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# Ribosomal DNA-ITS sequence polymorphism in the sugarcane rust, *Puccinia kuehnii**

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**The two highly divergent ITS types previously observed in isolates of *Puccinia kuehnii* were amplified from the same isolates through the use of ITS type-specific primers for PCR and are therefore considered as polymorphisms of ITS regions within the species. Although homology of sequences of one of the ITS types with other *Puccinia* species was of expected levels, significantly high homology of sequences of the other type with those of *Cronartium* members indicates that abnormal genetic events led to the occurrence of these polymorphic regions. These results indicate that ITS gene tree phylogeny may not reflect true species phylogeny in this group of rust fungi. Rather, D1/D2 region tree phylogeny, which was concordant with the differences in morphology with and among related rusts, more correctly reflects phylogenetic relationships of sugarcane and related grass rusts.**

Key Words——*Puccinia kuehnii*; ribosomal DNA ITS polymorphism; sugarcane rust.

The analysis of nucleotide sequences of the ribosomal RNA gene to determine phylogenetic relationships and genetic variations in fungi is widely established. The rDNA unit is organized into three genes coding for the ribosomal units and two internal transcribed spacer (ITS) regions (Hibbet, 1992). One of the reasons for the usefulness of the nuclear rDNA in phylogenetic analysis lies in its occurrence as tandem repeat arrays with several hundred copies per genome, allowing for easy accessibility and amplification by PCR (White et al., 1990; Bruns et al., 1991). Furthermore, rDNA genes, like other multigene families, are subject to concerted evolution, resulting in homogeneity among the different copies of the gene in the cell, and making them ideal phylogenetic markers (Hillis and Dixon, 1991; Li, 1997; Page and Holmes, 1998). However, despite the homogenizing effect of concerted evolution and although the gene cluster may be derived from a single ancestral unit, the different copies of the rDNA array within a single nucleus are all replicated independently, allowing, although rarely, independent mutation and crossover that can result in various levels of polymorphism in the gene cluster within individual cells (Fatehi and Bridge, 1998; Hillis and Dixon, 1991). These intraspecific or intragenomic variations are considered to occur at low rates, mostly as length variations in both non-transcribed and transcribed regions of the gene (Hillis and Dixon, 1991; Hillis and Davis, 1988). Heterogeneity resulting from sequence differences that do not lead to size variation will only be detected through sequencing and may affect RFLP patterns (Harlton et al., 1995; Fatehi and Bridge, 1998). Occurrence of multiple forms of ITS regions in a single individual has been reported in other organisms (Zijstra, et al, 1995; Baldwin et al., 1995; Vogler and DeSalle, 1994), and in fungi (Sanders et al., 1995; Harlton et al., 1995; O' Donnell and Cigelnick, 1997; Hijri et al., 1999; Antoniolli et al., 2000).

The authors have previously detected two highly divergent ITS sequence types in morphologically indistinguishable isolates of *P. kuehnii* Butler (Virtudazo et al., 2001a). However, despite the divergence in ITS sequences, all isolates were found to have identical sequences in the D1/D2 region of the nuclear LSU rDNA and hence belonged to a single cluster in the phylogenetic tree constructed from D1/D2 region sequences. Despite having identical D1/D2 sequences and morphological characters, the high divergence and the occurrence of two nucleotide differences in the conserved 5.8S rDNA region between the two ITS types may support separation into different populations within *Puccinia kuehnii*. However, the unusual homology of ITS sequences of *P. kuehnii* with *Cronartium* members shows a homoplastic pattern of evolution and indicates that the D1/D2 region tree, which clearly separated the morphologically distinguishable units, rather than the ITS region tree, reflects true species phylogeny in this group of rust fungi (Virtudazo et al., 2001a, b). Therefore, rather than showing support for interspecific divergence, the possibility that the two highly divergent sequences may have resulted from unusual genetic events leading to non-orthologous, possibly intragenomic polymorphisms in the species, seemed to be more likely and is further

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examined in this study.

## Materials and Methods

Isolates used in this study are listed in Table 1. ITS type-specific primers were designed from previously determined sequences and are shown in Table 2. They were tested for percentage homology and maximum matching with the target and non-target ITS types using Genetyx-Mac v. 10.1 (Software Development Co.) and were found highly specific to the respective target sequence type.

PCR was conducted with DNA extracted from urediniospores by the same method used in the original amplification with ITS 1F-ITS 4 primer pair (Virtudazo et al., 2001a). Amplifications were done in 100 $\mu$l PCR reaction mixtures containing different combinations of 2 $\mu$M type-specific primer pairs, 2.5 units of TaKaRa Ex Taq DNA polymerase (Takara, Japan), and the supplied dNTP mixture (containing 2.5 mM dNTPs) and Ex Taq reaction buffer (containing 2 mM $Mg^{2+}$). PCR was carried out using an ATTO Zymoreactor II (Atto Co., Japan) under the following conditions: 95°C for 3 min, then 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final step of 72°C for 10 min. The type-specific primers were also tested further on purified PCR products (ITS 1F-ITS 4 primer products previously used as templates in sequencing reactions) of isolates of both ITS types. Although the band sizes of the type-specific PCR products in agarose gel electrophoresis were consistent with predicted sizes computed from the location of the primers, showing that the target ITS type sequences were amplified, we further verified this by sequencing the type-specific PCR reaction products. Furthermore, we also conducted sequencing reactions using the original PCR products (ITS 1F-ITS4) as templates and the type-specific primers as sequencing primers. Sequencing was done according to previously reported procedures (Virtudazo et al., 2001a).

## Results and Discussion

### Amplification from DNA extracted from urediniospores

Table 2. Primers designed for specific amplification of ITS types I and II.

| Primer[a] | Primer sequence | *Tm*[b] (°C) |
|---|---|---|
| ITS type I | | |
| PK 1-1F | GTGTGCCTTTTTTGGTATAGCATC | 68 |
| PK 1-1B | AATGGGGGTTAGGAAGCTATT | 60 |
| PK 1-2F | CCCTTTATAAGTGACCCCCTTT | 64 |
| PK 1-2B | AATAAAGAATTGGAATGAGAGGG | 62 |
| ITS type II | | |
| PK 2-1F | AATATGGGGGAAACCTCATTA | 58 |
| PK 2-1B | GGCAGGTAACACCTTCCTTGATG | 70 |
| PK 2-2F | CCACATATATGAAAATGAATGTA | 58 |
| PK 2-2B | GTTAAGGGGGAGGAGGAACTTGA | 70 |

[a] F primers are forward primers, B primers are backward primers. Sequences are written 5′-3′.
[b] *Tm*'s were calculated by the formula: $Tm = 2(A+T)+4(G+C)$.

Initial PCR tests showed that ITS type I-specific primers did not yield amplification products, while ITS type II-specific primers yielded amplification products in isolates of both ITS types (Table 3). However, upon lowering the annealing temperature of the PCR reactions to 50°C, amplifications were detected using ITS type I-specific primers for isolates of both types (Fig. 1).

Amplification from both isolates using specific primers for ITS types I and II demonstrated that both ITS types are present in the same isolate. However, ITS type II is apparently preferentially amplified. This could be due to difference in copy number, with the ITS type II representing the major ITS form and occurring in higher copy number than the ITS type I. This agrees with the observation that most isolates of *P. kuehnii* exhibited the ITS type II and only a small percentage yielded ITS type I. It could also be due to amplification bias due to PCR selection, wherein primer efficiencies vary between the type-specific primers designed in this study (Wagner et al., 1994). Further studies, such as hybridization experiments, may provide insight into copy number differences between these two types.

**Amplification from original PCR products** Tests con-

Table 1. Isolates of ITS groups I and II used in the study.

| Isolate number | Locality | Date collected | Voucher specimen[a] | ITS Type (Virtudazo et al., 2001a) |
|---|---|---|---|---|
| PSPFS2-2 | Naha, Japan | 1997. 6.18 | TSH-R11164 | I |
| PSPFS3-2 | Miyako, Japan | 1997. 6.19 | TSH-R11165 | I |
| PSPFS20-2 | Naha, Japan | 1997. 6.18 | TSH-R11170 | I |
| PSP1211 | Naha, Japan | 1997. 6.18 | TSH-R11175 | I |
| PKUDS50-1 | Australia | 1935. 9.15 | BPI-79612 | I |
| PKUDS51-1 | Hawaii | 1916. 6.26 | BPI-79624 | I |
| PSPFS32-1 | Miyako, Japan | 1997. 6.19 | TSH-R11174 | II |
| PSPFS16-1 | Naha, Japan | 1997. 6.18 | TSH-R11169 | II |
| PSPFS19-1 | Amamioshima, Japan | 1996.10.29 | TSH-R11042 | II |
| PSP1163 | Amamioshima, Japan | 1996.10.29 | TSH-R11043 | II |

[a] TSH-, Mycological Herbarium, University of Tsukuba, Japan; BPI-, USDA National Fungus Collections, USA.

Table 3. Results of PCR using ITS type-specific primers[a].

| Primer pair | Predicted Prod. size (bp) | ITS type I isolates | | | | | | ITS type II isolates | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | P. sp 20 | P. sp 1211 | P. sp FS2 | P. sp FS3 | P. ku DS1 | P. ku DS2 | P. sp 19 | P. sp 1163 | P. sp FS32 |
| ITS type 1 primers | | | | | | | | | | |
| PK1-1F–PK1-1B | 484 | — | — | — | — | — | — | — | — | — |
| PK1-1F–PK1-2B | 297 | — | | — | — | — | — | | | |
| PK1-2F–PK1-2B | 223 | — | — | — | — | — | — | — | — | — |
| PK1-2F–PK1-1B | 410 | — | | — | — | — | — | | | |
| ITS1F–PK1-1B | 636 | — | — | | — | | | — | — | |
| ITS1F–PK1-2B | 449 | — | — | | — | | | — | — | |
| PK1-1F–ITS4 | 551 | — | — | | — | | | — | — | |
| PK1-2F–ITS4 | 477 | — | — | | — | | | — | — | |
| ITS type 2 primers | | | | | | | | | | |
| PK2-1F–PK2-1B | 417 | + | + | | + | — | — | + | + | + |
| PK2-1F–PK2-2B | 345 | + | | — | + | — | — | | | |
| PK2-2F–PK2-2B | 258 | + | + | | + | — | — | + | + | + |
| PK2-2F–PK2-1B | 330 | + | | — | + | — | — | | | |
| ITS1F–ITS4 | 704/678 | + | + | + | + | — | — | + | + | — |

[a] Band size matched predicted sizes for different primer pairs. Predicted product size is the size of the region amplified minus the primers. Blank means not tested.

ducted with purified PCR products (ITS 1F-ITS 4 primer products previously used as templates in sequencing reactions) of isolates of both ITS types yielded amplification with both primers for products that previously yielded the different ITS types (Fig. 2A, 2B). Results from sequencing of type-specific PCR products showed that

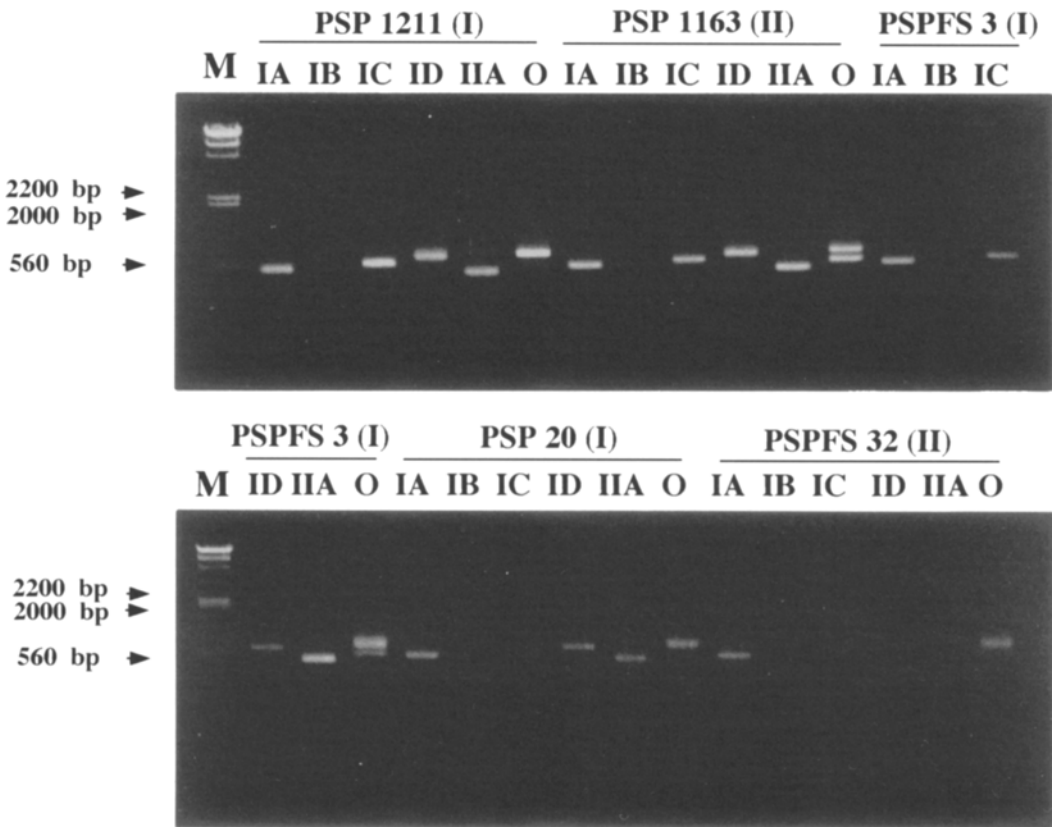


Fig. 1. Results of PCR at lowered annealing temperatures (50°C). Isolates PSP 1211, PSPFS3, PSP 20 are ITS type I isolates, while PSP 1163, PSPFS 32 are ITS type II isolates. Lanes IA to ID were amplified with ITS type I-specific primers (IA: PK1-1F, PK1-1B; IB: PK1-2F, PK1-2B; IC:PK1-1F, ITS 4; ID: ITS1F, PK1-1B), while lane IIA was amplified with ITS type II-specific primers (PK2-1F, PK2-1B). Lane O was amplified with the original primer pair, ITS1F, ITS 4.

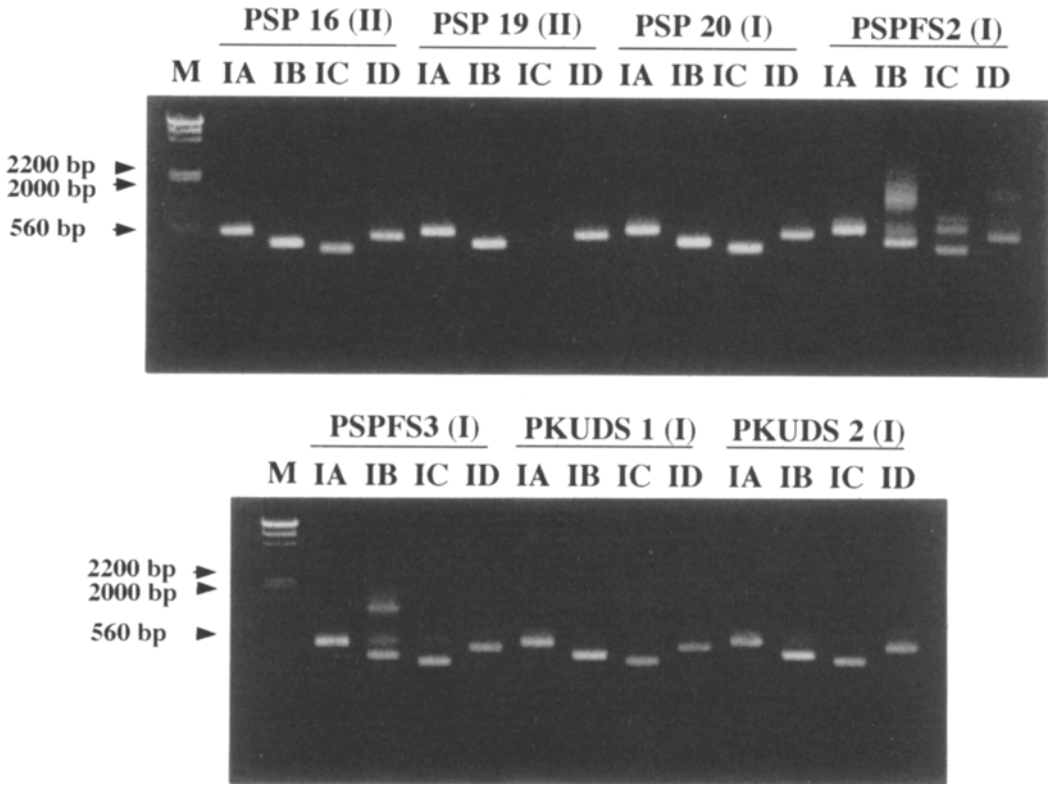


Fig. 2A. Results of PCR from original PCR products (amplified with primer pair ITS1F, ITS 4) using ITS type I-specific primers. Isolates PSP 20, PSPFS2, PSPFS3, PKUDS 1, PKUDS 2 are ITS type I isolates, while PSP 16 and PSP19 are ITS type II isolates. Lanes IA to ID were amplified with ITS type I-specific primers (IA: PK1-1F, PK1-1B; IB: PK1-1F, PK1-2B; IC:PK1-2F, PK1-2B; ID: PK1-2F, PK1-1B).

the type-specific PCR reaction products were true to the type. In cases where type I isolates yielded type II sequences, products amplified by the type II primers were indeed type II sequences, and vice versa.

Amplification of both types with templates obtained from the original PCR products of the ITS1F-ITS4 primer pair indicate that both types were amplified in the original PCR reaction but in unequal proportions. This is conceivable because the primer pair ITS1F-ITS4 amplifies both the ITS types. Thus, PCR products of isolates that yielded ITS type I in sequencing reactions also possessed small amounts of amplified ITS type II sequences, and vice versa. Although the two ITS sequence types differ in length by around 20 bp, they were not detected in agarose gel electrophoresis, possibly because one of the types was present in too small an amount to produce a detectable electrophoretic band. Thus, using these "mixed" amplification products as template for ITS type-specific PCR reactions resulted in amplification of sequences of both types from one supposedly "pure" PCR product. In many cases, however, PCR with the original primer pair ITS1F- ITS 4 resulted in distinctly double electrophoretic bands in agarose, from which subsequent sequencing analysis was not done.

**Homology with ITS sequences of other rusts** The phylogenetic tree (Fig. 3) showed that these two ITS types in *P. kuehnii* are highly divergent, and that divergence exceeds the level of variation normally found between and among related species. Homology search by BLAST of the DNA sequence database showed that ITS type II sequences were similar to ITS sequences of rusts of sugarcane and other hosts belonging to *Puccinia*. The ITS type I sequence, on the contrary, showed significantly high homology with ITS sequences of rust fungi belonging to the genus *Cronartium* rather than *Puccinia*. This type may have arisen through abnormal accidental insertions resulting from interspecies hybridization with rust from other lineages and thus represent non-orthologous, specifically xenologous sequences (O' Donnell and Cigelnik, 1997).

Members of *Puccinia*, belonging to Pucciniaceae, alternate on a wide range of host families, while members of *Cronartium*, belonging to Cronartiaceae, have a more limited host range. Although *Cronartium* aecial hosts are not hosts of *Puccinia*, certain telial host families such as Saxifragaceae, Scrophulariaceae and Gentanaceae are also hosts of some *Puccinia* members (Cummins and Hiratsuka, 1983; Hiratsuka et al., 1992). These common host families could have served as venues for the putative introgressive hybridization event between members of these two different rust lineages.

On the other hand, these polymorphisms could also be products of an ancient gene duplication event that occurred prior to the evolutionary radiation of the *Puccinia* lineage and may represent paralogous polymorphic sequences of rDNA (Sanderson and Doyle, 1992; O' Don-

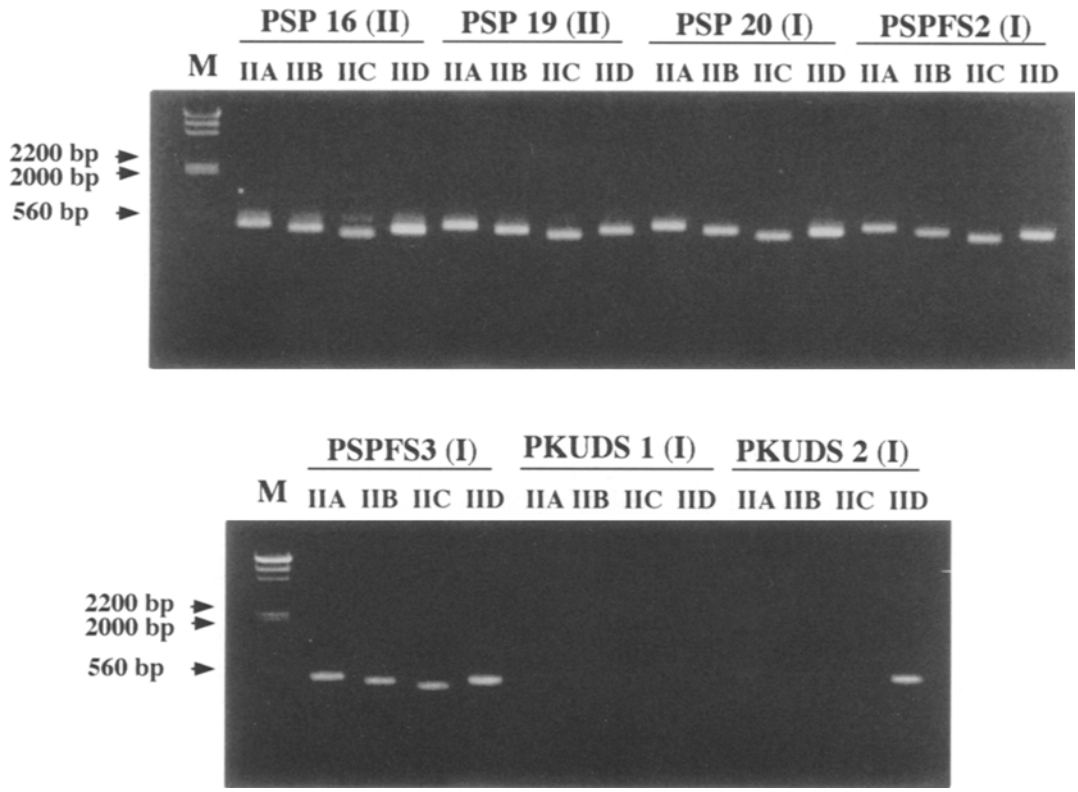


Fig. 2B. Results of PCR from original PCR products (amplified with primer pair ITS1F, ITS 4) using ITS type II-specific primers. Isolates PSP 20, PSPFS2, PSPFS3, PKUDS 1, PKUDS 2 are ITS type I isolates, while PSP 16 and PSP19 are ITS type II isolates. Lanes IIA to IID were amplified with ITS type II-specific primers (IA: PK2-1F, PK2-1B; IB: PK2-1F, PK2-2B; IC:PK2-2F, PK2-2B; ID: PK2-2F, PK2-1B).

nell and Cigelnick, 1997). Previously reported rDNA polymorphisms (Zijstra, et al., 1995; Suh et al., 1993; Vogler and DeSalle, 1994; Sanders et al., 1995; Harlton et al., 1995; O' Donnell and Cigelnick, 1997; Hijri et al., 1999; Antoniolli et al., 2000) are composed of a small number of nucleotide differences that either span the entire ITS region or occur in either of the two spacer regions. Divergence of the two nonorthologous sequences observed in this study is extremely high and even involves two nucleotide differences in the conserved 5.8S rDNA region. One of the nucleotide differences in the 5.8S rDNA region between the two types is shared with the other *Puccinia* species, while the other is shared with members of *Cronartium*. Unlike the D1/D2 region sequences, which were conserved among the isolates that showed different ITS types, the 5.8S rDNA region is apparently implicated in the putative hybridization or gene duplication events that brought about this heterogeneity.

Since they appear to be more ancient than the species in which they occur, they can also be considered as transspecies polymorphisms that were sorted among *Puccinia* and *Cronartium* lineages in such a way that coalescence of these polymorphic ITS types did not occur within the *Puccinia* lineage but prior to separation of *Puccinia* and *Cronartium* lineages (Doyle, 1992; Li, 1997; Page and Holmes, 1998). Since the 5.8S rDNA region is also heterogeneous and carries nucleotide substitutions shared by both lineages, its gene tree phylogeny may provide an insight into the coalescence of these two ITS types and phylogenetic relationships between these two rust lineages.

Analysis of members of Urediniomycetes placed *Puccinia* and *Cronartium* in distant clades in the phylogenetic trees (Sjamsuridzal et al., 1999). Furthermore, ontogeny and morphology of spermogonia and telia, which are considered to reflect evolutionary and phylogenetic relationships in rust fungi, are clearly differentiated in *Puccinia* and *Cronartium* members, which respectively form two distinct families of rust fungi, Pucciniaceae and Cronartiaceae, (Cummins and Hiratsuka, 1983). Pucciniaceae belong to Group V and Cronartiaceae to Group II based on spermogonial morphology and have telial Type IV and Type II, respectively (Hiratsuka and Cummins, 1963; Hiratsuka, 1988). These molecular and morphological evidences indicate that they are phylogenetically distant lineages. Thus, the extremely high homology of ITS type I to sequences of a quite unrelated lineage indicates a homoplastic pattern of evolution (Hillis and Dixon, 1991). If ITS type I sequences are products of an ancient gene duplication event synchronous to the divergence of the lineages that led to *Puccinia* and *Cronartium*, then these paralogous polymorphisms complicate the hierarchical relationships among rDNA genes within and between species of these two lineages (Sanderson and Doyle, 1992) and mask true species phylo-

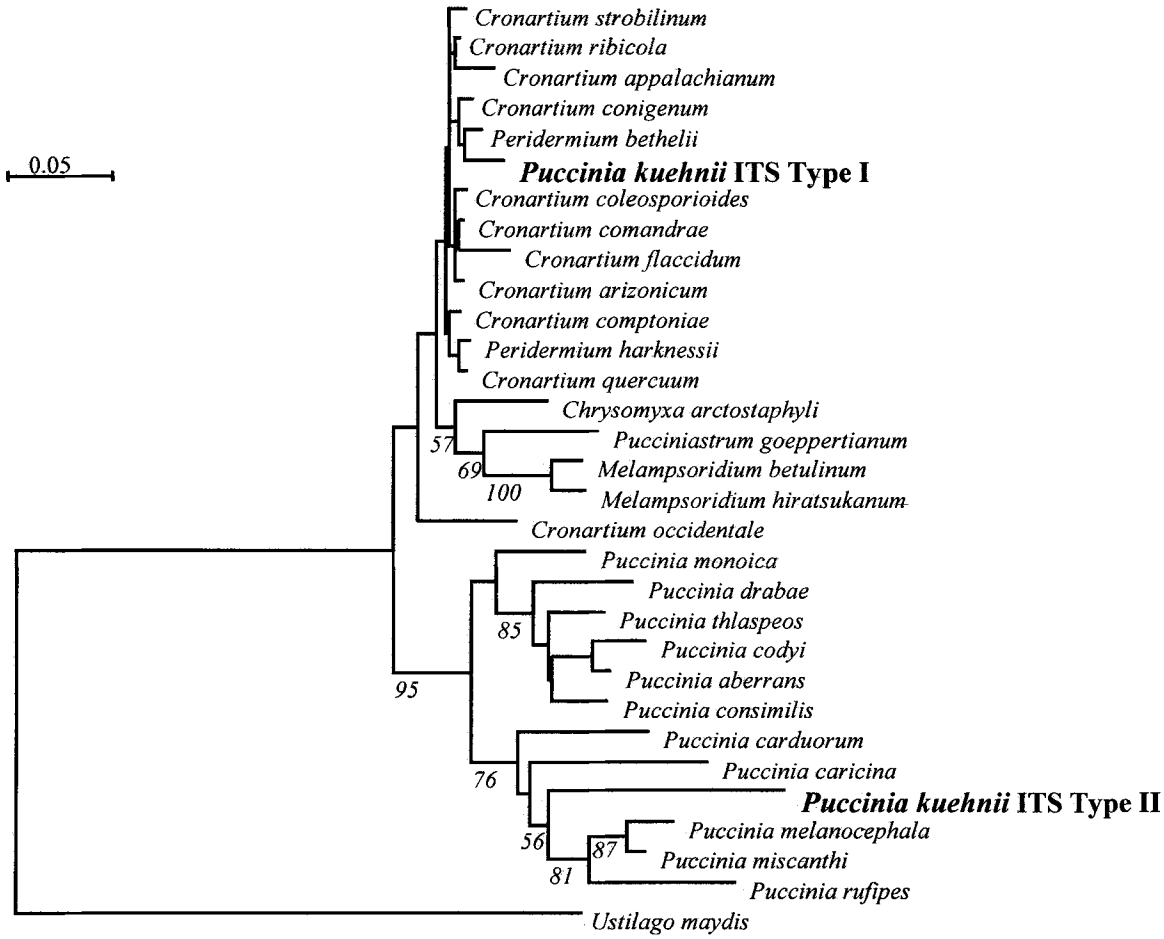


Fig. 3. Neighbor-joining tree inferred from sequences of ITS and 5.8S rDNA regions. Bootstrap support for 1000 resamplings is shown for branches with more than 50% support. Length of branches is proportional to number of base changes indicated by the scale above.

genetic relationships (Doyle, 1992).

Further evidence must be obtained to determine the exact origin of these highly divergent polymorphic sequences. In addition, the occurrence of similar homologous regions in rDNA or in other loci should be examined between other members of *Cronartium* and *Puccinia*.

ITS type I sequences apparently persisted over a long time, spanning several divergence and speciation events. O' Donnell and Cigelnik (1997) discussed the mechanisms by which intragenomic heterogeneity could have escaped the homogenizing effects of concerted evolution and become established across many speciation events in *Fusarium*. Among the factors that affect the rate of concerted evolution is the arrangement of the repeats in the genome (Graur and Li, 2000). Concerted evolution is least favorable when the repeats of an array are highly dispersed throughout the genome (Vogler and DeSalle, 1994; Li, 1997). The polymorphisms could be located in the chromosomes in such a way that they were highly diverged and inaccessible to inter-chromosomal conversions (Li, 1997; O' Donnell and Cigelnik, 1997).

Since the DNA was extracted from urediniospores, which are binucleate (n+n), it is also possible that these two types occur in different nuclei of the spore (Harlton et al., 1995) and thus represent intra-sporal heterogeneity similar to that observed in multinucleate spores of Glomales (Sanders et al., 1995; Hijri et al., 1999; Antoniolli et al., 2000).

This heterogeneity in ITS regions may have implications for the interpretation of phylogenetic relationships of these rusts based on rDNA ITS sequence data alone (O' Donnell and Cigelnik, 1997; Hillis and Davis, 1988). Non-orthologous rDNA loci are reported to possibly replace major orthologous loci during speciation and lead to disruptions in an evolutionary lineage (Dubcovsky and Dvorak, 1995). In addition, these rDNA polymorphisms may also sometimes go undetected by direct sequencing of PCR products (Wendel et al., 1995; Baldwin et al., 1995). The occurrence of rDNA polymorphisms shows that thorough sampling to detect possible polymorphisms and comparisons with other regions or gene loci that more appropriately reflect the species phylogeny must be done (Hillis and Dixon, 1991; Sanderson and Doyle, 1992; Doyle, 1992).

Our results showed that these two highly divergent ITS types occur in the same individual as polymorphic ITS forms and confirm previous speculations that ribosomal DNA polymorphism occurs in ITS regions of the sugar-

cane rust *Puccinia kuehnii*. They also coincide with the grouping together of these isolates into a single cluster based on sequences of the more conserved D1/D2 region of the LSU rDNA, and with results from comparative morphological studies showing that *Puccinia* sp. and *P. kuehnii* isolates could not be separated morphologically (Virtudazo et al., 2001b). They therefore support the grouping of these isolates into one species, *P. kuehnii* (Virtudazo et al., 2001a). In addition, this shows that the D1/D2 regions of the LSU rDNA of *P. kuehnii* is more appropriate for resolving phylogenetic relationships in sugarcane and other related rusts.

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## Literature cited

Antoniolli, Z. I., Schachtman, D. P., Ophel-Keller, K. and Smith, S. E. 2000. Variation in rDNA ITS sequences in *Glomus mossaea* and *Gigaspora margarita* spores from a permanent pasture. Mycol. Res. **104**: 708–715.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. **82**: 247–277.

Bruns, T. D., White, T. J. and Taylor, J. W. 1991. Fungal Molecular Systematics. Annu. Rev. Ecol. Syst. **22**: 525–564.

Cummins, G. B. and Hiratsuka, Y. 1983. Illustrated genera of rust fungi, revised ed. The American Phytopathological Society, Minnesota.

Doyle, J. J. 1992. Gene trees and species trees: molecular systematics as one-character state taxonomy. Syst. Bot. **17**: 144–163.

Dubcovsky, J. and Dvorak, J. 1995. Ribosomal RNA multigene loci: nomads of the Triticeae genomes. Genetics **140**: 1367–1377.

Fatehi, J. and Bridge, P. 1998. Detection of multiple rRNA-ITS regions in isolates of *Ascochyta*. Mycol. Res. **102**: 762–766.

Graur, D. and Li, W-H. 2000. Fundamentals of Molecular Evolution. 2nd ed. Sinauer Associates, Inc. Massachusetts.

Harlton, C. E., Levesque, C. A. and Punja, Z. K. 1995. Genetic diversity in *Sclerotium (Athelia) rolfsii* and related species. Phytopathology **85**: 1269–1281.

Hibbet, D. S. 1992. Ribosomal RNA and fungal systematics. Trans. Mycol. Soc. Japan **33**: 533–556.

Hijri, M., Hosnen, M., van Tuinen, D. and Dulieu, H. 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fung. Gen. Biol. **26**: 141–151.

Hillis, D. M. and Davis, S. K. 1988. Ribosomal DNA: intraspecific polymorphism, concerted evolution, and phylogeny reconstruction. Syst. Zool. **37**: 63–66.

Hillis, D. M. and Dixon, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quart. Rev. Biol. **64**: 411–453.

Hiratsuka, N., Sato, S., Kakishima, M., Kaneko, S., Sato, T., Hiratsuka, T., Katsuya, K., Hiratsuka, Y., Ono, Y., Harada, Y. and Nakayama, K. 1992. The rust flora of Japan. Tsukuba Shuppankai, Ibaraki.

Hiratsuka, Y. 1988. Ontogeny and morphology of teliospores (probasidia) in Uredinales and their significance in taxonomy and phylogeny. Mycotaxon **31**: 517–531.

Hiratsuka, Y. and Cummins, G. B. 1963. Morphology of spermogonia of the rust fungi. Mycologia **55**: 487–507.

Li, W-H. 1997. Molecular Evolution. Sinauer Associates, Inc. Masssachusetts.

O' Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogen. Evol. **7**: 103–116.

Page, R. D. and Holmes, E. C. 1998. Molecular evolution: a phylogenetic approach. Blackwell Science Ltd., Oxford.

Sanders, I. R., Alt, M., Groppe, K., Boller, T. and Wiemken, A. 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. **130**: 419–427.

Sanderson, M. J. and Doyle, J. J. 1992. Reconstruction of organismal and gene phylogenies from data on multigene families: concerted evolution, homoplasy, and confidence. Syst. Biol. **41**: 4–17.

Sjamsuridzal, W., Nishida, H., Ogawa, H., Kakishima, M. and Sugiyama, J. 1999. Phylogenetic relationships of rust fungi parasitic on ferns: Evidence from 18S rDNA sequence analysis. Mycoscience **40**: 21–27.

Suh, Y., Thien, L. B., Reeve, H. E. and Zimmer, E. A. 1993. Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. Am. J. Bot. **80**: 1042–1055.

Virtudazo, E. V., Nakamura, H. and Kakishima, M. 2001a. Phylogenetic analysis of sugarcane rusts based on sequences of ITS, 5.8S rDNA and D1/D2 region of LSU rDNA. J. Gen. Plant Pathol. **67**: 28–36.

Virtudazo, E. V., Nojima, H. and Kakishima, M. 2001b. Taxonomy of *Puccinia* species causing rust diseases on sugarcane. Mycoscience **42**: 167–175.

Vogler, A. P. and DeSalle, R. 1994. Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. Mol. Biol. Evol. **11**: 393–405.

Wagner, A., Blackstone, N., Cartwright, P., Dick, M., Misof, B., Snow, P., Wagner, G. P., Bartels, J., Murtha, M. and Pendleton, J. 1994. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst. Biol. **43**: 250–261.

Wendel, J. F., Schnabel, A. and Seelanan, T. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. USA **92**: 280–284.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols (Innis, M. A., Gelfrand, D. H., Sninsky, J. J. and White, J., eds.). pp. 315–322. Academic Press, San Diego, California.

Zijstra, C., Lever, A. E. M., Uenk, B. J. and Van Silfhuot, C. H. 1995. Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. Phytopathology **85**: 1231–1237.