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Multiple gene genealogies and AFLPs suggest cryptic speciation and long-distance dispersal in the basidiomycete *Serpula himantioides* (Boletales)

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Abstract

Serpula himantioides **(Boletales, Basidiomycota) produces thin resupinate basidiocarps on dead coniferous wood worldwide and causes damage in buildings as well. In this study, we present evidence for the existence of at least three phylogenetically defined cryptic species (referred to as Sib I–III) within the morphospecies *S. himantioides*, a conclusion based on analyses of sequence data from four DNA regions and amplified fragment length polymorphisms (AFLPS). A low degree of shared sequence polymorphisms was observed among the three lineages indicating a long-lasting separation. The AFLPs revealed two additional subgroups within Sib III. Results from mating studies were consistent with the molecular data. In Sib III, no correspondence between genetic and geographical distance was observed among isolates worldwide, presumably reflecting recent dispersal events. Our results indicate that at least two of the lineages (Sib II and Sib III) have wide sympatric distributions. A population genetic analysis of Sib III isolates, scoring sequence polymorphisms as codominant SNP markers, indicates that panmictic conditions exist in the Sib III group. This study supports the view that cryptic speciation is a common phenomenon in basidiomycete fungi and that phylogenetic species recognition can be a powerful inference to detect cryptic species. Furthermore, this study shows that AFLP data are a valuable supplement to DNA sequence data in that they may detect a finer level of genetic variation.**

Keywords: AFLP, biological species concept, cryptic species, genealogical concordance, phylogenetic species recognition, *Serpula himantioides*

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Introduction

With relatively few exceptions, the approximately 100 000 described fungi (Kirk *et al*. 2001) are diagnosed by morphological characters or other phenotypic traits. However, recent studies contain numerous examples of fungal morphospecies wherein reproductively and genetically isolated subgroups (sibling species) occur. This kind of cryptic speciation seems to be common in fungi, including basidiomycetes, and the records of cryptic species are on the rise, largely revealed by surveys of DNA sequence variation. For example, within the *Pleurotus ostreatus* complex, multiple biological species (8) and even more phylogenetically recognized species (phylospecies) were identified (Vilgalys & Sun 1994). The *Heterobasidion annosum* complex is another well-known example, where three intersterility groups and several genetically distinct phylospecies occur (Korhonen 1978; Johannesson & Stenlid 2003). Nine taxa were recently recognized within the corticoid *Hyphoderma setigerum* complex based on molecular, morphological and mating criteria (Nilsson *et al*. 2003).

Obviously, the number of species recognized within a fungal taxon often relies on which species concept is employed, e.g. a morphological (typological), biological (Mayr 1940) or a phylogenetic species concept. Taylor *et al*.

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(2000) advocated the use of analysing multiple unlinked genes as a criterion to identify phylogenetic species within the fungi and introduced the term genealogical concordance phylogenetic species recognition (GCPSR). Conflict among independent gene topologies can be caused by recombination among individuals within a species, and the transition from concordance to conflict determines the limits of species (Taylor *et al*. 2000). In recent years the GCPSR approach has been used to study cryptic speciation in, for example *Coccidioides immitis* (Koufopanou & Taylor 1997), *Aspergillus flavus* (Geiser *et al*. 1998), *Fusarium graminearum* and *Gibberella fujikuroi* (O'Donnell *et al*. 2000a, b), *Letharia* (Kroken & Taylor 2001), and *Neurospora* (Dettman *et al*. 2003a).

This study deals with the corticoid basidiomycete fungus *Serpula himantioides* (Fr.) P. Karst. (Coniophoraceae, Boletales), which is the sister species to the devastating dry rot fungus *Serpula lacrymans* (Wulfen) J. Schröt. The study was motivated by earlier observations of a low correlation between genetic divergence and geographical distance in a worldwide sample of the fungus that, hypothetically, could have been caused by widespread sympatric cryptic species, obscuring the relationship (Kauserud *et al*. 2004). Furthermore, the observation of intersterility between some isolates of *S. himantioides* and interfertility between others (Harmsen 1960) could indicate the existence of intersterility barriers and cryptic species. *S. himantioides* produces thin resupinate basidiocarps on dead coniferous wood and possesses a heterothallic (outcrossing) mating system (Harmsen 1960). Historically, there has been some confusion in the taxonomy of *S. himantioides* and allied taxa, and different forms have been ascribed different names, including *Merulius americanus* Burt and *Merulius silvester* O. Falck. Harmsen (1960) argued that these forms should be included in *S. himantioides* since only negligible differences in fruit body morphology were observed between them.

The main objective of this study was to determine whether cryptic species occur within the morphospecies *S. himantioides*, using the GCPSR approach. Gene-tree concordance between four DNA regions with different evolutionary rates [ITS and LSU nrDNA, and parts of the beta tubulin (*tub*) and heat stress protein (*hsp*) 80–1 genes] were analysed within 34 isolates of *S. himantiodies*. In addition, an amplified fragment length polymorphism (AFLP) analysis was carried out in order to test the concordance between the sequence data and genome-wide AFLP data and to determine whether the more sensitive AFLP markers could detect any further substructure. AFLP markers are known as highly replicable and able to give high resolution (Vos *et al*. 1995; Mueller & Wolfenbarger 1999). Furthermore, mating experiments were carried out and the sequence data generated enabled us to perform a population genetic analysis of a subsample of the isolates.

Materials and methods

Isolates

Table 1 lists the 34 isolates included in this study and their geographical origins. Thirty-one of the isolates were dikaryons and three monokaryons. Di-mon matings (matings between dikaryons and monokaryons) were carried out on 2% malt extract agar by pairing the mycelia and after 3–4 weeks they were inspected for clamp connections. Unfortunately, due to highly reduced growth rates, the monokaryotic isolates MUCL30856 and MUCL30857 could not be included in the mating experiments.

DNA sequencing

DNA was extracted from all isolates using the 2% cetyltrimethyl ammonium bromide (CTAB) miniprep method described by Murray & Thompson (1980) with minor modifications: DNA was resuspended in 100 μL sterile dsH_2O at the final step of extraction, and DNA templates were diluted 50-fold before polymerase chain reaction (PCR) amplification. PCR amplification was accomplished using the primers ITS4 and ITS5 (White *et al*. 1990) for the ITS nrDNA region, primers LROR and LR5 (White *et al*. 1990) for the partial nrDNA LSU region, primers B36F and B12R for the partial beta tubulin (*tub*) region (Thon & Royse 1999) and primers HspF and HspR for the partial heat stress protein (*hsp*) region (Johannesson *et al*. 2000). The *tub* and *hsp* sequences both included two partial and one complete exon and two introns (inferred by comparisons with data of previously published sequences of homologous genes of other fungi).

PCR was performed in 30-μL reactions containing 17.5 μL 50 × diluted template DNA and 12.3 μL reaction mix [final concentrations: 4 × 250 mm dNTPs, 0.625 mm of each primer, 2 mm $MgCl_2$ and 1 unit DyNazyme™ II DNA polymerase (Finnzymes)] on a Biometra thermocycler. The PCR amplification programme was as follows: 4 min at 94 °C, followed by 37 cycles of 30 s at 94 °C, 35 s at 54 °C, 72 °C for 40 s, and a final extension step at 72 °C for 10 min before storage at 4 °C. PCR products and cycle-sequencing products were purified using the ExoSAP-IT and Auto-Seq96™ Dye Terminator Clean-up Kits, respectively (Amersham Biosciences). Sequences were generated on a MegaBACE™ 500 DNA Analysis System (Amersham Biosciences), using the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) according to the producer's directions, using the PCR primers as sequencing primers. Both strands were analysed in all isolates.

AFLP analysis

Twenty-eight of the isolates were subjected to an AFLP analysis and four of these were run twice from independent

Table 1 Isolates included in the study and their geographical origin. The first isolate (SL1) represents *Serpula lacrymans* and was used as outgroup in the phylogenetic analyses. Information about lineages (Sib I–III) the isolates belong to, the nuclear condition of the isolates (n + n = dikaryon, n = monokaryon), the number of heterozygous sequence positions observed and isolates included in the AFLP analysis, are provided together with GenBank Accession nos for the four regions sequenced. In the AFLP column, ++ indicates isolates run twice as controls. For isolate HHB-17587, only freeze-dried mycelium was available.

Isolate	Origin	Sib	Nuc	Het	AFLP	LSU	ITS	*tub*	*hsp*
SL1	Norway		n + n	0		AM076559	AJ518881	AJ518064	AM076435
MUCL30798	?	I	n + n	1	+	AM076526	AM076492	AJ557382	AM076436
HHB-17587	USA (Alaska)	I	n + n†	0	–	AF518648	AM076493	AM076412	AM076437
MUCL30795	Canada (BC)	II	n + n	2	+	AM076527	AM076494	AJ557380	AM076438
82–90/7	Norway	II	n	0	+	AM076528	AM076495	AM076420	AM076439
FSC-31*	USA (Beltsville)	II	n + n	2	+	AM076529	AM076496	AM076825	AM076440
TH-Nor	Norway	II	n + n	1	–	AM076530	AM076497	AM076414	AM076441
MUCL30528	Belgium	IIIa	n + n	4	+	AM076531	AM076498	AJ518086	AM076442
MUCL30796	Canada (BC)	IIIb	n + n	3	+	AM076532	AM076499	AJ557377	AM076443
MUCL30797	England	IIIa	n + n	2	+ +	AM076533	AM076500	AJ518087	AM076444
MUCL40854	Denmark	IIIa	n + n	9	+	AM076534	AM076501	AM076417	AM076445
MUCL30855	UK	IIIa	n + n	1	+	AM076535	AM076502	AJ518088	AM076446
MUCL30856	Denmark	IIIa	n	0	+	AM076536	AM076503	AJ557379	AM076447
MUCL30857	Denmark	IIIa	n	0	+	AM076537	AM076504	AJ518089	AM076448
MUCL31289	Belgium	IIIa	n + n	4	+	AM076538	AM076505	AJ518090	AM076449
MUCL38575	Zimbabwe	IIIa	n + n	0	+	AM076539	AM076506	AJ557373	AM076450
MUCL38576	Zimbabwe	IIIa	n + n	1	+	AM076540	AM076507	AM076430	AM076451
MUCL38935	UK	IIIa	n + n	1	+	AM076541	AM076508	AM076423	AM076452
MUCL38979	France	IIIa	n + n	6	+	AM076542	AM076509	AM076427	AM076453
MUCL39729	Belgium?	IIIa	n + n	9	+ +	AM076543	AM076510	AM076425	AM076454
MUCL30794*	Belgium	IIIb	n + n	1	+	AM076544	AM076511	AM076415	AM076455
S6	?	IIIa	n + n	8	+	AM076545	AM076512	AM076431	AM076456
S19	?	IIIa	n + n	4	+	AM076546	AM076513	AM076413	AM076457
P99/M213	USA (Wisconsin)	IIIa	n + n	4	+	AM076547	AM076514	AM076432	AM076458
P218	Germany	IIIa	n + n	2	+	AM076548	AM076515	AM076434	AM076459
P278	Netherlands	IIIa	n + n	9	+	AM076549	AM076516	AM076416	AM076460
P283	Germany	IIIa	n + n	12	+ +	AM076550	AM076517	AM076424	AM076461
P288	Germany	IIIa	n + n	0	+	AM076551	AM076518	AM076418	AM076462
P291	New Zealand	IIIa	n + n	3	+	AM076552	AM076519	AM076429	AM076463
CCBAS110*	Czech Republic	IIIa	n + n	2	+ +	AM076553	AM076520	AM076419	AM076464
NZFS 1436*	New Zealand	IIIa	n + n	3	+	AM076554	AM076521	AM076421	AM076465
2024PS	Sweden	IIIa	n + n†	9	–	AM076555	AM076522	AM076428	AM076466
DSM5043	Germany	IIIa	n + n	5	–	AM076556	AM076523	AM076433	AM076467
DSM6419	Denmark?	IIIa	n + n	6	–	AM076557	AM076524	AM076426	AM076468
ATCC36335*	?	IIIa	n + n	6	–	AM076558	AM076525	AM076422	AM076469

*wrongly accessioned as *Serpula lacrymans* in the FSC, MUCL, CCBAS, NZFS and ATCC culture collections.
†dikaryon established from inbred polyspore culture.

DNA extracts as controls (Table 1). The AFLP analysis was performed essentially as described by Vos *et al*. (1995). The restriction-ligation samples consisted of 5.5 μL undiluted DNA extract and 5.5 μL restriction-ligation master mix: 0.405 μL milliQ-H_2O, 1.100 μL NaCl (0.5 M), 1.000 μL *Eco*RI adaptor pair (10 μM), 1.000 μL *Mse*I adaptor pair(10 μM), 0.020 μL *Mse*I (50 U/μL), 0.125 μL *Eco*RI (40 U/μL), 1.100 μL T4 DNA buffer 10X, 0.550 μL BSA (0.1%), 0.200 T4 DNA Ligase (primers from MWG-Biotech; *Mse*I from New England Biolabs, other reagents from Roche). The samples were incubated for 3 h at 37 °C on an ABI 9700 (Applied Biosystems) thermocycler, and thereafter diluted 10 times in milliQ-H_2O and mixed thoroughly.

The preselective amplification reactions were performed in 25-μL reaction volumes, consisting of 3 μL dilutions from the restriction-ligation step, 14.9 μL milliQ-H_2O, 0.5 μL *Eco*RI preselective primer (10 μM), 0.5 μL *Mse*I preselective primer (10 μM), 2.0 μL dNTPs solution (10 mM), 2.5 μL *Taq* buffer, 1.5 μL $MgCl_2$ (25 mM), and 0.1 μL Ampli*Taq* (primers from MWG-Biotech; other reagents from Applied

Biosystems). Only the core *Eco*RI/*Mse*I primer sequences were used during the preselective amplifications. The preselective amplifications were run under the following conditions on an ABI 9700 thermocycler: 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C, and with a final 10 min elongation step at 72 °C. Aliquots of 10 µL of the preamplification products were tested for visible smears on a 1.0% agarose gel in 1X TBE buffer. The gel was stained with ethidium bromide, and examined under UV light. Smear product in the 100–1500 base pair range indicated successful amplifications. The remaining preamplification products were diluted 20 times in milliQ-H_2O and mixed thoroughly.

The selective amplifications were conducted with *Eco*RI and *Mse*I primers with two additional nucleotides. Initially, 36 different primer combinations were tested on four *Serpula lacrymans* isolates for selective amplification. Five primer combinations, giving high quality chromatograms, were chosen for further analyses (*Eco*RI-AT + *Mse*I-TA/GT/GC/GA/CA). The *Eco*RI-primer were 5′-labelled with the fluorescent dye 6-FAM. The selective amplification reactions were performed in 10-µL reaction volumes, consisting of 2 µL dilutions from the preselective amplification step, 4.64 µL milliQ-H_2O, 0.20 µL *Eco*RI-AN selective primer (10 µM), 0.20 µL *Mse*I-CN selective primer (10 µM), 0.08 µL BSA (0.1%), 0.80 µL dNTPs solution (10 mM), 1.00 µL *Taq* buffer, 1.00 µL $MgCl_2$ (25 mM), and 0.08 µL Ampli*Taq* (primers from MWG-Biotech; BSA from Roche; other reagents from Applied Biosystems). The selective amplifications were run under the following conditions on an ABI 9700 thermocycler: 10 min at 95 °C followed by 13 cycles of 30 s at 94 °C, 60 s at 65–56 °C (temperature decreased by 0.7 °C for each of the 13 cycles), and 60 s at 72 °C, followed by 23 cycles of 30 s at 94 °C, 60 s at 56 °C, and 60 s at 72 °C, and with a final 10-min elongation step at 72 °C.

The AFLP products were prepared for electrophoresis as follows: 11.8 µL of sample loading solution (HiDi formamide) was mixed with 1.5 µL 6-FAM-labelled AFLP products and 0.2 µL of a GENESCAN ROX 500 Size Standard Kit (Applied Biosystems). The samples were denatured for 5 min at 95 °C, and thereafter placed on ice. After a 1-min centrifugation of the reaction plate at rcf = 5900xg, electrophoresis was performed using an ABI 3100 genetic analyser (Applied Biosystems) with POP4 polymer, default settings, and a 35-s injection time. The peak profiles were first checked and aligned using ABI GENESCAN analysis software version 3.7 (Applied Biosystems) and then viewed and scored as binary characters in GENOGRAPHER 1.6.0 (http://hordeum.oscs.montana.edu/genographer). A binary AFLP data matrix was constructed for principal co-ordinate (PCO) and NJ analyses. In the four isolates that were run twice as controls, between 0 and 3 differences (out of 275 scored and 261 polymorphic markers) were obtained between the replicates. Thus, a very high reproducibility occurred in our AFLP analysis.

PCR-RFLP analysis

PCR-RFLP analyses of amplicons from the 26 dikaryotic Sib III isolates were performed as a control for the interpretations of sequence polymorphisms as codominant bi-allelic markers. WEBCUTTER 2.0 (online at www.firstmarket.com/cutter) was used to detect restriction enzymes that could cut at polymorphic sites and separate between homo- and heterozygous genotypes (and thus reveal codominant information for these sites). The restriction enzyme *Aci*I were found to cleave ITS at two different polymorphic sites (situated in ITS1 and ITS2, respectively) and *Hpy*CH4IV was found to cleave the *tub* amplicons at one polymorphic site (cf. Supplementary material). ITS1 and ITS2 amplicons were generated from the 26 dikaryotic Sib III isolates using primers designed by White *et al*. (1990) and the same PCR conditions as for the entire ITS region. For restriction analyses, 10 µL of ITS1, ITS2 and *tub* amplicons were digested in 25-µL volumes containing 16.5 µL H_2O, 2.5 µL buffer and 0.5 µL enzyme (*Aci*I for ITS1 and ITS2 amplicons and *Hpy*CH4IV for the *tub* amplicons), following the manufacturers instructions (New England Biolabs). The cleaved amplicons were run on 1.5% agarose gels and stained with ethidium bromide and visualized over a UV transilluminator.

Statistical analyses

Sequence chromatograms were corrected and sequences were aligned manually in BIOEDIT (Hall 1999). Five different sequence data sets were constructed, including single-gene alignments of the LSU, ITS, *tub* and *hsp* sequences and one alignment with all these sequences concatenated. In two positions in the ITS alignment, a single base-pair deletion and a nucleotide (e.g. '–/T') occurred in the same position within dikaryotic isolates (causing a nonsense sequence downstream after this position). This has been indicated as 'X' in the Supplementary material. The gap ('–') was converted to an ordinary character prior to phylogenetic analysis [e.g. '–/T' (='X') to 'C/T' (= 'Y')]. The dry rot fungus *Serpula lacrymans*, which is the closest relative to *Serpula himantioides*, was used as an outgroup in the phylogenetic analyses.

Model comparison for all single gene data was performed using likelihood-ratio tests in the MODELTEST program (Posada & Crandall 1998).The 'GTR' model was chosen for the LSU matrix, 'HKY + G' for ITS and *tub* and 'K80 + G' for *hsp*. The best-fitting evolutionary models were implemented in the phylogenetic analyses of single gene and concatenated data applying Bayesian inferences with MRBAYES version 3.0 (Ronquist & Huelsenbeck 2003). Inferences of combined

sequence alignments were done by partitioning the data and applying the best-fitting model for each gene and unlinking the following parameters: gamma shape, branch lengths, switching rate for the covariotide model, stationary substitution matrix and the character state frequencies. The Markov chain Monte Carlo (MCMC) chains were run for 2 000 000–5 000 000 generations, and trees were saved each 100 generation, in all counting 20 000–50 000 trees. Burn-in was set to 500 000 generations based on visual inspection of the stationary phase of the MCMC chains. Trees saved after burn-in were used for calculating the consensus tree and posterior probability values. Convergence of the MCMC chains were tested by repeating the Bayesian inference from different starting trees, generated randomly by the program. Tree topologies, mean likelihood scores and the posterior-probability values showed almost identical results from independent runs, suggesting that the MCMC had run long enough to converge. Since the branch lengths were unlinked for all partitions in the concatenated data, the consensus tree was estimated using all trees saved after burn-in from each partition.

Maximum-parsimony (MP) analyses were performed in PAUP* version 4.02b (Swofford 1999), treating characters as unordered with equal weights. Gaps were treated as a fifth character in the MP analyses (recent studies have shown that gaps represent reliable phylogenetic information — cf. Kawakita *et al*. 2003). The heuristic search option, with the tree-bisection–reconnection (TBR) branch swapping algorithm and the random addition sequence option with 100 replicates to find multiple islands, was employed for all searches for most parsimonious tree(s). Sites possessing two nucleotides (e.g. C/T = Y) where treated as 'polymorphism'. All other settings were default. Bootstrap support for branching topologies (Felsenstein 1985) was determined with the same parameter settings using 1000 search replicates, except that simple addition of sequences was used and no branch swapping was performed (due to limited computational power).

The PCO analysis of the AFLP data was performed in NTSYSPC (Rholf 1997), using Jaccard's coefficient for similarity. Neighbour-joining (NJ) analysis of the AFLP data was conducted in PAUP*, using the 'total character difference' distance measurement. Population genetic analyses of the SNP genotypes were performed in ARLEQUIN version 2.0 (Schneider *et al*. 2000).

Results

Phylogenetic analyses of DNA sequences

Among the 34 isolates investigated, 13 variable sites were present in the LSU alignment (877 bp), 28 in ITS (568 bp), 47 in *tub* (456 bp), and 43 in *hsp* (416 bp), respectively. All the DNA polymorphisms observed are listed in the Supplementary material. Twenty-eight out of the 31 dikaryotic isolates possessed between 1 and 12 sequence positions with two nucleotides in the same position, apparently reflecting heterozygous sites. In the three monokaryotic isolates, no sequence positions with two nucleotides were observed (cf. Supplementary material). Phylogenetic analyses of the LSU, ITS, *tub*, *hsp* and the concatenated data, employing Bayesian inferences (see above) and using *Serpula lacrymans* as outgroup, gave largely similar topologies (Fig. 1). The 34 *Serpula himantioides* isolates grouped consistently into three groups (Sib I–III), with the exception of isolates 82–90/7 and MUCL38575, that switched positions in one of the trees. Analyses based on the MP criterion gave highly congruent topologies (bootstrap support values given in Fig. 1). Noteworthy, little correspondence was observed between genetic and geographical distance in the Sib III group.

Table 2 summarizes the number of shared and fixed sequence polymorphisms among the three lineages. A very low proportion of shared polymorphisms occurred among lineages (1.3–2.4%) and all three lineages shared a polymorphism in only one position out of the 131 polymorphic sites (cf. Supplementary material).

AFLP analysis

From a subset of 28 isolates (cf. Table 1) we obtained 261 variable AFLP markers that could be scored unambiguously as present or absent in each isolate. Thirty-nine of these

Table 2 The number of shared and fixed sequence polymorphisms among the three lineages Sib I–III

Comparisons	Variable sites	Fixed sites	Variable in one of the lineages	Shared polymorphisms*	Different polymorphisms†
I–II	84	24 (28.6%)	57 (67.9%)	2 (2.4%)	1 (1.2%)
I–III	110	23 (20.9%)	85 (77.3%)	2 (1.8%)	—
II–III	80	18 (22.5%)	59 (73.8%)	1 (1.3%)	1 (1.3%)

*Polymorphisms occurring within both lineages.
†Different polymorphisms occurring within each lineage (e.g. A/T vs. A/G).

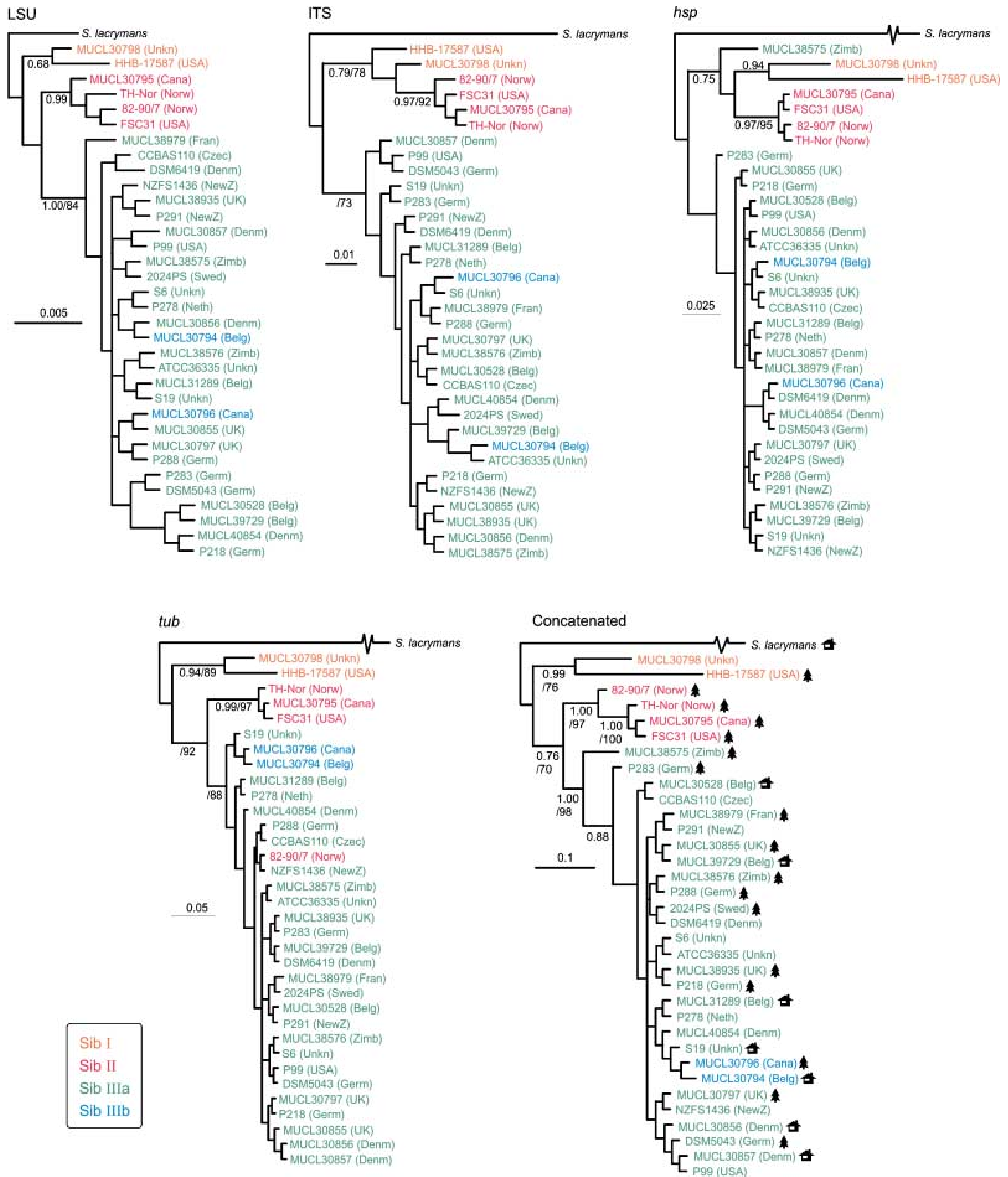

Fig. 1 Phylogenies derived from the LSU, ITS, *tub*, *hsp* and the concatenated data set using Bayesian analyses. Bayesian posterior probabilities (BPP) and bootstrap support values from maximum-parsimony analyses are given below branches. The 34 *Serpula himantioides* isolates were largely grouped into three different subgroups, indicated in colours. Blue colour represents two isolates belonging to a subgroup (Sib IIIb) recognized in the AFLP analysis (cf. Fig. 2). The symbols (tree and house) indicate whether the isolates were derived from a natural environment (forest) or from a human-made construction.

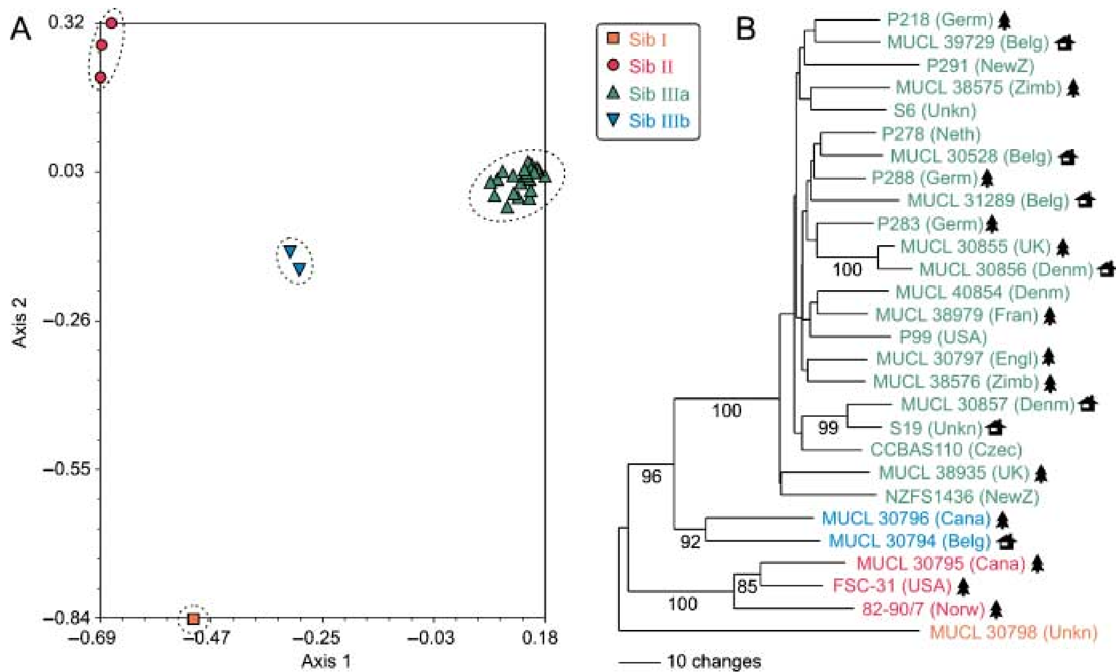

Fig. 2 (A) Diagram from a principal coordinate (PCO) analysis of AFLP data from 28 isolates of *Serpula himantioides*. The variation is distributed along the first two PCO axes. (B) Neighbour-joining (NJ) analysis of the same AFLP data. In both analyses, the 28 investigated isolates grouped into four distinct clusters; Sib I, Sib II, Sib IIIa and Sib IIIb. Within Sib IIIa, no correlation between genetic and geographical distance was observed. The symbols (tree and house) indicate whether the isolates were derived from a natural environment (forest) or from a human-made construction.

markers were singletons, i.e. present or absent in a single isolate. In Fig. 2(A), a plot from a PCO analysis of the isolates is shown, where the AFLP variation is distributed along the two first PCO axes. The isolates grouped into four clearly separated groups. In addition to the three groups recognized in the phylogenies above (Sib I–III), the AFLP markers divided the Sib III group into two well-separated subgroups, referred to as Sib IIIa and Sib IIIb. A corresponding NJ tree of the 28 isolates is shown in Fig. 2(B), where the same four groups were recognized and well supported. As in the sequence phylogenies, little correspondence was observed between geographical and genetic distance within the Sib III group, both in the PCO plot and the NJ tree. This was also the case when the AFLP data from the Sib IIIa group was analysed separately (data not shown).

Mating studies

Compatible di-mon matings were observed between the homokaryotic 82–90/7 isolate from Norway (Sib II) and the isolates MUCL30795, FSC-31 and TH-Nor, also appearing in the Sib II group (cf. Figs 1 and 2). Incompatible di-mon matings were observed between 82 and 90/7 and the 26 dikaryotic isolates of the Sib III group and MUCL30798 from the Sib I group.

Population genetic analysis

A population genetic analysis of the 26 dikaryotic isolates included in the Sib III group was carried out, using sequence polymorphisms as codominant SNP markers. A total of 44 polymorphic sites were found among these isolates and inferred as homo- or heterozygous based on the presence of a single peak or double peaks in polymorphic positions in the sequence chromatograms. As a control, ITS1, ITS2 and *tub* amplicons were included in the PCR–RFLP analyses, using three polymorphic sites (cf. supplementary material) as targets for the restriction enzymes *Aci*I (ITS) and *Hpy*CH4IV (*tub*). The resulting restriction patterns corresponded with the interpretations of the sequence chromatograms (data not shown). Thirteen SNP markers, where the frequency of the most infrequent allele was more than 10%, were selected for further analyses (indicated in supplementary material). No significant deviations from Hardy–Weinberg (HW) expectations were observed, except at one locus. Heterozygote deficits were observed in 10 out of the 13 SNPs (Table 3). When analysing the European Sib III material separately ($n = 21$), no significant deviations from HW expectations occurred. The 13 SNP markers represent largely replicates and not independent data due to their occurrences in the same sequences. In 48% of the cases, significant linkage disequilibria

Table 3 Results from population genetic analysis of the 26 heterokaryotic isolates in the Sib III group. Observed (H_O) and expected (H_E) levels of heterozygosity in the 13 loci are given in the same order as appearing in the supplementary material

Locus	H_O	H_E	*P* value
LSU1	0.19	0.25	1.00
*tub*1	0.17	0.41	0.03
*tub*2	0.23	0.38	0.20
*tub*3	0.19	0.25	1.00
*tub*4	0.54	0.46	0.14
ITS1	0.19	0.30	0.37
ITS2	0.12	0.25	0.19
ITS3	0.15	0.33	0.08
ITS4	0.19	0.25	1.00
ITS5	0.19	0.30	0.37
ITS6	0.35	0.38	0.63
*hsp*1	0.27	0.24	1.00
*hsp*2	0.27	0.24	1.00

were observed among SNPs from the same DNA regions (LSU/ITS, *tub* and *hsp*).

Discussion

The results from the four sequenced loci (LSU, ITS, *tub* and *hsp*) in conjunction with AFLP data clearly identify three well-differentiated genetic lineages within the morphospecies *Serpula himantioides*. The phylogenies from the four sequence data sets and the AFLPs were highly concordant. This, together with the very low proportion of shared sequence polymorphisms between lineages (cf. Table 2), indicates a long-lasting separation not compatible with a hypothesis of a recent split between the lineages. In newly formed sibling species, a higher degree of shared polymorphisms is to be expected (Avise & Wollenberg 1997). One exception was observed in the Norwegian 82–90/7 isolate that clustered with the Sib III isolates in the *tub* tree, but otherwise clustered in the Sib II group. A second example involves the Sib III isolate MUCL38575 from Zimbabwe that occured in a divergent position in the *hsp* tree (cf. Fig. 1). Lineage sorting of similar alleles into different lineages followed by incomplete fixation of allelic variants may explain these observations. An alternative explanation is that introgression may have occurred between the lineages. However, since these isolates (82–90/7 and MUCL38575) did not take more intermediate positions in the PCO plot (cf. Fig. 2A), which is to be expected with hybrid isolates, we find it implausible that introgression has happened.

Species delimitation by the GCPSR approach rests on the concordance of multiple gene genealogies from independent loci. In this context, a species is a group of organisms all of whose genes coalesce more recently with each other than with those of any organism outside the group. Preferably, more unlinked loci could have been analysed (LSU and ITS are linked within the nrDNA repeat) and more isolates could have been included in the Sib I and Sib II groups, which could have strengthened our conclusions further.

The AFLP data separated the Sib III group into two subgroups (Sib IIIa and Sib IIIb), a pattern not clearly recognized by the sequence data. However, the Bayesian analysis of the concatenated data set also grouped the two Sib IIIb isolates (MUCL30794 and MUCL30796) together (although in a separate position compared to the AFLP data). The two subgroups could, hypothetically, represent two more recently diverged lineages only detected by the sensitive AFLP markers. Our results emphasize that DNA fingerprinting methods, such as AFLP, may provide an important supplement to sequence data in order to unveil genetic complexity within taxa. AFLPs have proved to work well also in other organism groups to investigate species boundaries (e.g. Parsons & Shaw 2001; Allender *et al*. 2003). The different properties of the two applied methods (AFLP and sequencing) apparently make them a good combination to analyse inter- and intraspecific genetic boundaries. While the AFLPs reflect changes on a genomic level, sequence data reflect changes in some very limited DNA regions. Given the effort required to generate microsatellite markers for new taxa, the combined 'AFLP and sequencing strategy' might be a superior method where no microsatellite markers exist.

The population genetic analysis of the inferred SNP data showed that the genotype distributions in the Sib III group, both at regional and global scales, largely corresponded to HW expectations, indicating panmictic conditions in Sib III. However, the sample size from non-European continents was far too low to conclude whether HW expectations exist at a global scale. Nevertheless, the results so far suggest a geographically large interbreeding population in the Sib III lineage. A full concordance was observed between the PCR-RFLP restriction patterns (data not shown) and the interpreted SNP genotypes, which demonstrates the usefulness of sequence chromatograms in scoring codominant SNP data in dikaryotic/diploid organisms (cf. Morin *et al*. 2004).

Our results further support the observations made by Harmsen (1960), that mating compatibility exists between some isolates of *S. himantioides* while other isolates are incompatible. Unfortunately, we had only one monokaryon of *S. himantioides* available that could be used in mating studies. Mating experiments are a prerequisite to analyse the concordance between phylogenetic and biological species boundaries. For example, in a comprehensive study, Dettman *et al*. (2003b) observed high concordance between biological and phylogenetic species recognition in *Neurospora*. Nevertheless, the limited results from our mating experiments indicate that mating barriers at least exist between Sib II and Sib I/Sib III, suggesting a

correspondence between biological and phylogenetic species boundaries. However, more extensive mating experiments are needed to resolve this issue within the *S. himantioides* complex.

In general, genetic isolation precedes the divergence of morphological character states. Therefore, it is not expected that recent genetically isolated species will show phenotypic differences. Several examples from the literature have demonstrated that gross-morphological variation, such as fruit body size and shape do not clearly reflect reproductive barriers of phylogenetically defined species (Brasier 1987; Vilgalys & Sun 1994; Nilsson *et al*. 2003). Likewise, little morphological divergence seems to have been accumulated between the various *S. himantiodies* lineages. Among the earlier described forms of *S. himantioides*, including *Merulius americanus* and *Merulius silvestre*, no significant differentiation in spore size and shape occur, and only slight differences in fruit body morphology have been recognized (Harmsen 1960). Whether these forms correspond to the different genetic lineages uncovered in this study, can only be addressed through genetic analysis of herbarium-type material. Our molecular data indicate a long-existent separation of the lineages; hence under the guidance of the obtained phylogenies, diagnostic morphological characters may be elucidated *in posteriori* analyses of the lineages.

Whether ecological differences exist between the phylogenetic species is not yet known. Some degree of differentiation in the biology of cryptic species is actually predicted by ecological competition theory. At least Sib II and Sib III seem to exist sympatrically in Europe and North America, and equal competitors cannot coexist in the long term. However, the current sympatry of Sib II and Sib III could merely reflect recent human-mediated dispersal events (see below). In the root rot pathogen *Heterobasidion annosum*, three morphologically similar sibling species are known to coexist in Europe, sometimes in sympatry (Korhonen 1978), and niche differentiation to different substrate types has taken place, possibly as a result of historic competition.

Whether the isolates were derived from houses (or other human made constructions) or from nature had no impact on their phylogenetic placement within the Sib III clade (cf. Figs 1, 2). Not surprisingly, it seems to be a continuous influx from nature to buildings (and possibly vice versa), and that no special form has adapted to human-made environments. In Sib II, all the four isolates were derived from nature, which indicate that this group do not so often attacks buildings. However, more data are necessary to conclude on this topic.

Within the Sib III lineage, a remarkable lack of correlation was observed between geographical and genetic distance, even on a global scale. Isolates from New Zealand, Africa, and North America did not deviate significantly from the larger European sample, neither in sequence nor in AFLP data (Figs 1 and 2). This rather unexpected pattern could reflect either natural intercontinental long-distance dispersal, or alternatively, that Sib III individuals in recent time may have been transported by humans between continents, e.g. on infected timber or wooden boats. Human-mediated intercontinental dispersal has been reported in some fungi, e.g. in *Armillaria mellea* (Coetzee *et al*. 2001), *Amylostereum areolatum* (Slippers *et al*. 2001), *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (Brasier & Buck 2001). In the case of *S. himantoides,* we find it likely that the isolates from, e.g. New Zealand and Africa have been introduced by humans. For example, the two isolates from Zimbabwe (MUCL38575 and MUCL38576) were obtained in a *Pinus* plantation on non-native substrates. Interestingly, in the *hsp* locus, the MUCL38575 isolate from Zimbabwe deviated significantly from the other Sib III isolates and included four nucleotides otherwise only occurring in Sib I and Sib II (see Supplementary material). The second isolate from Zimbabwe (MUCL38576), obtained on a closely neighbouring log in the same plantation had an *hsp* sequence similar to isolates from Europe and New Zealand. Noteworthy, a European dikaryotic Sib III isolate (P283) included the divergent *hsp* nucleotides observed in MUCL38575, in addition to the common nucleotides occurring in other Sib III isolates (cf. Supplementary material).

Interestingly, the two isolates separated into Sib IIIb by the AFLP data and partially by the concatenated sequence data set (MUCL30794 and MUCL30794), were derived from two different continents: Europe and North America. Thus, our data indicate that two widespread subgroups occur sympatrically within Sib III. However, more data are necessary to conclude on this topic.

Cryptic speciation, as uncovered in *S. himantioides* using the GCPSR approach, may be a common phenomenon in fungi. GCPSR is hitherto probably the most powerful way to recognize and delimit species in fungi, especially since mating studies are difficult to conduct in many fungal taxa. However, our analyses show that AFLP is a valuable supplement to the GCPSR approach and might recover patterns not detected by multilocus sequence data.

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2768/MEC2768sm.htm

Supplementary material. Polymorphic nucleotide positions observed in the four sequenced DNA regions. Grey shading indicates positions where only one of the lineages is polymorphic. * = monokaryotic isolates. † = fixation in all lineages has occurred. Π = lineages share polymorphisms. ψ = polymorphism segregates among lineages. ‡ = different polymorphisms occur in different lineages. ↓ = positions used in the population genetic analysis. In ITS, 'X' indicates heterozygous positions where a single base pair deletion occurs in one nucleus and a nucleotide in the other (e.g.: -/T). Frames mark alleles discussed in the text.

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