	ca2	SP
NNSI3-2-1	*Setaria italica*	Japan	1984	rd5	ac1	be1	ca2	SP
IN77-16-1-1	*Setaria italica*	India	1977	rd5	ac1	be1	ca2	SP
IN77-20-1-1	*Setaria italica*	India	1977	rd5	ac1	be1	ca2	SP
KANSV1-4-1	*Setaria viridis*	Japan	1975	rd5	ac1	be1	ca2	SP
NI913	*Setaria viridis*	Japan	1974	rd5	ac1	be1	ca2	SP
NNPM1-2-1	*Panicum miliaceum*	Japan	1984	rd5	ac1	be1	ca2	SP
NRPM1-1-1	*Panicum miliaceum*	Japan	1990	rd5	ac1	be1	ca2	SP
STPM1-3-2	*Panicum miliaceum*	Japan	1981	rd5	ac1	be1	ca2	SP
STPM4-2-2	*Panicum miliaceum*	Japan	1981	rd5	ac1	be1	ca2	SP
SZPM1-1-1	*Panicum miliaceum*	Japan	1978	rd5	ac1	be1	ca2	SP
YNPM4-1-1	*Panicum miliaceum*	Japan	1983	rd5	ac1	be1	ca2	SP
NI922	*Panicum bisulcatum*	Japan	1974	rd5	ac1	be1	ca2	SP
G10-1	*Eleusine coracana*	Japan	1977	rd6(AB274423)	ac1	be3(AB274456)	ca2	TELE1
Z2-1	*Eleusine coracana*	Japan	1977	rd6	ac1	be3	ca2	TELE1
MZ5-1-6	*Eleusine coracana*	Japan	1976	rd6	ac1	be3	ca2	TELE1
Ken15-15-1	*Eleusine coracana*	Japan	1976	rd6	ac1	be3	ca2	TELE1
SZEC1-1-1	*Eleusine coracana*	Japan	1978	rd8(AB274425)	ac1	be3	ca2	TELE2
GFEC1-5-1	*Eleusine coracana*	Japan	1977	rd8	ac1	be3	ca2	TELE2
IN77-31-1-1	*Eleusine coracana*	India	1977	rd6	ac1	be3	ca2	TELE1
IN77-39-1-2	*Eleusine coracana*	India	1977	rd6	ac1	be3	ca2	TELE1
NP10-17-4-1-3	*Eleusine coracana*	Nepal	1975	rd6	ac1	be3	ca2	TELE1
NP10-28-1-1-1	*Eleusine coracana*	Nepal	1975	rd6	ac1	be3	ca2	TELE1
NI1006	*Eleusine africana*	Japan	1975	rd6	ac1	be3	ca2	TELE1
NI1011	*Eleusine boranensis*	Japan	1975	rd6	ac1	be3	ca2	TELE1
IN77-36-1-1	*Eleusine indica*	India	1977	rd6	ac1	be3	ca2	TELE1
Br58	*Avena sativa*	Brazil	1990	rd7(AB274424)	ac1	be3	ca2	As
Br3	*Triticum aestivum*	Brazil	1990	rd6	ac1	be4(AB274457)	ca2	TELE3
Br7	*Triticum aestivum*	Brazil	1990	rd6	ac1	be3	ca2	TELE1
Br8	*Triticum aestivum*	Brazil	1990	rd6	ac1	be4	ca2	TELE3
Br48 (IMI368172)	*Triticum aestivum*	Brazil	1990	rd6	ac1	be4	ca2	TELE3
Br49 (IMI368173)	*Triticum aestivum*	Brazil	1990	rd6	ac1	be3	ca2	TELE1
Br115.7	*Triticum aestivum*	Brazil	1992	rd6	ac1	be3	ca2	TELE1
Br116.5	*Triticum aestivum*	Brazil	1992	rd6	ac1	be3	ca2	TELE1
Br118.2D	*Triticum aestivum*	Brazil	1992	rd6	ac1	be4	ca2	TELE3
TP1	*Lolium perenne*	Japan	1997	rd6	ac1	be3	ca2	TELE1
TP2	*Lolium perenne*	Japan	1997	rd6	ac1	be3	ca2	TELE1
AK1	*Lolium perenne*	Japan	1998	rd6	ac1	be3	ca2	TELE1
LW3	*Lolium perenne*	Japan	1999	rd6	ac1	be3	ca2	TELE1
FI5	*Lolium perenne*	Japan	1998	rd6	ac1	be3	ca2	TELE1
WK3-1	*Lolium perenne*	Japan	1996	rd6	ac1	be3	ca2	TELE1
Br35	*Brachiaria plantaginea*	Brazil	1990	rd5	ac1	be4	ca2	Bp1
Bp3a	*Brachiaria plantaginea*	Brazil	1996	rd5	ac1	be4	ca3(AB274472)	Bp2
NI986	*Eragrostis lehmanniana*	Japan	1975	rd8	ac1	be3	ca2	TELE2
NI919 (MAFF305509)	*Leersia oryzoides*	Japan	1974	rd9(AB274426)	ac2(AB274440)	be5(AB274458)	ca4(AB274473)	Lo
Br37	*Setaria geniculata*	Brazil	1990	rd9	ac2	be6(AB274459)	ca5(AB274474)	Sg
NI981 (MAFF305501)	*Cenchrus ciliaris*	Japan	1975	rd10(AB274427)	ac3(AB274441)	be7(AB274460)	ca6(AB274475)	CE1
Br36	*Cenchrus echinatus*	Brazil	1990	rd10	ac3	be8(AB274461)	ca7(AB274476)	CE2
Br38	*Echinochloa colonum*	Brazil	1990	rd10	ac3	be8	ca7	CE2
Dig41	*Digitaria sanguinalis*	Japan	1990	rd11(AB274428)	ac4(AB274442)	be9(AB274462)	ca8(AB274477)	Dssh1
NI907	*Digitaria sanguinalis*	Japan	1974	rd12(AB274429)	ac4	be9	ca8	Dssh2
IBDS4-1-1	*Digitaria sanguinalis*	Japan	1985	rd11	ac4	be9	ca8	Dssh1
NI980	*Digitaria smutsii*	Japan	1975	rd12	ac4	be9	ca8	Dssh2
Br29 (IMI368175)	*Digitaria horizontalis*	Brazil	1990	rd12	ac4	be9	ca8	Dssh2
Br33	*Digitaria horizontalis*	Brazil	1990	rd13(AB274430)	ac4	be9	ca9(AB274478)	Dssh3
IBZL3-1-1	*Zizania latifolia*	Japan	1985	rd14(AB274431)	ac5(AB274443)	be10(AB274463)	ca10(AB274479)	Zl1
KYZL201-1-1	*Zizania latifolia*	Japan	2003	rd15(AB274432)	ac6(AB274444)	be10	ca11(AB274480)	Zl2
HYZiM101-1-1-1	*Zingiber mioga*	Japan	1990	rd16(AB274433)	ac7(AB274445)	be11(AB274464)	ca12(AB274481)	Zm1
HYZiM201-0-1	*Zingiber mioga*	Japan	2002	rd17(AB274434)	ac8(AB274446)	be12(AB274465)	ca12	Zm2
HYZiM202-1-2	*Zingiber mioga*	Japan	2003	rd16	ac9(AB274447)	be11	ca12	Zm3
HYZiM201-1-1	*Zingiber mioga*	Japan	2003	rd17	ac10(AB274448)	be12	ca12	Zm4
INA-B-92-45	*Sasa* sp.	Japan	1992	rd18(AB274435)	ac11(AB274449)	be13(AB274466)	ca13(AB274482)	Ss
INA-B-93-19	*Phyllostachys bambusoides*	Japan	1993	rd19(AB274436)	ac12(AB274450)	be14(AB274467)	ca14(AB274483)	Pb
HYKB202-1-2	*Kyllinga brevifolia*	Japan	2003	rd20(AB274437)	ac13(AB274451)	be15(AB274468)	ca15(AB274484)	Kb1
FKKB201-1-5	*Kyllinga brevifolia*	Japan	2003	rd20	ac14(AB274452)	be15	ca15	Kb2
FKKB201-3-2	*Kyllinga brevifolia*	Japan	2003	rd20	ac14	be15	ca15	Kb2
HYCI201-1-1	*Cyperus iria*	Japan	2002	rd21(AB274438)	ac15(AB274453)	be16(AB274469)	ca16(AB274485)	Ci

[a] Accession numbers in public culture collections are shown in parentheses. (MAFF, Microorganisms Section of the NIAS Genebank, National Institute of Agrobiological Sciences, Tsukuba, Japan; IMI: CABI Bioscience, UK Centre, Egham, UK.)

[b] Accession numbers in GenBank are shown in parentheses.

Table 2 - Characteristics of the four gene regions sequenced

	Number of				
Gene encoding	aligned sites	polymorphic sites	gaps	uninformative sites	informative sites
rDNA-ITS	494	150	60	20	70 (14.2%)
5.8S	158	3	0	1	2 (1.3%)
ITS1	140	68	30	5	33 (23.6%)
ITS2	196	79	30	14	35 (17.9%)
Actin	336	222	76	13	133 (39.6%)
exon	71	8	0	0	8 (11.3%)
intron	265	214	76	13	125 (47.2%)
β-Tubulin	519	142	52	19	71 (13.7%)
exon	427	57	0	18	39 (9.1%)
intron	92	85	52	1	32 (34.8%)
Calmodulin	533	345	121	33	191 (35.8%)
exon	153	28	0	7	21 (13.7%)
intron	380	317	121	26	170 (44.7%)
Total	1882	857	304	86	467 (24.8%)

Table 3 - Characteristics of informative sites in the four gene regions of the four cryptic species

	Number of informative sites									
Gene encoding	(T,L)[a]	(C,D)	(T,D)	(L,C)	(L,D)	(T,C)	(L,C,D)	(T,C,D)	(T,L,D)	(T,L,C)
rDNA-ITS	3	2	1	1	3	3	0	1	0	2
Actin	4	4	2	1	3	3	1	2	0	0
β-tubulin	7	7	4	5	3	3	2	2	1	3
Calmodulin	6	7	4	5	1	1	6	5	5	6
Total	20	20	11	12	10	10	9	10	6	11

[a] The four representative genotypes, TELE1, Lo, CE, and Dssh1, are abbreviated to T, L, C, and D, respectively. Genotypes that share the same nucleotide is parenthesized. For example, (T,L) includes sites at which T and L share the same nucleotide such as [Zl1(C,D)(T,L)],[(Zl1,C),D,(T,L)], [(Zl1,D),C,(T,L)], [(Zl1,C,D),(T,L)], [(Zl1,T,L),(C,D)]. Among these sites, [Zl1(C,D),(T,L)], [(Zl1,C,D),(T,L)], [(Zl1,T,L),(C,D)] are also counted in (C,D).