A widely distributed ITS polymorphism within a biological species of the ectomycorrhizal fungus *Hebeloma velutipes*

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The ectomycorrhizal fungus *Hebeloma velutipes* consists of two biological species (BSP 16 and 17). Within BSP 17 a dikaryon was found with two divergent types of the ribosomal Internal Transcribed Spacer (ITS1 and 2). The two ITS types segregated in monokaryotic progeny of that dikaryon, showing that these different ITS types represent different alleles at homologous rDNA loci in the two nuclei. RFLP analysis of a number of strains of BSP 17 showed that the polymorphism is widespread in Europe. There was no deficiency of the heterokaryotic type, demonstrating that ITS divergence in this species is not correlated with reduced intercompatibility. A strain from North America, not assigned to a biological species, showed the same polymorphism. Cladistic analysis of the two ITS sequences showed that they were not sister groups. One of the ITS types formed a monophyletic group together with the ITS type of BSP 16, the other type formed a clade with the ITS type of *H. incarnatulum* (BSP 18). BSP 16 and 17 showed partial intercompatibility. However, several lines of evidence suggest that the polymorphism of BSP 17 is not the result of frequent and continuing hybridisation with BSP 16. Instead, we give arguments for the hypothesis that the polymorphism evolved in allopatry and that the two types have come together relatively recently. The results of the polymorphism indicate a potential problem for molecular identification of fungal species based on ITS fingerprinting. The results also show that no generalisations are possible about the relation of speciation (the formation of BSP) and nuclear ITS divergence.

INTRODUCTION

Species of the genus *Hebeloma* (*Agaricales*, *Basidiomycota*) form ectomycorrhizas with a large number of tree species under a wide range of environmental conditions. The genus is considered taxonomically difficult and the taxonomic status of several taxa is unclear. This taxonomic complexity is due to the widespread existence of cryptic species, i.e. reproductively isolated populations, which behave as biological species (BSP) but which cannot be recognised morphologically. In the *H. crustuliniforme* complex intercompatibility tests have shown the existence of at least 20 BSP in northwestern Europe (Aanen & Kuyper 1999). Within that complex at large, 2 BSP could be assigned to the morphospecies *H. velutipes* (BSP 16 and 17) and one to the closely related morphospecies *H. incarnatulum* (BSP 18; numbering of BSP according to Aanen & Kuyper 1999). In an analysis of the phylogeny of the *H. crustuliniforme* complex, based on ITS sequences, one dikaryon of one of the BSP of *H. velutipes* (BSP 17) produced a double signal at 19 nucleotide positions. Two monosporic isolates derived from that collection had single sequences that differed at these 19 positions (Aanen *et al*. 2000a).

The presence of two divergent ITS types within a single dikaryon raises several questions, that were addressed in this study. First of all we studied the distribution of the polymorphism within BSP 17 and determined whether the occurrence of mixed sequences is exceptional. Multigene families, such as the ribosomal DNA, commonly show identical sequences within a genome. The process of homogenisation of copies within a genome has been termed concerted evolution (Arnheim *et al*. 1980). The two mechanisms proposed as responsible for the homogenisation are gene conversion and unequal crossing over (Elder & Turner 1995). However, the factors leading to and the rates of homogenisation are not well understood. Another question that is addressed is whether the different ITS types are located within nuclei or between nuclei. If differences reside between nuclei recombination between nuclear types is apparently low, and both types have undergone independent evolutionary histories. If the different types reside in different nuclei, a question that follows is whether their distribution is consistent with Hardy–Weinberg equilibrium. If the Hardy–Weinberg equilibrium cannot be rejected, (extensive) ITS divergence would not necessarily be a first step towards speciation (the formation of BSP). Furthermore we determined whether both ITS sequences of BSP 17 form a clade, i.e. possess a unique

Table 1. The collections used in the study.

Collection number*	Geographic origin	Host tree genera	BSP	ITS sequence (+ or −)	ITS RFLP (+ or −)	ITS type (Ia or Ib)†	mit. RFLP (+ or −)
European strains							
D9502	Wijster, Drenthe, Netherlands	*Betula*	16	+	+	IB/IB	+
D9541	Roden, Drenthe, Netherlands	*Carpinus*	16		+	IB/IB	
D9542	Roden, Drenthe, Netherlands	*Fagus, Carpinus*	16		+	IB/IB	
D9543	Roden, Drenthe, Netherlands	*Fagus, Quercus, Corylus*	16		+	IB/IB	+
M9545	Roden, Drenthe, Netherlands	*Fagus*	16		+	IB	+
D9407	Terschelling, Friesland, Netherlands	*Fagus*	17		+	IA/IB	+
D9432	Assen, Drenthe, Netherlands	*Quercus*	17		+	IA/IB	+
D9504	Borgsjö, Medelpad, Sweden	*Betula*	17	+	+	IA/IB	+
M9504-1-2	Borgsjö, Medelpad, Sweden	*Betula*	17	+	+	IA or IB	
M9504-3-10	Borgsjö, Medelpad, Sweden	*Betula*	17		+	IA or IB	
M9516	Roth, Bayern, Germany	*Picea, Pinus*	17		+	IA	+
M9522	Hilpoltstein, Bayern, Germany	*Betula*	17		+	IB	+
M9524	Hilpoltstein, Bayern, Germany	*Pinus*	17		+	IB	+
D9526	Roth, Bayern, Germany	*Fagus*	17		+	IA/IA	+
M9529	Roth, Bayern, Germany	*Pinus*	17		+	IB	
D9532	Kemnathen, Bayern, Germany	*Quercus, Salix*	17		+	IB/IB	+
M9534	Mauk, Bayern, Germany	*Pinus*	17		+	IA	+
D9535	Mauk, Bayern, Germany	*Pinus*	17	+	+	IA/IA	+
D9540	Roden, Drenthe, Netherlands	*Carpinus*	17	+	+	IA/IA	
M9556	Havelte, Drenthe, Netherlands	*Quercus, Betula, Salix*	17		+	IA	+
D9623	Dwingeloo, Drenthe, Netherlands	*Quercus, Betula*	17		+	IA/IA	+
D9625	Wijster, Drenthe, Netherlands	*Fagus*	17		+	IB/IB	+
D9639	Lac de Rouges Truites, Jura, France	*Picea*	17		+	IA/IA	+
D9642	Lac de Rouges Truites, Jura, France	*Salix*	17	+		IA/IA	
D9643	Lac de Rouges Truites, Jura, France	*Fagus, Quercus, Carpinus*	17		+	IB/IB	+
D9647	Lac de Rouges Truites, Jura, France	*Salix*	17		+	IA/IA	+
D9653	Lac de Rouges Truites, Jura, France	*Picea, Salix*	17		+	IA/IA	+
D9527	Roth, Bayern, Germany	*Pinus*	18	+			
North American strains							
DS166	Oregon, USA	*Pseudotsuga*	?	+		IA/IB	
D7650	Oregon, USA	*Pinus, Tsuga*	?	+		IA/IA	
Outgroup							
D9514	Roth, Bayern, Germany	*Pinus, Picea*		+			

* Strains starting with M are monokaryotic and with D are dikaryotic.

† IA/IB indicate that both types were present in a strain (always dikaryons). IA or IB indicates that either IA or IB was present (in monokaryons M504-3 to M504-10).

most recent common ancestor, compared to the sequences of BSP 16 and 18. If so, ITS divergence took place after the last speciation event. If not, speciation could be independent of ITS divergence. The answer to this question allows us to discriminate between models of divergence-first (speciation as a consequence of genetic divergence) and incompatibility-first (speciation as the cause of genetic divergence) (Aanen *et al*. 2000b). If both nuclear sequences do not form a monophyletic group, another question is whether the polymorphism is a consequence of frequent and continuing hybridisation between the two BSP of *H. velutipes*. If so, traces of hybridisation, including traces of cytoplasmic exchange should be expected. An alternative explanation is a rare hybridisation between both BSP followed by introgression of the BSP 16 into BSP 17.

We addressed these questions by analysing ITS sequences and fingerprints of collections belonging to the morphospecies *H. velutipes* (BSP 16 and 17) in detail, by determining the frequencies of the various ITS types within dikaryons and monokaryotic progeny of a mixed dikaryon by multiple fingerprinting, and by studying fingerprints of a mitochondrial gene as a tracer for cytoplasmic exchange.

MATERIALS AND METHODS

Strain collection

Carpophores belonging to the *Hebeloma velutipes* group (members of the *H. crustuliniforme* complex, showing the combination of dextrinoid spores and cylindrico-clavate, non-capitate cheilocystidia, and usually with a distinctly bulbous stipe) were collected in a variety of habitats, with a variety of host trees, in Europe in 1994, 1995, and 1996. Only carpophores growing closely together were considered to belong to the same mycelium. In cases of doubt, only one carpophore was collected. Table 1 gives data on all collections used in this study. For each collection, dikaryotic mycelium was regenerated from excised sporocarp context tissue and

monokaryons were obtained using spore germination (Aanen & Kuyper 1999). Sporocarps were dried and preserved in the Wageningen herbarium (WAG) for future reference. Data on ecology (including potential host trees) were recorded. Fungal cultures are preserved at the Centraalbureau voor Schimmelcultures, Utrecht (CBS). Two North American culture collections were obtained from D. McKay (Corvallis, Oregon). They were provided as *H. crustuliniforme*, but molecular data (see Table 2) unambiguously show that they belong to the *H. velutipes* group. As an outgroup *H. sinapizans* was used (Aanen *et al*., 2000a).

DNA isolation, sequencing, and phylogenetic analysis

Direct sequencing was performed and DNA was isolated from dikaryotic or monokaryotic cultures. For methods of DNA isolation, PCR and sequencing of the ITS1 and 2 region, refer to Aanen *et al*. (2000a). In addition to the sequences already published elsewhere (Aanen *et al*. 2000a), sequences were determined for two American cultures. Since these were dikaryons, we have not tested sexual intercompatibility relationships of these, and hence these cultures could not be assigned to a BSP. As sequencing primers ITS 1, ITS 3, and ITS 4 were used (White *et al*. 1990). Sequences were deposited in GenBank. The aligned sequences have been deposited to TreeBASE (HTTP://herbaria.harvard.edu/treebase) as SN514 and are also available from the first author upon request.

Sequences were aligned manually using a matrix created in PAUP* 4.0 (Swofford, test version, 1998). Phylogenetic relationships were inferred from the aligned sequences using parsimony with PAUP. Gaps were treated as missing data. The Branch and Bound option in PAUP was used to find most parsimonious trees. Clade stability was assessed by 1000 bootstrap replications (Hillis & Bull 1993), with setting Branch and Bound. Decay indices (Bremer 1988, Donoghue *et al*. 1992) were calculated from PAUP tree files using the program Autodecay (version 4.0; Eriksson 1998) and PAUP. Other measures (tree length, sequence divergence, consistency and retention indices) were calculated using PAUP.

Part of the mitochondrial small subunit rDNA (SSU) was amplified using primers MS 1 and MS 2 (White *et al*. 1990). For one strain of BSP 16 and 17 (D9545: BSP 16; M9524: BSP 17) the sequence of this PCR product was determined, using MS 1 as sequencing primer.

RFLP analysis

Choice of restriction enzymes for ITS–RFLP markers was based on initial sequence analyses. *Hae*III and *Hin*fI were used to study the ITS of a large sample of cultures belonging to both BSP 16 and 17. Furthermore, 10 monokaryotic progeny of the dikaryon (D9504), where the mixture of both ITS-types was initially observed, were analysed by ITS–RFLP, using *Hae*III, to study the inheritance of the polymorphism.

*Tru*9I was used to study the mitochondrial SSU of a sample of strains belonging to both BSP 16 and 17.

Between 1 and 2 µg of amplified DNA were digested for 3 h with 5 to 10 units of various restriction enzymes (*Hae*III, *Hin*fI, *Tru*9I, Pharmacia) according to the manufacturer's instructions. The restriction fragments were size fractionated using 1.5% agarose gel electrophoresis. Gels were stained with ethidium bromide, and photographed under UV light. A 100 bp ladder (Promega) was used as a size marker.

Table 1 also indicates whether monokaryons or dikaryons were analysed for nuclear and mitochondrial sequences or RFLP patterns.

RESULTS

ITS sequences

The total length of ITS 1, 5.8S, and ITS2 was 611 nucleotides for all isolates, the length of the aligned sequences 613 nucleotides. The sequences of M9504–1 and D9535 (BSP 17) were identical. The electropherograms of D9504 (BSP 17) and S166 showed mixed peaks at 19 and 17 positions respectively. Using the monokaryons of D9504 (M9504–1, M9504–2), single sequences were obtained that differed at these 19 positions. After excluding the sequences with mixed peaks, one most parsimonious tree (with CI = 1.0) was found (Fig. 1). Two monophyletic groups (IA and IB, corresponding to ITS type IA and IB) were found. However, sequences M9504–1 and M9504–2 did not form a monophyletic group. M9504–2 formed a clade with the ITS of BSP 16, and M9504–1 was a member of the same clade as the ITS of BSP 18. It is therefore clear that both ITS types of BSP 17 have independent evolutionary histories.

The total number of variable nucleotide positions were 26 (Table 2). The monophyletic groups of Fig. 1 differed at 17 positions (10 in ITS1 and 7 in ITS2). The remaining nine

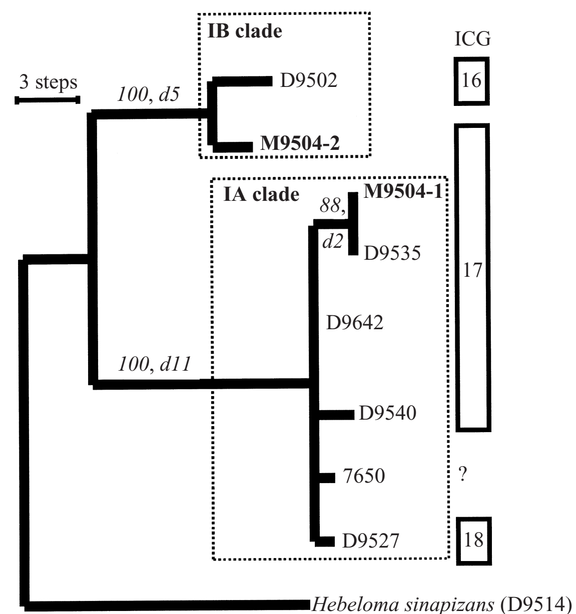

Fig. 1. Phylogenetic relationships between strains BSP 16, 17 and 18 of *Hebeloma velutipes*, based on ITS 1and 2 sequences. Single most parsimonious tree of length 48 (C.I. = 1.0, R.I. = 1.0; length = 20 using only informative characters) using *H. sinapizans* as an outgroup. Indicated are bootstrap values and decay indices (preceded by d). On the right, the BSP to which isolates belong is indicated.

Table 2. Variable positions in ITS 1 and 2.

		ITS 1														ITS 2											
Nucleotide Position Strain	BSP	30	31	38	92 *	96 *	133	137	138	162	163 *	178 *	208	216	232	414	415	421	446	467	507 *	545 *	558 *	565 *	583	591	609 *
D9502	16	T	C	G	T	G	T	A	T	G	G	–	T	C	C	C	T	C	C	G	T	G	T	G	C	C	C
M9504–2	17	T	C	G	C	G	T	A	T	G	G	–	T	C	C	C	T	C	C	G	C	G	C	G	C	C	T
D9504	17	T	C	G	C	G	T	A	T	G	G	–	T	C	C	C	T	C	C	G	C	G	C	G	C	C	T
		C	T	A			C	G	C	A			C	A	T	A	C	T	T	A		C			T	G	C
M9504–1	17	C	T	A	C	G	C	G	C	A	G	–	C	A	T	A	C	T	T	A	C	C	C	G	T	G	C
S166	?	T	C	G	C	G	T	A	T	G	G	–	T	C	C	C	T	C	C	G	C	G	C	G	C	C	C
		C	T	A			C	G	C	A			C	A	T	A	C	T	T	A					T	G	
7650	?	C	T	A	C	G	C	G	C	A	T	–	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C
D9642	17	C	T	A	C	G	C	G	C	A	G	–	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C
D9535	17	C	T	A	C	G	C	G	C	A	G	–	C	A	T	A	C	T	T	A	C	C	C	G	T	G	C
D9540	17	C	T	A	C	G	C	G	C	A	G	A	C	A	T	A	C	T	T	A	C	G	C	–	T	G	C
D9527	18	C	T	A	C	A	C	G	C	A	G	–	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C

–, Indel in alignment.
*, Difference within clade IA or IB.

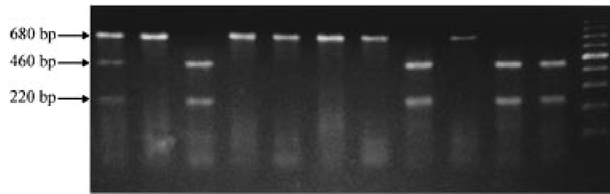

Fig. 2. RFLPs of ITS PCR—products digested with *Hae*III of strain D504 (lane 1) and 10 monokaryotic progeny of the same strain (M504-1–M504-10; lane 2–11). Lane 12: 100 bp ladder. The mixed pattern of D504 segregates in the monokaryotic progeny.

nucleotide differences were within the monophyletic groups (* in Table 2). The electropherogram of S166 had mixed peaks at all of these 17 positions. As no monokaryons of this collection were available, no further analysis of the polymorphism could be made.

Sequence analysis showed a polymorphism at position 216 (ITS1) allowing discrimination of both groups with *Hae*III. Members of clade IA had an A at this position and lacked the restriction site, resulting in a band of approximately 680 bp. Members of clade IB had a C and possessed the restriction site, resulting in two bands of approximately 220 and 460 bp. Sequence analysis also showed a polymorphism at position 591 (ITS2) allowing discrimination of both groups with *Hin*fI. Members of clade IA had a G at this position and lacked the restriction site, resulting in two bands of approximately 340 bp. Members of clade IB had a C at this position and possessed the restriction site, resulting in three bands of approximately 340, 250, and 90 bp.

Nuclear fingerprinting

PCR products of 10 monokaryotic progeny of D9504 were analysed for ITS–RFLP using *Hae*III (Fig. 2). Four monokaryons had the type IB and six monokaryons IA. No mixed patterns were observed, indicating segregation of both types in monokaryons.

Table 3. Expected and observed heterodikaryotic types within BSP17 and significance testing using the G test.

	IAIA	IAIB	IBIB
Expected frequency	0.419	0.457	0.124
Expected numbers	5.86	6.39	1.74
Observed numbers	8	3	3

$G = 3.54$, $0.10 < P < 0.25$.

Four dikaryons and one monokaryon of BSP 16 were studied, giving a total of 9 nuclei. All nuclei possessed only type IB. Fourteen dikaryons and 6 monokaryons of BSP 17 were studied, giving a total of 34 nuclei. Both type IA and IB were found. Frequency of type IA was 65% (22/34), of type IB 35% (12/34). Three dikaryons were heterokaryotic, namely D9407, D9432, and D9504. We compared expected Hardy–Weinberg equilibrium frequencies with observed frequencies of the different homokaryotic and heterokaryotic types (Table 3). The difference between observed and expected frequencies was not significant ($P > 0.1$), demonstrating: 1. that extensive ITS divergence has apparently not led to reduced compatibility and 2. that there is no reduction in fitness of such dikaryons. However, our sample size is small, so we can not exclude the possibility of finding a significant deviation from Hardy–Weinberg equilibrium when a larger sample size is studied.

Nuclei that were classified as type IA or IB using *Hae*III were always classified as the same type using HinfI. This indicates that we failed to indicate recombination between ITS1 and 2 in our sample.

Mitochondrial fingerprinting

The mitochondrial sequences of BSP 16 and 17 differed at three nucleotide positions; these three positions were in three Tru9I sites (TTAA). Members of BSP 16 are predicted to have a large band of 160 bp, members of BSP 17 a large band of 182 bp. Both strains of BSP 16 possessed a band of 160 bp, and all 16 strains of BSP 17 had a band of approximately 180

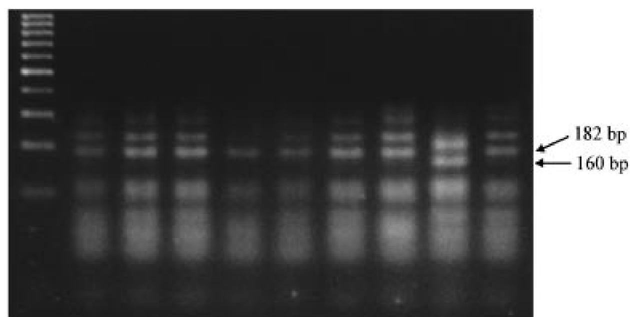

Fig. 3. RFLPs of MS1-2 PCR-products digested with *Tru*9I of different strains belonging to BSP 16 (lane 9) and strains belonging to BSP 17 (other lanes). Lane 1: 100 bp ladder. BSP 16 and 17 have different RFLP patterns.

bp. The presence of a unique mitochondrial type (Fig. 3) in each BSP means that we failed to detect cytoplasmic exchange.

DISCUSSION

Nature of the polymorphism

Polymorphisms in the ITS region in the same individual have been reported in plants (Baldwin *et al.* 1995), beetles (Vogler & DeSalle 1994), and nematodes (Zijlstra *et al.* 1995). rDNA polymorphisms in multinucleate fungi, such as members of the *Glomales*, have been reported by Lloyd MacGilp *et al.* (1996), Lanfranco, Delpero & Bonfante (1999), Pringle, Moncalvo & Vilgalys (2000), and Redecker *et al.* (1999). The various ITS types within a single spore of *Gigaspora margarita* clustered closely together and formed a monophyletic group (Lanfranco *et al.* 1999). However, claims by Hijri *et al.* (1999) on the very large variation in ITS sequences of *Scutellospora castanea* were later demonstrated to be incorrect (Redecker *et al.* 1999). The situation reported here for *Hebeloma velutipes*, where the various ITS sequences of a single BSP (and even of a single dikaryon) did not have a unique most recent common ancestor, is clearly different.

rDNA polymorphism in dikaryotic fungi can either result from differences within nuclei, or from differences between nuclei. In the first case there can be allelic differences between multiple rDNA loci or within allele heterogeneity in ITS type, while in the latter case there can be different alleles at homologous rDNA loci that reside in different nuclei. The ITS polymorphism found in *H. velutipes* results from different ITS types residing on homologous chromosomes in the different nuclei of a dikaryon. This conclusion is based on the ITS–RFLP analysis of 10 monokaryotic progeny of D9504, which all received only one ITS type. Presence of polymorphisms in three further dikaryons, but never in monokaryons tested, supports this explanation.

This ITS polymorphism in *H. velutipes*, consisting of orthologous ITS sequences, is also different from the situation in *Fusarium*, studied by O'Donnell & Cigelnik (1997). They observed a polymorphism in the ITS2 within single individuals of *Fusarium* species. They showed that the lack of congruence between a species phylogeny based on three gene sequences (the mitochondrial ssu rDNA, the nuclear 28S rDNA and the β-tubulin gene, which gave more or less concordant phylogenies) and one based on ITS2 sequences was due to non-orthologous ITS2 sequences. Within each species of *Fusarium* two ITS2 types were present, one in high copy number and one in low copy number. Direct sequencing yielded only the sequence of the dominant type. Since the relative copy abundance had shifted several times in the course of evolution, non-orthologous sequences could be obtained when doing direct sequencing. Phylogenies based on orthologous ITS 2 types did not show incongruence.

Crossing over could result in monokaryons having mixed arrays of rDNA. However, no examples have been found of these arrays in monokaryons, so no recombination between the two different ITS types has been observed. Moreover, crossing over between ITS1 and 2 could result in nuclei having ITS 1 of type Ia or Ib and ITS 2 of the other type. In all cases analysed, monokaryons having ITS 1 of type Ia or Ib had ITS 2 of the corresponding type, so no recombination between ITS 1 and 2 has been found. Taken together, these results show that recombination between these divergent types is rare.

Meiotic recombination within rDNA is thought to be suppressed in many eukaryotes including larger fungi (Petes & Pukkila 1995). Iraçabal & Labarère (1994) found no recombination between different ribosomal types in *Pleurotus cornucopiae*. However, recombination between divergent intergenic spacer (IGS) types in the rDNA has been shown in *Laccaria bicolor* (Selosse *et al.* 1996).

Polymorphisms within BSP and even within single dikaryons may be less exceptional than is generally assumed. Jonsson (1998) mentioned heterogeneity in amplified ITS products from dikaryons as one of the explanations for the observation that the sum of lengths of the RFLP bands exceeded that of the PCR products. We know of several cases in which direct sequencing of ITS PCR products was not successful (D. K. Aanen, unpubl.). This could be explained by two different ITS copies with minor length mutations in the PCR mixture.

Molecular divergence and the formation of biological species

Even though no evidence of recombination within or between the ITS sequences was found, concerted evolution is expected to homogenise the rDNA repeats in the long term. Maintenance of an ancestral polymorphism in BSP 17 is for that reason implausible. It is far more likely that the two divergent types have come together relatively recently in evolutionary history and that they have diverged in allopatry (i.e. in the absence of gene flow). In Europe strains with different ITS types did not show either geographical or ecological differentiation. A dikaryotic strain from North America, which could not be assigned to a BSP, also possessed the polymorphism in all of the 17 variable positions in which both ITS types in Europe differed. Assuming that originally only one of the two ITS types was found on one continent and the other type on the other, the present occurrence of the polymorphism in North America and Europe could be explained by two independent long-distance dispersal events. A possibility for migration from North America to Europe

could be the migration as an ectomycorrhizal symbiont of *Pseudotsuga menziesii*, which has been widely planted in Europe.

The phylogenetic analysis based on the ITS sequences (Fig. 1) demonstrated that both ITS sequences of BSP 17 did not have a unique most recent common ancestor. The more frequent sequence of BSP 17 formed a monophyletic group with the morphospecies *H. incarnatulum* (BSP 18), whereas the less frequent sequence of BSP 17 formed a clade with BSP 16. This phylogenetic pattern demonstrates that BSP could be paraphyletic or polyphyletic and that a biological and a phylogenetic species concept would not necessarily be congruent (see also Aanen *et al.* 2000b).

Aanen & Kuyper (1999) noted that BSP 16 and 17 showed partial intercompatibility, with 3.6% of the interspecies pairings showing clamp-connections. Could the ITS polymorphism in BSP 17 be the consequence of hybridisation between both BSP? Although hybridisation between biological species in the *Basidiomycota* has not gained wide acceptance, Newcombe *et al.* (2000) recently found an example of a naturally occurring *Melampsora* hybrid which showed intermediate character states for a range of different characters, including host preference, molecular, and morphological characters. Moreover, Aanen *et al.* (2000b) found evidence in the *H. crustuliniforme*/*H. alpinum* species group for a hybridogenous origin of one of the BSP. A number of lines of evidence point against frequent and continuing hybridisation between BSP 16 and 17: (1) although partial intercompatibility has been found between BSP 16 and 17, the resulting dikaryons showed reduced performance, as was evident from aberrant clamp-connections, aberrant hyphal morphology, lack of backwards nuclear migration into the monokaryon, and reduced dikaryotic growth rates (Aanen & Kuyper 1999); (2) individual isolates could always be unambiguously assigned to a single BSP, whereas in the face of frequent hybridisation we would expect to have isolates that cannot be unambiguously assigned to one BSP because of high levels of intercompatibility with two BSP; (3) BSP 16 had only one ITS type, whereas in the case of frequent hybridisation we would expect both types to be present in BSP 16 as well; and finally, (4) both BSP have unique mitochondrial sequences and fingerprints (Fig. 3) indicating that no recent cytoplasmic exchange occurred between the two BSP. However, we can not exclude that the rare type in BSP 17 may have originated from a hybridisation event followed by introgression of the BSP 16 type into BSP 17. An alternative explanation for the ITS polymorphism is that speciation is independent of ITS divergence. Aanen *et al.* (2000b) contrasted models of 'divergence-first' (speciation as a consequence of genetic divergence) with 'incompatibility-first' (speciation as the cause of genetic divergence). These models predict different relationships between the degree of incompatibility between strains and the relative age of their most recent common ancestor. In an analysis of the 5 BSP of the *H. crustuliniforme*/*H. alpinum* group, support for both models was obtained for different BSP. However, the level of ITS divergence within and between these 5 BSP (maximally 5 nucleotides within and 8 between) was substantially lower than the ITS divergence within BSP 17 of *H. velutipes* (20 nucleotides). Clearly, as yet generalisations about the relationship between levels of intercompatibility and ITS divergence have no firm basis.

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REFERENCES

Aanen, D. K. & Kuyper, Th. W. (1999) Intercompatibility tests in the *Hebeloma crustuliniforme* complex in northwestern Europe. *Mycologia* **91**: 783–795.

Aanen, D. K., Kuyper, Th. W., Boekhout, T. & Hoekstra, R. F. (2000 a) Phylogenetic relationships in the genus *Hebeloma* based on ITS 1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex. *Mycologia* **92**: 269–281.

Aanen, D. K., Kuyper, Th. W., Mes, T. H. M. & Hoekstra, R. F. (2000 b) The evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme* aggregate (basidiomycetes) in northwestern Europe: a phylogenetic approach. *Evolution* **54**: 1192–1206.

Arnheim, N., Krysta, M., Schmickel, R., Wilson, G., Ryder, O. & Zimmer, E. (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and ape. *Proceedings of the National Academy of Sciences, USA* **77**: 7323–7327.

Baldwin, B. G., Saunderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. & Donoghue, M. J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Bremer, K. (1988) The limits of amino-acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**: 795–803

Bruchet, G. (1970) Contribution à l'étude du genre *Hebeloma* (Fr.) Kummer. *Bulletin Mensuel de la Société Linnéan de Lyon* **39** (Suppl.): 3–132.

Buckler, E. S. & Holtsford, T. P. (1996) *Zea* systematics: ribosomal ITS evidence. *Molecular Biology and Evolution* **13**: 612–622.

Donoghue, M. J., Olmstead, R. G., Smith, J. F. & Palmer, J. D. (1992) Phylogenetic relationships of *Dipsacales* based on rbcL sequences. *Annals of the Missouri Botanical Garden* **79**: 333–345.

Elder, J. F. & Turner, B. J. (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review of Biology* **70**: 297–320.

Eriksson, T. (1998) *Autodecay*. Version 4.0. [Program distributed by the author.] Department of Botany, University of Stockholm, Stockholm.

Hijri, M., Hosny, M., van Tuinen, D. & Dulieu, H. (1999) Intraspecific ITS polymorphism in *Scutellospora castanea* (*Glomales*, *Zygomycota*) is structured within multinucleate spores. *Fungal Genetics and Biology* **26**: 141–151.

Hillis, D. M. & Bull, J. J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.

Iraçabal B. & Labarère, J. (1994) Restriction site and length polymorphism of the rDNA unit in the cultivated basidiomycete *Pleurotus cornucopiae*. *Theoretical and Applied Genetics* **88**: 824–830.

Jonsson, L. (1998) *Community structure of ectomycorrhizal fungi in Swedish boreal forests*. PhD thesis, Swedish University of Agricultural Sciences, Uppsala.

Lanfranco, L., Delero, M. & Bonfante, P. (1999) Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. *Molecular Ecology* **8**: 37-45.

Lloyd MacGilp, S. A., Chambers, S. M., Dodd, J. C., Fitter, A. H., Walker, C. & Young, J. P. W. (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. *New Phytologist* **133**: 103–111.

Newcombe, G., Stirling, B., McDonald, S. & Bradshaw, H. D. jr (2000) *Melampsora* ×*columbiana*, a natural hybrid of *M. medusae* and *M. occidentalis*. *Mycological Research* **104**: 261–274.

O'Donnell, K. & Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.

Petes, T. D. & Pukkila, P. J. (1995) Meiotic sister chromatid recombination. *Advances in Genetics*. **33**: 41–62.

Pringle, A., Moncalvo, J.-M. & Vilgalys, R. (2000) High levels of variation in ribosomal DNA sequences within and among species of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. *Mycologia* **92**: 259–268.

Redecker, D., Hijri, M., Dulieu, H. & Sanders, I. R. (1999) Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of Internal Transcribed Spacers reported from *Scutellospora castanea* are of ascomycete origin. *Fungal Genetics and Biology* **28**: 238–244.

Sanders, I. R., Alt, M., Groppe, K., Boller, T. & 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 Phytologist* **130**: 419–427.

Selosse, M.-A., Costa, G., Di Battista, C., Le Tacon, F. & Martin, F. (1996) Meiotic segregation and recombination of the intergenic spacer of the ribosomal DNA in the ectomycorrhizal basidiomycete *Laccaria bicolor*. *Current Genetics* **30**: 332–337.

Swofford, D. L. (1998) *PAUP: Phylogenetic analysis using parsimony.* Test version * 4.0. Smithsonian Institution, Washington DC.

Vogler, A. P. & DeSalle, R. (1994) Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. *Molecular Biology and Evolution* **11**: 393–405.

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

Zijlstra, C., Lever, A. E. M., Uenk, B. J. & van Silfhout, C. H. (1995) Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* **85**: 1231–1237.

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