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CHARACTERIZATION OF APHYLLOPHORALES CAUSING BROWN AND WHITE ROT IN WOODS: ANALYSIS OF ITS RESTRICTION POLYMORPHISM (PCR/RFLP)

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Abstract:

Intraspecific polymorphism exists for many fungus species, making their macroscopic identification difficult. Easily usable molecular markers providing access to information about variability have boosted the development of fungus diversity analyses. For this study, on wood-decaying Aphyllophorales, the main problem to be solved consisted in detecting any existence of intraspecific polymorphism in these fungi, in order to complete and clarify their identification. In order to characterize 98 strains in the CIRAD collection, we developed a rapid discriminant analysis method based on analysing the diversity of partial ribosomal DNA sequences independently of the macro- and microscopic morphological traits of the fruiting bodies required for the identification of genera and species. Adaptation of the Qiagen kit for higher plants made it possible to extract fungal DNA from a few milligrams of mycelium; with most of the strains studied, these DNA extracts enabled us to obtain an ITS amplificate of a size varying from 550 to 895 base pairs. We also found a large margin of error, of around 12%, for the DNA molecular weight readings on gel, leading us to prefer direct sequencing for precise determination of amplificate lengths. PCR-RFLP showed a good ability to characterize a given strain by providing different profiles obtained using several restriction enzymes. We thus demonstrated that studying ITS diversity using PCR-RFLP is a discriminant method making it possible to distinguish between strains. However, this method calls for the compilation of a database whose use remains partly subjected to interpretation, which cannot be used to work on a material unknown to the database. This simple, rapid technique is efficient and perfectly suitable for monitoring known strains in controlled trials.

Key words: *PCR/RFLP; wood fungi; fibrous rot; cubic rot; ribosomal DNA; molecular taxonomy; internal transcribed spacer.*

INTRODUCTION

Intraspecific polymorphism exists for many fungus species, making their macroscopic identification difficult (Stalpers 1976). Easily usable molecular markers providing access to information about variability have boosted the development of fungus diversity analyses. For this study, on wood-decaying Aphyllophorales, the main problem to be solved consisted in detecting any existence of intraspecific polymorphism in these fungi, in order to complete and clarify their identification. To do that, an ideal marker needs to be independent from environmental conditions, discriminant and neutral (Bruns *et al.* 1991). Use of molecular biology techniques based on the polymerization chain reaction of DNA (PCR) developed by Bruns *et al.* (1991) makes it possible to amplify portions of the nuclear genome of fungi. The most widely used portions of DNA for genetic studies on Basidiomycetes are situated in the nuclear ribosomal operon (Gardes *et al*. 1991, Gardes and Bruns 1993). In Basidiomycetes, these genes are organized in at least 200 copies and a length of around 9,000 base pairs. It is formed by three genes involved in the structure of ribosomes: 18 S (1,800 base pairs), 5,8 S (120 base pairs) and 28 S (3,200 base pairs) separated by two non-encoding regions called ITS (*Internal Transcribed Spacer*). Each of the copies is separated by an IGS (*Inter-Genic Spacer*) or, in the case of Basidiomycetes, an additional ribosomal 5 S gene is intercalated. Some portions of that DNA are selected to study diversity at different taxonomic levels (Bruns *et al*. 1991). In fact, the

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ribosomal operon contains some portions that vary little, enabling interspecific studies at genus and family level, and some portions that are highly variable, enabling studies between close species or of intraspecific variability (Bruns *et al.* 1991, Hillis *et al*. 1991).

The CIRAD laboratory holds 500 strains of fungi responsible for decay in tropical and temperate woods; most of these strains were determined between 1952 and 1982 based on morphological and anatomical traits of the fruiting bodies from which they came. Some species are represented by dozens of strains, such as *Pycnoporus sanguineus, Trametes versicolor, Donkioporia expansa* and *Spongipellis spumeus* without it being possible to know whether that multiplicity corresponds to true genetic diversity. It soon appeared that using molecular techniques for better knowledge of these strains would be beneficial for the good conservation, management and use of this biological heritage. Indeed, molecular tools can be used to work at several taxonomic levels, including families, genera and intraspecific variability (White *et al*. 1990; Bruns *et al.* 1991, Gardes and Bruns 1993). To do that, it is necessary to implement different methods, such as obtaining mycelium free of any trace of exogenous DNA, DNA purification by successive stages, PCR amplification of the target sequence, use of restriction enzymes capable of cleaving the DNA so as to generate interpretable restriction profiles (Zaremski *et al*. 1998) and ITS sequencing. The sequences obtained are then compared with sequences in databases (NCBI) using NCBI BLASTn. The comparison with sequences displaying high or even total homologies (varying from 80 to 100%) could provide some indications on the taxonomic position and diversity of these Basidiomycetes (Jasalavich *et al*. 2000, Moreth and Schmidt 2000, Göller *et al*. 2003).

Thus, to complete the characterization of our strains and explore any intraspecific diversity among Basidiomycetes, all of which are Aphyllophorales decaying wood by brown and white rot, in the CIRAD strain collection, we chose to use PCR/RFLP and DNA sequencing.

METHOD AND MATERIALS

CIRAD strain collection

The CIRAD Forest Department has a collection of wood-decaying fungus cultures (Fig. 1). The collection is primarily the culmination of surveys and isolations carried out during work trips between 1952 and 1982. It mostly contains wood-decaying Basidiomycetes from hot regions; some species collected in France have been added to it to broaden our frame of reference.

All the identifications were made from fruiting bodies collected in the field, not from the traits of cultures, or morphological and anatomical traits of the vegetative system alone.

Fig. 1.
Partial view of the collection of strains (Aphyllophorales) in the CIRAD Laboratory.

Biological material

Ninety-eight strains of Basidiomycetes, all belonging to the order of the Aphyllophorales, were chosen from the CIRAD collection. Their characteristics are presented in Table 1. These Basidiomycetes were identified using conventional classification criteria, such as morphology and the usual physiological and biochemical characteristics. All these strains were chosen for their easy culturing, their representativeness of the diversity in the collection (white or brown rot, tropical or temperate fungus) and the reality of fungal attacks in timber.

Table 1

List of the 98 fungus species studied, reference number, strain origin and type of rot.

Name Fungus	N° Réf.	Origine	Rot*
Abortiporus biennis	4399	Bois indéterminé, France, 1970	F
Abortiporus biennis	4354	H.D.	F
Amylostereum areolatum	9026	*Picea abies*, Allemagne, 1978	F
Antrodia serialis	153	Bois indéterminé, Afrique du Sud, 1981	C
Bjerkandera adusta	147	*Pterocarpus soyauxii*, Cameroun, 1981	F
Cerrena unicolor	3948	*Pycnanthus angolensis*, Cameroun, 1953	F
Climacocystis borealis	526	H.B.	F
Coniophora puteana	CBS.132.70	C.B.S.	C
Daedaleopsis confragosa	4033	H.B.	F
Donkioporia expansa	4415	H.B.	F
Donkioporia expansa	3021	H.B.	F
Favolus alveolaris	975	Bois indéterminé, Côte d'Ivoire, 1975	F
Favolus mori	3705	H.B.	F
Fibroporia vaillantii	4491	H.D.	C
Fistulina hepatica	CBS.161.30	C.B.S.	C
Flavodon flavus	5988	H.B.	F
Fomes fomentarius	4549	*Betula* sp., France, 1970	F
Fomes fomentarius	3470	*Quercus* sp., France, 1978	F
Fomes fomentarius	4547	H.B.	F
Fomitopsis officinalis	4343	H.B.	C
Fomitopsis officinalis	543	H.D.	C
Fomitopsis pinicola	4311	H.B.	C
Fomitopsis pinicola	4295	H.B.	C
Ganoderma adspersum	4229	H.B.	F
Ganoderma applanatum	CBS 175.30	C.B.S.	F
Ganoderma lucidum	4301	*Quercus* sp., France, 1982	F
Ganoderma resinaceum	4361	H.B.	F
Ganoderma resinaceum	4351	H.B.	F
Ganoderm resinaceum	4143	H.B.	F
Ganoderma sp.	4356	H.B.	F
Ganoderma sp.	4355	H.B.	F
Gloeophyllum abietinum	155	*Picea* sp., France, 1980	C
Gloeophyllum odoratum	590	H.D.	C
Gloeophyllum trabeum	CBS.900.73	C.B.S.	C
Hericium coralloides	CBS 206.29	C.B.S	F
Hericium coralloides	9910	H.B.	F
Heterobasidion annosum	2031	H.B.	F
Heterobasidion annosum	4278	H.B.	F
Hexagonia hydnoides	E 749	Bois indéterminé, Guyane, 1982	F
Hexagonia nitida	CBS.212.53	C.B.S.	F
Hexagonia nitida	4419	H.B.	F
Hymenochaete tabacina	701	H.B.	C
Inonotus radiatus	486	H.B.	F
Irpex flavus	1004	H.B.	F
Laetiporus sulphureus	440	H.B.	C
Lenzites betulinus	CBS.102.11	C.B.S.	F
Meripilus giganteus	301	Bois indéterminé, France, 1975	F
Meripilus giganteus	4357	H.D.	F
Merulius tremellosus	6550	Ouvrage hydraulique, France 1978	F
Merulius tremellosus	CBS.217.56	C.B.S.	F
Oligoporus caesius	5921	H.D.	C

Oligoporus caesius	4119	H.D.	C
Oligoporus caesius	4502	H.D.	C
Oligoporus stipticus	332	H.D.	C
Oxyporus latemarginatus	CBS.298.33	C.B.S.	F
Oxyporus latemarginatus	4211	Bois indéterminé, Madagascar, 1971	F
Oxyporus latemarginatus	279	Bois indéterminé, Madagascar, 1971	F
Oxyporus latemarginatus	365	H.B.	F
Oxyporus sp.	4370	*Terminalia superba*, France, 1979	F
Perenniporia fraxinea	190	H.D.	F
Perenniporia medulla-panis	4597	*Prunus avium*, france, 1978	F
Perenniporia subacida	985	Bois indéterminé, Guyane, 1975	F
Perenniporia subacida	549	H.D.	F
Perenniporia subacida	4553	H.D.	F
Phellinus ferreus	3618	*Teminalia superba* , Côte d'Ivoire, 1975	F
Phellinus pini	202	H.B.	F
Phellinus pomaceus	300	H.D.	F
Phellinus punctatus	3918	H.D.	F
Phellinus torulosus	223	H.D.	F
Phlebiopsis roumeguerei	6570	*Quercus*, Drôme, 1970	F
Podoscypha bolleana	5430	Bois indéterminé,Côte d'Ivoire, 1967	F
Podoscypha petalodes	4317	Bois indéterminé, Pakistan, 1962	F
Podoscypha ursina	7034	H.B.	F
Polyporus melanopus	900	H.D.	F
Polyporus melanopus	3602	H.D.	F
Polyporus melanopus	182	H.D.	F
Poria placenta	CBS.384.82	C.B.S.	C
Rigidoporus lineatus	1194	*Vochysia* sp., Guyane, 1982	F
Rigidoporus ulmarius	3027	H.B.	F
Serpula lacrymans	CBS 235.33	C.B.S.	C
Spongipellis spumeus	314	H.D.	F
Spongipellis spumeus	986	H.D.	F
Spongipellis spumeus	4566	H.B.	F
Spongipellis spumeus	988	H.D.	F
Spongipellis spumeus	154	H.D.	F
Spongipellis spumeus	349	H.D.	F
Stereum rugosum	3837	*Quercus ilex*, Ardèche 1960	F
Stereum sanguinolentum	CBS.529.50	C.B.S.	F
Trametes odorata	4166	H.D.	F
Trametes odorata	166	H.D.	F
Trametes odorata	534	H.D.	F
Trametes pubescens	288	*Betula* sp., France, 1970	F
Trametes versicolor	171	*Fagus sylvatica*, France, 1970	F
Trametes versicolor	34671	Bois indéterminé, Portugal, 1969	F
Trametes versicolor	CBS.737.85	C.B.S.	F
Trametes versicolor	32745	*Fagus sylvatica* , France, 1979	F
Trametes zonatus	287	Bois indéterminé, Madagascar 1970	F
Xylobus frustulatus	4420	H.B.	F

Type of rot; C: cubic; F: fibrous; H.D.: Herbier A. DAVID; H.B.: Herbier J. BOIDIN; C.B.S.: Centraal Bureau voor Schimmelcultures, Pays-Bas.*

Restriction polymorphism (RFLP)

The ITS restriction reactions were carried out with 15µl of amplificate and 5µl of a mixture of 2.5µl ultra-pure H_2O, 2µl of a reagent corresponding to the enzyme and 5 units of enzyme. The reactions took place at 37°C overnight with the enzymes AluI (5'AG^CT3'), Sau96 (5'G^GNCC3') and for 1 hour at 60°C for

TaqI (5'T^CGA3').

For each of the stages described below (DNA purification, ITS amplification and ITS digestion by different restriction enzymes), the DNA was visualized in UV after electrophoresis on agarose gel containing ethidium bromide (ETB) at 2µg.ml^{-1}. The control gels were done in TBE 1X with different types of agarose and different migration conditions depending on the cases.

Deposits were placed in the wells of the different gels in 13µl of a mixture comprising 10µl of the solution (purified DNA, PCR products or amplified DNA digestion products) and 3µl of loading buffer (bromophenol blue, 87% glycerol, 0.5M EDTA, xylene cyanol FF). Molecular weight markers VIII and XIII (Bœhringer Mannheim) were placed either side of the gel to estimate the number of base pairs of the fragments obtained.

RESULTS

ITS amplification and restriction polymorphism (RFLP) analysis

It was possible to estimate the length of the amplified fragments for 88 ITS amplificates obtained on the 98 studied strains (Table 2). Ten strains could not be amplified; these displayed a coloured product after DNA extraction. The strains were: *Bjerkandera adusta*, *Flavodon flavus*, *Fomes fomentarius*, *Ganoderma resinaceum*, *Oligoporus caesius*, *Phellinus pomaceus*, *Poria placenta*, *Trametes versicolor*, *Trametes zonatus* and *Xylobolus frustulatus*.

The evaluation of the number of ITS base pairs for the different strains revealed ITS with a length of between 560 and 895 bp. Forty-five percent of the ITS studied had a length of between 600 and 650 bp, 16% between 650 and 675 bp and 17% between 725 and 750 bp. The remaining 20% of strains were divided up, outside the previously mentioned classes, between 550 and 895 bp for the length of their ITS.

Within the same species, we found some differences in molecular weights of 40 to 100 base pairs. Those differences corresponded to a margin of error of around 12% for DNA molecular weight readings on agarose gel.

Table 2 shows only the sum of the base pairs of the fragments obtained for each strain by the restriction enzymes TaqI, Sau96A and AluI. This sum was obtained by adding together the multitude of DNA fragments of variable sizes generated by enzymatic digestion then separated on agarose gel. To date, all these data constitute a library of restriction profiles per strain and per restriction enzyme.

The sum of the numbers of base pairs of the fragments of each restriction profile led to the identification of three situations (Table 2):

a. The sum of the numbers of base pairs of the fragments was equal to the number of base pairs of the undigested PCR product; this involved 6% of the strains.

b. The sum of the numbers of base pairs of the fragments was lower than the number of base pairs of the undigested PCR product; this involved 54% of the strains.

c. The sum of the number of base pairs of the fragments was higher than the number of base pairs of the undigested PCR product; this concerned 24% of the strains.

In addition, we found that 16% of the strains were not cleaved by the restriction enzymes.

Table 2

Reference number of the strain, species, number of base pairs of the amplified ITS (88 strains) and sums of the numbers of base pairs of the fragments obtained for each strain with restriction enzymes TaqI, Sau96A and AluI

The strains not cleaved by the restriction enzymes are indicated by nd (nd: not digested).

Strain reference number	Species	Sum of fragments obtained with enzymes			nbp of ITS
		Taq I	Sau 96A	Alu I	
4354	*Abortiporus biennis*	663	790	712	725
4399	*Abortiporus biennis*	498	345	600	625
9026	*Amylostereum areolatum*	456	640	600	600
153	*Antrodia serialis*	601	420	908	640
948	*Cerrena unicolor*	638	735	650	705
526	*Climacocystis borealis*	1586	759	1112	640
CBS 132.70	*Coniophora puteana*	nd	nd	nd	740
4033	*Daedaleopsis confragosa*	472	370	540	560

4415	*Donkiopora expansa*	1709	735	580	585
3021	*Donkiopora expansa*	552	565	360	625
975	*Favolus alveolaris*	468	630	364	685
3705	*Favolus mori*	498	620	490	625
4491	*Fibroporia vaillantii*	724	970	789	655
CBS 161.30	*Fistulina hepatica*	871	nd	1354	895
4549	*Fomes fomentarius*	607	715	600	660
4547	*Fomes fomentarius*	775	665	800	650
4343	*Fomitopsis officinalis*	632	670	635	685
543	*Fomitopsis officinalis*	nd	635	600	625
4311	*Fomitopsis pinicola*	nd	nd	nd	665
4295	*Fomitopsis pinicola*	470	490	550	605
4229	*Ganoderma adspersum*	nd	510	364	740
CBS 175.30	*Ganoderma applanatum*	nd	nd	nd	580
4301	*Ganoderma lucidum*	576	976	416	640
4361	*Ganoderma resinaceum*	576	680	344	725
4351	*Ganoderma resinaceum*	542	1280	960	710
4355	*Ganoderma* sp.	nd	nd	nd	625
4356	*Ganoderma* sp.	nd	840	nd	615
155	*Gloeophyllum abietinum*	601	660	780	635
590	*Gloeophyllum odoratum*	912	680	505	640
CBS 900.73	*Gloeophyllum trabeum*	576	nd	1180	640
CBS 206.29	*Hericium coralloides*	457	670	640	635
9910	*Hericium coralloides*	nd	450	600	610
4278	*Heterobasidion annosum*	nd	490	nd	625
2031	*Heterobasidion annosum*	539	nd	830	640
E 749	*Hexagonia hydnoides*	591	660	436	675
4419	*Hexagonia nitida*	594	525	478	705
CBS 212.53	*Hexagonia nitida*	562	1380	600	655
701	*Hymenochaete tabacina*	467	720	550	565
486	*Inonotus radiatus*	nd	nd	nd	765
1004	*Irpex flavus*	nd	350	600	615
440	*Laetiporus sulphureus*	900	nd	930	615
CBS 102.11	*Lenzites betulinus*	535	345	623	610
301	*Meripilus giganteus*	599	260	740	635
4357	*Meripilus giganteus*	966	685	1025	645
6550	*Merulius tremellosus*	590	nd	635	685
CBS 217.56	*Merulius tremellosus*	841	560	755	780
5921	*Oligoporus caesius*	nd	180	860	745
4119	*Oligoporus caesius*	634	485	594	725
332	*Oligoporus stipticus*	446	375	466	640

CBS 298.33	*Oxyporus latemarginatus*	578	nd	nd	705
279	*Oxyporus latemarginatus*	579	720	416	640
365	*Oxyporus latemarginatus*	596	nd	nd	655
4211	*Oxyporus latemarginatus*	580	780	740	640
4370	*Oxyporus* sp.	630	650	1100	740
190	*Perenniporia fraxinea*	550	1270	1000	625
4597	*Perenniporia medulla-panis*	565	670	nd	640
4553	*Perenniporia subacida*	556	624	nd	675
549	*Perenniporia subacida*	356	1260	580	665
985	*Perenniporia subacida*	523	645	850	635
3618	*Phellinus ferreus*	nd	640	530	650
202	*Phellinus pini*	494	715	1270	710
3918	*Phellinus punctatus*	344	nd	650	750
223	*Phellinus torulosus*	568	680	590	655
6570	*Phlebiopsis roumeguerei*	222	520	630	640
5430	*Podoscypha bolleana*	583	595	1283	810
4317	*Podoscypha petalodes*	1234	650	640	670
7034	*Podoscypha ursina*	528	720	800	745
182	*Polyporus melanopus*	401	nd	nd	735
900	*Polyporus melanopus*	488	360	612	690
3602	*Polyporus melanopus*	655	750	475	740
1194	*Rigidoporus lineatus*	603	650	960	600
3027	*Rigidoporus ulmarius*	425	380	600	600
CBS 235.33	*Serpula lacrymans*	586	705	1150	670
986	*Spongipellis spumeus*	617	705	640	715
349	*Spongipellis spumeus*	572	435	620	725
988	*Spongipellis spumeus*	441	855	540	565
314	*Spongipellis spumeus*	511	485	750	640
154	*Spongipellis spumeus*	nd	600	640	650
4566	*Spongipellis spumeus*	545	600	790	755
3837	*Stereum rugosum*	nd	385	600	615
CBS 529.50	*Stereum sanguinolentum*	479	685	650	660
4166	*Trametes odorata*	622	nd	612	665
166	*Trametes odorata*	nd	575	nd	740
534	*Trametes odorata*	532	320	577	740
288	*Trametes pubescens*	568	535	410	615
171	*Trametes versicolor*	401	555	357	640
BS 737.85	*Trametes versicolor*	411	345	590	625
34671	*Trametes versicolor*	423	600	940	725

After sequencing, we obtained 53 nucleotide sequences with ITS lengths of between 555 and 653 bp.

We found differences in ITS length of around 10 bp in certain genera, such as *Cerrena, Trametes, Oxyporus, Ganoderma* and *Phellinus*. These results confirmed those obtained after PCR/RFLP for certain strains, particularly for the genera *Fomes, Ganoderma, Heterobasidion, Meripilus, Oxyporus, Perenniporia, Polyporus, Spongipellis* and *Trametes*. This margin of error could be used as a tool for differentiating and discriminating between our strains. Indeed, automatic sequencing remains a reproducible method, whilst PCR/RFLP remains a qualitative, not particularly reliable method if it is wished to compare ITS lengths (estimation of molecular weights after visualization of agarose gels).

DISCUSSION

Analysis of ITS restriction polymorphism (RFLP) and size

The length of the amplified fragments varied from 550 to 895 base pairs. Some similar results have been obtained with numerous other fungus species (Henrion *et al.* 1992, Gardes and Bruns 1993). The differences in ITS length between strains can be used as an initial characterization element, though without being able to give an indication of the type of rot. In addition, this evaluation of the number of ITS base pairs for the different strains revealed the following situations: 47% of the ITS studied had a length of between 600 and 650 bp, 16% between 650 and 675 bp and 17% between 725 and 750 bp. The remaining 20% of strains were divided fairly regularly between 550 and 895 bp (outside the aforementioned classes) for their ITS length.

In view of these results, we suggest that the polymorphism detected was due to mutations at the enzyme restriction sites (restriction polymorphism). In fact, the PCR/RFLP technique is based on detecting variability in the nucleotide sequence of genomic DNA after digestion by restriction enzymes.

We believe that an evolution mechanism, probably based on uneven crossing-over, regularly homogenized the copies of ribosomal DNA and was opposed to their divergence by an accumulation of mutations (Srivasta and Schlessinger 1991).

We found that molecular weight readings on agarose gel for the amplificates exhibited imprecision amounting to around 12%. The charge of a molecule of DNA is proportional to its length and the frictional forces acting upon it during migration are linked to its length. Consequently, the longer a molecule is, the more slowly it will migrate. Thus, in theory, gel electrophoresis can be used to separate a mixture of DNA molecules according to their sizes. The dimension of the pores in the gel and their uniformity are decisive parameters for the resolution power of a gel which, at best, can only be a few dozen base pairs. In this work, all the electrophoresis operations were carried out under similar conditions, migrated with the same voltage and with the same equipment.

For 10 strains, we found it was impossible to obtain an amplificate, presuming that each strain had its particularities, as the fungus varied as much in the quality of the DNA obtained as in the quantity of DNA. We assumed that the pigments remaining after DNA extraction would inhibit Taq polymerase by blocking the elongation phase. A similar situation was encountered with the genus *Pisolithus*. Adding high doses of Bovine Serum Albumin, up to 1mg/ml, in the PCR reagents makes it possible to contain such inhibiting effects. It would be feasible to carry out these PCR reactions, eliminating the pigments remaining after the conventional DNA extraction protocol, by adding 1mg/ml of Bovine Serum Albumin as required.

The sum of the numbers of base pairs for the fragments of each restriction profile led to the identification of three situations, which could be interpreted as follows. The first case matched the theory perfectly: the sum of the numbers of base pairs for the fragments of each restriction profile corresponded to the molecular weight of the ITS. In the second case, it can be suggested that the restriction enzyme used generated small fragments likely to be "lost" in the gel during migration, or that the enzyme used generated two fragments or more with an identical molecular weight. The possible interpretations for the third case are more complex and we put forward two hypotheses. First hypothesis: the strains studied were, in fact, mixtures of several individuals possessing an ITS with the same number of base pairs. Second hypothesis: the strains studied were dikaryotic and heterozygous, at least for this portion of ribosomal DNA. This second hypothesis can explain, at most, a doubling of the number of base pairs compared to the number of base pairs of the initial undigested fragment. In some cases, we found sums that were between 2 and 3 times the number of base pairs of the undigested fragment. In those cases, we can suggest that different families (from 2 to 3 at least) of ribosomal genes are able to exist side by side inside the same thallus. Such a situation had already been suspected for another group of fungi: the red milk caps (Guerin-Laguette 1998).

PCR/RFLP can be used to rapidly reveal the variability of a nucleotide sequence. However, this method calls for the compilation of a large database of personal information that is difficult to exchange due to the great sensitivity of the PCR reaction (DNA concentration, amplification conditions) and imprecision in the band size readings; this largely limits the development possibilities for this method for analysing diversity in new populations. PCR/RFLP therefore seems to be a tool that needs to be limited, for example, to follow-up work in the laboratory or *in situ,* of strains that are already known and listed in databases.

CONCLUSION

In order to characterize the strains in the CIRAD collection, we developed a rapid discriminant analysis method based on analysing the diversity of partial ribosomal DNA sequences independently of the macro- and microscopic morphological traits of the fruiting bodies required for the identification of genera and species.

Adaptation of the Qiagen kit for higher plants made it possible to extract fungal DNA from a few milligrams of mycelium; with most of the strains studied, these DNA extracts enabled us to obtain an ITS amplificate of a size varying from 550 to 895 base pairs. We also found a large margin of error, of around 12%, for the DNA molecular weight readings on gel, leading us to prefer direct sequencing for precise determination of amplificate lengths. PCR-RFLP showed a good ability to characterize a given strain by providing different profiles obtained using several restriction enzymes. We thus demonstrated that studying ITS diversity using PCR-RFLP is a discriminant method making it possible to distinguish between strains. However, this method calls for the compilation of a database whose use remains partly subjected to interpretation, which cannot be used to work on a material unknown to the database. This simple, rapid technique is efficient and perfectly suitable for monitoring known strains in controlled trials.

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