J Mol Evol (2007) 64:285–298
DOI: 10.1007/s00239-005-0179-z

JOURNAL OF MOLECULAR EVOLUTION

Evolution of *Pleopsidium* (Lichenized Ascomycota) S943 Group I Introns and the Phylogeography of an Intron-Encoded Putative Homing Endonuclease

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Received: 17 July 2005 / Accepted: 17 October 2006 [*Reviewing Editor*: Dr. Manyuan Long]

Abstract. The sporadic distribution of nuclear group I introns among different fungal lineages can be explained by vertical inheritance of the introns followed by successive losses, or horizontal transfers from one lineage to another through intron homing or reverse splicing. Homing is mediated by an intron-encoded homing endonuclease (HE) and recent studies suggest that the introns and their associated HE gene (HEG) follow a recurrent cyclical model of invasion, degeneration, loss, and reinvasion. The purpose of this study was to compare this model to the evolution of HEGs found in the group I intron at position S943 of the nuclear ribosomal DNA of the lichen-forming fungus *Pleopsidium*. Forty-eight S943 introns were found in the 64 *Pleopsidium* samples from a worldwide screen, 22 of which contained a full-length HEG that encodes a putative 256-amino acid HE, and 2 contained HE pseudogenes. The HEGs are divided into two closely related types (as are the introns that encode them) that differ by 22.6% in their nucleotide sequences. The evolution of the *Pleopsidium* intron-HEG element shows strong evidence for a cyclical model of evolution. The intron was likely acquired twice in the genus and then transmitted via two or three interspecific horizontal transfers. Close geographical proximity plays an important role in intron-HEG horizontal transfer because most of these mobile elements were found in Europe. Once acquired in a lineage, the intron-HEG element was also vertically transmitted, and occasionally degenerated or was lost.

Key words: Group I intron mobility — Homing endonuclease gene (HEG) — Ribosomal RNA — Lichen-forming fungus *Pleopsidium* — Phylogeography — Ancestral state reconstruction

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Introduction

Group I introns are autocatalytic RNAs (or ribozymes) that interrupt a wide range of organellar and nuclear genes in eukaryotes, eubacteria, phages, and viruses (Cech 1985; Lambowitz and Belfort 1993; Bhattacharya 1998; Cannone et al. 2002; Haugen et al. 2005a). The survival of these introns in the host genome is ensured by their ability to self-splice at the RNA level, thus restoring the host gene function (reviewed by Dujon 1989; Haugen et al. 2005a). This self-splicing ability is conferred by a conserved secondary structure consisting of about 10 paired elements, P1–P10 (Michel and Westhof 1990; Adams et al. 2004).

Group I introns are particularly abundant in certain lineages of fungi (Haugen et al. 2004, 2005a), especially in some lichen-forming fungi (DePriest and Been 1992; Gargas et al. 1995; Stenroos and DePriest 1998; Grube et al. 1999; Bhattacharya et al. 2002; Martín et al. 2003; Piercey-Normore et al. 2004),

whereas they are absent from other lineages (see Simon et al. 2005). Detailed phylogenetic analyses have provided strong evidence for group I intron vertical transfer followed by losses (Johansen et al. 1992; Bhattacharya et al. 1994; Haugen et al. 2004; Müller et al. 2001; Simon et al. 2005). However, the sporadic distribution at different positions and across taxa has also been interpreted as evidence for lateral or horizontal transfer (Hibbett 1996; Friedl et al. 2000; Simon et al. 2005).

Two processes can contribute to the spread of group I introns by horizontal transfer (Dujon et al. 1989; Belfort and Perlman 1995). The well-documented intron homing process occurs at the DNA level and is initiated by an intron-encoded homing endonuclease (HE). After recognizing a 15- to 40-bp DNA sequence on an intron-less allele (Bryk et al. 1993; Dalgaard et al. 1994), the HE will catalyze a double-stranded DNA break at or near the intron insertion site (Belfort and Roberts 1997; reviewed by Chevalier and Stoddard 2001). The DNA break is repaired using the intron-containing allele as a template, thus copying the HE gene-containing intron (HEG^+) into the intron-less allele and creating a homozygous HEG^+ cell (Dujon 1989; Belfort and Perlman 1995; for review see Kowalski and Derbyshire 2002). The high specificity of the homing process prevents nonspecific and possibly lethal double-strand breaks throughout the host genome. Nevertheless, HEs are relatively tolerant to some specific nucleotide substitutions within the recognition site (Wittmayer et al. 1998; Jurica and Stoddard 1999), allowing the continued propagation of HEGs within the host (Bryk et al. 1995; Aagaard et al. 1997; Argast et al. 1998), and maximizes the potential for transfer between closely related host species. The alternative process for intron mobility involves the reverse splicing of an excised intron RNA into a second RNA molecule lacking the intron (Woodson and Cech 1989; Roman and Woodson 1995, 1998; Roman et al. 1999). Reverse splicing is followed by reverse transcription of the recombinant RNA and a recombination event that ensures the stable integration of the intron into the host genome. Reverse splicing is less specific than homing (4- to 6-nt [nucleotide] recognition site), thus providing a greater opportunity for intron translocation within and among genes, as well as for intergenomic propagation (Roman and Woodson 1998; Cousineau et al. 2000). However, the role of reverse splicing in intron spread remains unclear, as it has not yet been demonstrated in genetic crosses.

The majority of HEs are categorized into four families (reviewed by Belfort and Perlman 1995), with the His-Cys box family found exclusively in nuclear group I introns. If HEs have the ability to move in a ribozyme-independent fashion (Loizos et al. 1994; Haugen et al. 2004, 2005b), the insertion of HEGs within group I introns is advantageous for both the intron and the HEG. The ribozyme ensures the correct excision of the HEG-containing intron, leaving intact the transcript of the invaded gene, while the HE ensures the effective spread of the intron (Haugen et al. 2005a). In 1999 Goddard and Burt postulated that introns associated with HEGs follow a recurrent cycle of invasion by horizontal transmission, degeneration, and loss, followed by reinvasion (see also Koufopanou et al. 2002). Once an intron becomes fixed in a population, and no empty insertion sites remain to be cut, the HEG can be expected to degenerate due to less selection pressure to maintain HE activity (assuming there is no benefit to the host) and to the potential cost of enzyme production to the host cell (Goddard and Burt 1999). To prevent its extinction, the intron-HEG element may colonize an intronless population of the same or closely related species and start a new cycle. Alternatively, these elements could permanently integrate into the genome if they offer a selective advantage to the host. For example, in some cases within fungal mitochondrial genomes, HEs have secondarily adapted into a maturase that facilitates the splicing of their encoding introns and contributes to the host's regulation of the invaded gene (reviewed by Caprara and Waring 2005).

To date, about 43 His-Cys box HEs have been documented (reviewed by Galburt and Jurica 2005), including 6 enzymes with demonstrated activity, 17 predicted full-length ORFs, and 20 pseudogenes. Fifteen of these documented HEs are from nonlichenized fungi, whereas only one is from a lichen-forming fungus, *Pleopsidium chlorophanum* (Acarosporaceae, Lecanoromycetes, Ascomycota). The HEG of *P. chlorophanum* is located in an IC1 group I intron at position S943 (relative to the *E. coli* gene) of the small subunit nuclear ribosomal DNA (nrDNA), hypothetically encodes a 256-amino acid protein, and is arranged in an antisense orientation with respect to nrDNA transcription (Haugen et al. 2004). The antisense orientation of HEGs in nuclear ribosomal genes raises interesting questions regarding the expression of these enzymes, as protein-coding genes are normally transcribed into RNAs by RNA polymerase II, and rRNA genes are transcribed by RNA polymerase I. In their study, Lin and Vogt (1998) demonstrated that the I-*Ppo*I HE (which is encoded by an HEG on the sense strand) is expressed from an RNA polymerase I derived RNA. They also found strong evidence for I-*Ppo*I being translated from a full-length excised intron RNA. Although mechanisms involved in the expression of nuclear HEs from an antisense orientation have not yet been discussed in the literature, presumably the expression must come as a result of transcription from separate promoters.

The sequencing of additional SSU nrDNA for worldwide representatives of *Pleopsidium* revealed the presence of an HEG in different individuals and species. The abundance of this HEG in populations of *Pleopsidium* suggests that this lichen could be a good model for understanding HEG-driven group I intron spread across broadly dispersed populations and among species (e.g., Müller et al. 2001). The main objective of this study was to analyze the evolution of group I intron-encoded HEGs in populations of *Pleopsidium* throughout the world and to establish whether it followed the recurrent cyclical model of acquisition, degeneration, and loss proposed by Goddard and Burt (1999). For this purpose, it was important to estimate the proportion of horizontal versus vertical transfers from a common ancestor followed by several losses involved in the HEG presence/absence pattern found in the *Pleopsidium* phylogeny. In the case of horizontal transfer, we wanted to determine whether the HEGs moved between taxa within the genus, or were acquired from more distantly related species, and whether the HEGs moved independently between taxa or together with the group I intron that encodes it. Finally, we wanted to estimate the geographical distribution of the HEG, and determine the effect of taxon geographical proximity on gene transfer.

Materials and Methods

Taxon Sampling

A total of 64 *Pleopsidium* specimens, representing all four species included in this genus, were screened for the presence/absence of HEGs at position S943 in the SSU nrDNA: 26 were from Europe, 21 from Asia, 10 from North America, 6 from Antarctica, and 1 from Africa (Table 1). An additional 27 specimens from other genera of the family Acarosporaceae were screened for the presence of HEGs (only taxa containing the S943 intron or used in the host phylogeny are shown in Table 1).

DNA Isolation and Amplification

DNA was isolated using the Puregene Kit (Gentra Systems) following the manufacturer's protocol for filamentous fungi. When the amount of material available was too small for conventional DNA isolation, only a single apothecium or areole was taken from the specimen. The apothecium/areole was crushed between two microscope slides, which were coated with Sigmacoat (Sigma-Aldrich No. SL2) to prevent binding of DNA to the glass. The crushed apothecium/areole was resuspended in 25 μl of distilled water, and amplification by polymerase chain reaction (PCR) was performed using 1 μl of this crude DNA extraction.

Of the 64 *Pleopsidium* specimens screened for the presence/absence of the HEG within the group I intron S943, 51 were sequenced for the internal transcribed spacer of the nuclear ribosomal DNA (ITS nrDNA) and 34 for the nuclear protein-coding gene RNA polymerase II subunit (*RPB2*) (Table 1). Amplification of the ~600-bp ITS nrDNA was performed using the primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993). The 2.1-kb fragment (from conserved regions 5–11; Liu et al. 1999) of *RPB2* was amplified using fungal-specific primers (Liu et al. 1999; Reeb et al. 2004) as well as *RPB2*-1208F (5′-ARAARCGSYTRGAYYTRGC-3′) and *RPB2*-3281R (5′- CGCTGRTAGTAIGTIGGDCC-3′) based on *RPB2* sequences from species classified within the Acarosporaceae. PCR conditions, PCR product cleaning, and sequencing reactions were performed as by Reeb et al. (2004).

PCR Assay for the Presence of Group I Introns and HEGs at Position S943

We designed a PCR assay as suggested by Hibbett (1996) to amplify the nrDNA repeats containing the S943 group I intron or the S943 HEG$^+$ group I intron, even when these repeat-containing introns were present at low copy numbers (e.g., when DNA was partially degraded). Each specimen was subjected to three types of PCR (see Fig. 1): (i) a standard amplification with the nssu1002R and NS22 primers; (ii) a group I intron-specific amplification using the nssu1002R-PLES943.3R primer pair, where PLES943.3R overlaps the three last nucleotides of the S943 intron; and (iii) an HEG-specific amplification using nssu1002R-PLECHL1 and nssu1002R-PLECHL2 primer pairs. If PCR products were obtained for these group I intron- and HEG-specific amplifications, additional PCRs were carried out to complete the missing part of the intron/HEG, using the specific primers shown in Fig. 1. The nssu1002R primer was designed to amplify an Acarosporaceae-specific region of length 27 bp, about 100 bp upstream of the S943 insertion site (Fig. 1). Three sizes of PCR products were expected from the standard PCR: a 350-bp fragment in the absence of an intron, an 800- to 1000-bp fragment if an S943 HEG$^-$ group I intron was present, and a 1700-bp fragment if a group I intron-encoding HEG was amplified. An additional 300–400 bp was expected in the presence of an S934 group I intron upstream from the S943 insertion site or of an S1052 group I intron downstream of the S943 insertion site. All PCR products of different sizes recovered from a single PCR reaction were systematically sequenced and products that did not display the Acarosporaceae-specific region were considered contaminants (Miadlikowska et al. 2005) and excluded from further experiments and analyses.

Host Phylogeny Reconstruction

Analyses to reconstruct the host phylogeny were carried out on two data sets: a 59-individual data set using ITS nrDNA sequences and a 39-individual data set for which both ITS nrDNA and *RPB2* were sequenced. Outgroups for both data sets were chosen based on a phylogenetic reconstruction including all *Pleopsidium* specimens as well as 27 specimens from other genera classified in the Acarosporaceae (results not shown). Both ITS and *RPB2* sequences were aligned using MacClade 4.06 (Maddison and Maddison 2003). Delimitation of the ambiguously aligned regions was performed according to Lutzoni et al. (2000). All maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) analyses were implemented with PAUP* 4.0b10 (Swofford 2002), and all Bayesian analyses with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Models of evolution were selected by ACI in Modeltest 3.06 (Posada and Crandall 1998).

The 59-individual ITS data set (431 sites) was analyzed using three methods. First, weighted MP was used where nonambiguously aligned regions were subjected to a step matrix and ambiguously aligned regions were recoded using INAASE v.2.3b (Lutzoni et al. 2000; see setting of analyses in Reeb et al. 2004). Second, Bayesian posterior probabilities (PP) were computed under one model of evolution (GTR + I + G). Four chains were run simultaneously for 5 million generations, and trees were sampled every 100th generation. A majority rule consensus tree was gener-

Table 1. *Pleopsidium* and other specimens from the Acarosporaceae included in this study indicating presence/absence of their respective S943 introns and HEGs

Specimen	Origin and voucher information	PCR product recovered			ITS Accession number	*RPB2* Accession number
		350 bp	800–1000 bp	1700 bp		
PLEOPSIDIUM						
	Europe					
Pleopsidium chlorophanum 103	Austria, Osttirol, Keller 15669 (Herb. General Ch. Keller)	Y	?	**DQ525531**	**DQ525482**	-
Pleopsidium chlorophanum 8	Austria, Steiermark, Hafellner 8 Oct 1995 (DUKE)	?	?	**DQ525532**	**DQ525480**	**DQ525461**
Pleopsidium chlorophanum 124	Austria, Styria, Hafellner & Miadlikowska 18.08.2002/1 (DUKE)	N	N	**DQ525533**	**DQ525483**	**DQ525440**
Pleopsidium chlorophanum 129	Austria, Styria, Reeb VR13-VIII-98/5 (DUKE)	N	?	AY316151	**DQ525477**	AY641064
Pleopsidium chlorophanum 79	Austria, Tirol, Sipman 31993 (B 600082871)	Y	N	**DQ525534**	-	-
Pleopsidium flavum 83	Bulgaria, Pirin-Gebirge, Scholz 2 Aug 1984 (B 600125577)	Y	?	N	**DQ525516**	-
Pleopsidium chlorophanum 80	Finland, Lapponia, Sipman, Tan & Reiniko 23368 (B 600094244)	Y	?	**DQ525535**	**DQ525489**	-
Pleopsidium flavum 63	France, Ariege, Faron 11-7-1993 (Herb. C. Roux)	N	N	**DQ525536**	-	-
Pleopsidium flavum 133	France, Hautes-Alpes, Reeb VR9-VII-98/28 (DUKE)	Y	?	**DQ525537**	**DQ525508**	**DQ525464**
Pleopsidium chlorophanum 121	Italy, Friuli, Tretiach 24628 (TSB)	N	N	**DQ525538**	**DQ525473**	-
Pleopsidium flavum 122	Italy, Sardegna, Tretiach 8857 (TSB)	Y	**DQ525571**	N	-	-
Pleopsidium chlorophanum 10	Norway, Hordaland, Hafellner & Ochsenhofer 14501 (O lichens 10350)	?	?	**DQ525539**	**DQ525479**	**DQ525460**
Pleopsidium chlorophanum 132	Norway, Hordaland, Reeb VR8-VIII-02/4 (DUKE)	N	**DQ525565**	N	**DQ525501**	**DQ525444**
Pleopsidium chlorophanum 131	Norway, Hordaland, Reeb VR8-VIII-02/5 (DUKE)	N	N	**DQ525540**	**DQ525475**	**DQ525443**
Pleopsidium chlorophanum 130	Norway, Hordaland, Reeb VR8-VIII-02/8 (DUKE)	N	N	**DQ525541**	**DQ525474**	**DQ525442**
Pleopsidium chlorophanum 106	Norway, Oppland, Haugan SK00-117 (O Lichens 89195)	N	N	**DQ525542**	**DQ525484**	**DQ525438**
Pleopsidium chlorophanum 1	Norway, Troms, Timdal 4008 (O lichens 10401)	?	?	**DQ525543**	**DQ525478**	**DQ525459**
Pleopsidium flavum 128	Poland, Tetra National Park, Reeb VR22-VIII-99/4b (DUKE)	?	?	**DQ525544**	**DQ525507**	**DQ525466**
Pleopsidium flavum 126	Spain, Girona, Reeb VR2-IX-00/15 (DUKE)	Y	N	**DQ525545**	**DQ525521**	-
Pleopsidium chlorophanum 127	Spain, Mont Seny, Hladun & Gaya 2002/09/09 (DUKE)	Y	N	**DQ525546**	**DQ525486**	-
Pleopsidium flavum 125	Spain, Soria, Reeb VR31-VIII-00/1 (DUKE)	N	N	**DQ525547**	**DQ525515**	**DQ525441**
Pleopsidium chlorophanum 41	Sweden, Torne Lappmark, Hertel 34.491 (M 0062532)	Y	inc **DQ525570**	**DQ525548**	**DQ525487**	-
Pleopsidium chlorophanum 81	Sweden, Torne Lappmark, Hertel & Poelt 9.8.1980 (B 600125574)	Y	N	N	-	-
Pleopsidium flavum 98	Switzerland, Grisons, Scheidegger Oct. 1991 (Herb. Helveticum Scheidegger)	N	?	pseudo **DQ525549**	**DQ525514**	**DQ525458**
Pleopsidium chlorophanum 91	Switzerland, Lepontinische Alpen, Scheidegger 7195 (Herb. Helveticum Scheidegger)	Y	N	N	-	-
Pleopsidium flavum 11	Turkey, Trabzon, John & Zeybek 6.167 (B 600125572)	Y	**DQ525572**	inc **DQ525578**	**DQ525517**	-
	Asia					
Pleopsidium discurrens 111	China, Tibet, Obermayer 5127 (GZU 65-96)	Y	N	N	**DQ525510**	**DQ525463**
Pleopsidium gobiense 142	Iran, Sohrabi 603 (Herb. Sohrabi)	Y	N	N	**DQ525490**	**DQ525446**
Pleopsidium gobiense 40	Kazakhstan, Vost, Moberg & Nordin n.K21:11 (M 0062472)	N	**DQ525573**	N	**DQ525496**	**DQ525452**
Pleopsidium flavum 67	Malaysia, Sabah, Sipman 31140 (B 600085452)	Y	N	N	-	-
Pleopsidium chlorophanum 144	Mongolia, Arkhangay, Reeb VR23-VII-04/4 (DUKE)	N	**DQ525569**	N	**DQ525498**	**DQ525448**
Pleopsidium flavum 76	Mongolia, Bulgan Aimak, Huneck MVR 78-44 (B 600095260)	Y	N	N	-	-
Pleopsidium gobiense 19	Mongolia, Gobi Altai, Golubkova & Zogt 431 (DUKE)	N	**DQ525575**	**DQ525550**	**DQ525497**	**DQ525469**
Pleopsidium gobiense 143	Mongolia, Khov Aimak, Reeb VR14-VII-04/6 (DUKE)	N	**DQ525568**	N	**DQ525494**	**DQ525447**
Pleopsidium gobiense 68	Mongolia, ömnögobi Aimak, Huneck MVR 88-47 (B 600095250)	?	**DQ525576**	N	**DQ525492**	-
Pleopsidium gobiense 72	Mongolia, ömnögobi Aimak, Huneck MVR 88-304 (B 600095255)	N	**DQ525574**	N	**DQ525493**	-
Pleopsidium gobiense 66	Mongolia, Töv Aimak, Huneck MVR 83-182 (B 600095247)	Y	N	N	-	-
Pleopsidium gobiense 145	Mongolia, Töv Aimak, Reeb VR29-VII-04/6 (DUKE)	N	N	**DQ525552**	**DQ525522**	**DQ525449**

(Continued)

Table 1. Continued

Specimen	Origin and voucher information	PCR product recovered			ITS Accession number	*RPB2* Accession number
		350 bp	800–1000 bp	1700 bp		
Pleopsidium chlorophanum 149	Mongolia, Töv Aimak, Reeb VR29-VII-04/3 (DUKE)	Y	**DQ525583**	N	**DQ525523**	**DQ525450**
Pleopsidium chlorophanum 150	Mongolia, Töv Aimak, Reeb VR30-VII-04/2 (DUKE)	Y	N	pseudo **DQ525553**	**DQ525524**	**DQ525451**
Pleopsidium cf. *chlorophanum* 18	Russia, Altai Republic, Lunke 97 (DUKE)	Y	N	N	**DQ525512**	**DQ525470**
Pleopsidium chlorophanum 119	Russia, Baikal, Nimis & Tretiach 25181 (TSB)	Y	N	N	**DQ525488**	-
Pleopsidium gobiense 120	Russia, Baikal, Nimis & Tretiach 25345 (TSB)	N	**DQ525567**	N	**DQ525495**	-
Pleopsidium chlorophanum 51	Russia, Central Siberia, Zhurbenko 9585 (M 0062485)	N	N	**DQ525551**	**DQ525476**	**DQ525456**
Pleopsidium sp. 12	Russia, Yakutiya, Zhurbenko 92515 (DUKE)	Y	N	N	**DQ525513**	**DQ525471**
Pleopsidium flavum 75	Tajikistan, Mogoltan-Geb., Kubrajov 30.5.1974 (B 600125570)	Y	N	N	-	-
Pleopsidium gobiense 74	Tajikistan, South Kuramin, Kubrajov 19.7.1987 (B 600125571)	Y	N	N	**DQ525491**	**DQ525457**
	North America					
Pleopsidium chlorophanum 45	US, Arizona, Apache Co., Hertel 40.159 (M 0062490)	N	**DQ525559**	N	**DQ525506**	-
Pleopsidium chlorophanum 49	US, Arizona, Apache Co., Hertel 40.177 (M 0062489)	Y	N	N	**DQ525518**	**DQ525455**
Pleopsidium cf. *chlorophanum* 14	US, Arizona, Cochise Co., Nash 26 (DUKE)	N	**DQ525557**	N	**DQ525504** **DQ525511**	**DQ525468**
Pleopsidium flavum 46	US, Arizona, Coconino Co., Hertel 39.984 (M 0062614)	Y	N	N	**DQ525500**	**DQ525453**
Pleopsidium chlorophanum 50	US, Arizona, Coconino Co., Hertel 40.300 (M 0062486)	N	**DQ525556**	N	**DQ525505**	-
Pleopsidium chlorophanum 48	US, Arizona, Pima Co., Hertel 40.029 (M 0062488)	N	**DQ525558**	N	**DQ525520**	-
Pleopsidium cf. *chlorophanum* 15	US, California, Riverside Co., Riefner 87-426 (CANL 103835)	N	**DQ525561**	N	**DQ525503**	**DQ525467**
Pleopsidium chlorophanum 28	US, Colorado, Clear Creek Co., Shushan sl-3446 (DUKE)	N	**DQ525560**	N	**DQ525509**	-
Pleopsidium flavum 116	US, Oregon, Jefferson Co., Reeb VR10-VIII-00/9 (DUKE)	N	**DQ525562**	N	**DQ525502**	**DQ525465**
Pleopsidium chlorophanum 135	Greenland, Uummannaq, Hansen EHS-03.302 (C 17381)	N	**DQ525566**	N	**DQ525499**	**DQ525445**
	Antartica					
Pleopsidium chlorophanum 138	Dronning Maud Land, Engelskjøn 10 Feb 1985 (BG L-500158)	Y	N	N	-	-
Pleopsidium chlorophanum 136	Dronning Maud Land, Engelskjøn 13 Feb 1985 (BG L-500713)	Y	N	N	**DQ525485**	-
Pleopsidium chlorophanum 109	MacRobertson Land, Filson & Austin 6.2.1974 (O Lichens 125486)	Y	N	N	-	-
Pleopsidium chlorophanum 137	Vestfjella, Haugerud & Winsnes Feb 1979 (BG L-500879)	Y	inc **DQ525579**	inc **DQ525577**	-	-
Pleopsidium chlorophanum 123	Victoria Land, Bargagli 577 (TSB)	Y	N	N	**DQ525481**	**DQ525462**
Pleopsidium chlorophanum 113	Victoria Land, Bargagli 612 (TSB)	Y	**DQ525582**	N	-	-
	Africa					
Pleopsidium cf. *chlorophanum*. 115	South Africa, Western Cape Province, Feuerer 18.2.2003 (DUKE)	Y	N	N	**DQ525472**	**DQ525439**
Other ACAROSPORACEAE						
Acarospora bullata	Italy, Piemonte, Reeb VR 8-VII-98/6 (DUKE)	N	AY640980	N	-	-
Acarospora cervina	France, Gard, Reeb VR 6-VII-98/11 (DUKE)	N	AY640982	N	-	-
Acarospora clauzadeana	Spain, Andalousia, Scheidegger 4.1988 (Herb. Helveticum Scheidegger)	N	AY640987	N	-	-
Acarospora complanata	Canada, Québec, Reeb VR 10-VIII-97 st.4.1/2 (DUKE)	N	AF356653	N	-	-
Acarospora fulvoviridula	Germany, Saxony-Auhalt, Scholz 03.06.1999 (Herb. Peter Scholz)	N	**DQ525564**	N	**DQ525530**	**DQ525436**
Acarospora hilaris	France, Var, Ménard 1 Fev. 1998 (Herb. Thierry Ménard)	N	AY640983	N	-	-
Acarospora laqueata	France, Gard, Reeb VR 6-VII-98/14 (DUKE)	N	AY640984	N	-	-
Acarospora macrospora	Norway, Oslo, Timdal 3186 (O Lichens 33416)	N	AY640985	N	-	-
Acarospora molybdina	Norway, Finnmark, Timdal 4733 (O Lichens 33418)	Y	N	N	**DQ525525**	**DQ525435**

(Continued)

Table 1. Continued

Specimen	Origin and voucher information	PCR product recovered			ITS Accession number	*RPB2* Accession number
		350 bp	800–1000 bp	1700 bp		
Acarospora scabrida	France, Haut-Rhin, Reeb VR 17-VII-98/3 (DUKE)	N	**DQ525563**	N	**DQ525526**	**DQ525437**
Acarospora schleicheri	France, Bouches-du-Rhône, Reeb VR 5-VII-98/30 (DUKE)	Y	N	N	**DQ525529**	AY641026
Acarospora sp. 47	US, Arizona, Gila Co., Hertel 39.701 (M 0062487)	N	**DQ525580**	N	**DQ525519**	**DQ525454**
Acarospora sp. 112	Russia, Southern Siberia Altay, Nimis 23/6/1990 (TSB)	Y	**N**	N	**DQ525527**	
Acarospora sp. 114	US, California, Sequoia National Park, Nimis 4255 (TSB)	N	**DQ525581**	N	**DQ525528**	-
Glypholecia scabra	France, Alpes-de Haute-Provence, Reeb VR 8-VII-98/19 (DUKE)	N	AY640991	N	-	-
Glypholecia scabra 148	Norway, Oppland, Haugan, Rui & Timdal 8031 (O Lichens 15084)	N	**DQ525555**	N	-	-
Glypholecia scabra 34	Spain, Girona, Reeb VR 2-IX-00/12 (DUKE)	N	**DQ525554**	N	-	-
Polysporina cf. *simplex*	US, Georgia, Coffee Co., Lay 99-0351 (Herb. Elisabeth Lay)	N	AY641000	N	-	-

Note. Voucher information shows country, region, name of collector, collection number, and herbarium acronym (in parentheses) with herbarium accession number if available. GenBank accession numbers in boldface correspond to sequences generated for the present study. GenBank accession numbers not in boldface were submitted in previous papers (Lutzoni et al. 2001; Haugen et al. 2004; Reeb et al. 2004). Only sequences from the S943 site that contained group I introns were submitted to GenBank. 350 bp: PCR fragment without S943 group I intron. 800–1000 bp: PCR fragment with S943 HEG^- group I intron. 1700 bp: PCR fragment with S943 HEG^+ group I intron. -: DNA markers not sequenced. N: PCR fragment of given size not found at position S943. ?: a PCR fragment for the corresponding size was detected but could not be sequenced. Y: PCR fragment of given size found at position S943. inc: incomplete sequencing of group I intron with or without HEG. pseudo: HEG lacking a single nucleotide or with a major deletion, thus expected to be nonfunctional.

ated from the 40,000 post-burn-in trees. Third, Bayesian bootstrap proportions (B-BP) were used where phylogenies were reconstructed (2 million generations) using one model of evolution on 100 independent nonparametric bootstrap data sets (generated with p4-0.81, written by Peter G. Foster, The Natural History Museum, London, UK, 2004). B-BP were obtained from a majority rule consensus of 1 million trees (100 reps. × 10,000 post-burn-in trees per run; for discussion on B-BP, see Reeb et al. 2004).

The 39-individual ITS + *RPB2* data set (448 + 2028 sites) was subjected to four types of analyses: (i) weighted MP with non-ambiguously aligned regions subjected to four step matrices (ITS and *RPB2* first-, second-, and third-codon positions) and ambiguously aligned regions recoded using INAASE and arc 1.5 (program written by F. Kauff and available at http://www.lutzonilab.net/pages/download.shtml#Ambiguous; see setting of analyses in Reeb et al. 2004); (ii) Bayesian analyses under four models of evolution (one per partition); (iii) ML under one model of evolution for the combined data set; and (iv) B-BP as described above, except that four models of evolution were used to run the analyses.

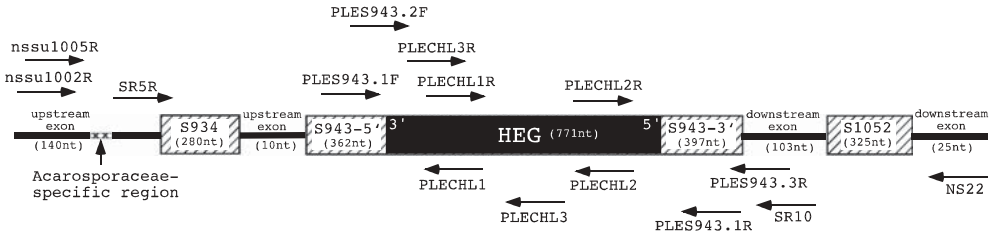
While reconstructing the phylogeny, we noticed an incongruency between the ITS and the *RPB2* data sets at one node of the tree. NJ with ML distance (NJ-ML) and Bayesian inference showed *Pleopsidium chlorophanum* 149 and 18 to be monophyletic in the ITS data set (84% bootstrap proportion [BP] and 99% PP, respectively). In the *RPB2* data set, *P. chlorophanum* 149 groups with *P. chlorophanum* 150 with high support values (96% BP and 100% PP). After verification of both the sequences and the specimens, we decided to combine the two data sets, keeping in mind that the *RPB2* data set had a greater influence on the combined phylogenetic reconstruction than the ITS data set. Nevertheless, this topological difference did not affect our general conclusions on HEG evolution.

Reconstruction of Ancestral States on Host Phylogenies

Ancestral character states for the presence/absence of the HEG in the S943 group I intron of *Pleopsidium* were reconstructed at selected nodes on 4000 trees drawn randomly (using the program rt.py written by F. Kauff and available upon request from F.L.) from the pool of 1 million post-burn-in trees generated by the 100 bootstrapped Bayesian analyses (40 post-burn-in trees per bootstrap analysis) on the 39-individual combined and 59-individual ITS data sets. The reconstruction was done using the ML optimality criterion in Mesquite 1.5 (Maddison and Maddison 2004a, b) as well as the Lasrdisc margin global and joint global criteria with character state at the root set to be equal, at equilibrium, or optimized (a module in Mesquite 1.5; Jackson 2004). An ancestral state was assigned to a node if its raw likelihood was higher by at least 2 log units than the likelihood value of the other ancestral state (default in Mesquite 1.5). Results for a given node were considered to be not sensitive to phylogenetic uncertainty when at least 90% of the 4000 trees under investigation were recovered with a given character state at that node. None of the 22 additional Acarosporaceae outgroups screened were found with an HEG, therefore the number of outgroups used would not significantly influence the reconstruction.

S943 Group I Intron Phylogenies

To assess monophyly of the S943 group I intron, a data set of 70 group I introns was gathered. This selection included two classes of introns (IC and IE) from six insertion sites of the SSU nrDNA. In addition, a phylogeny for the S943 group I intron was reconstructed based on 63 intron sequences found within members of the family Acarosporaceae. Both data sets were aligned manually with MacClade 4.06 (Maddison and Maddison 2003) using the intron



Primer name	Sequence	Position	Reference
PLECHL1	5'-CCGTCATGGATTTGGGTCTT-3'	2003–1984	This study
PLECHL1R	5'-AAGACCCAAATCCATGACGG-3'	1984–2003	This study
PLECHL2	5'-CCTTTCAGAGCGTCATCAGC-3'	2637–2618	This study
PLECHL2R	5'-GCTGATGACGCTCTGAAAGG-3'	2618–2637	This study
PLECHL3R	5'-ATGATAACCATCTGGGACAAC-3'	1934–1954	This study
PLECHL3	5'-TGCAGATATACCTGCATCAGC-3'	2232–2212	This study
PLES943.1F	5'-ACACCTAACCTGCGGGAA-3'	1695–1712	This study
PLES943.1R	5'-ACTGGATTAAAGCTCAAG-3'	3054–3037	This study
PLES943.2F	5'-CAGAGATTAGATGGGTGTG-3'	1842–1860	This study
PLES943.3R	5'-ATTAAGCCGCAGGCTCCCGT-3'	3106–3087	This study
nssu1002R	5'-ATACCGTCGTAGTCTTAACC-3'	1394–1413	This study
nssu1005R	5'-CCGTCGTAGTCTTAACCAT-3'	1397–1415	This study
SR5R	5'-AATTGACGGAAGGGCAC-3'	1533–1549	This study
SR10	5'-CCCGTGTTGAGTCAAA-3'	3121–3106	This study
NS22	5'-AATTAAGCAGACAAATCACT-3'	3237–3218	Gargas and Taylor (1992)

Fig. 1. The nuclear SSU rDNA region amplified to detect S943 group I introns and associated HEGs in *Pleopsidium*. Group I introns were recorded at three insertion sites of the target region: positions S934, S943, and S1052. An HEG, when found in the S943 group I intron, occurred in an antisense orientation (denoted by their 3′ and 5′orientation). An Acarosporaceae-specific region of 27 nt (5′-ggacggtgttactattttgacccgttc-3′) is found at the 5′ end of the targeted region (positions 1043–1069 relative to the SSU nrDNA gene of *Pleopsidium chlorophanum*; GenBank accession no. AY316151). The number of nucleotides, in parentheses, corresponds to the maximum length found for a given portion of the targeted region. Primers used for symmetric PCR amplifications and cycle sequencing reactions of the S943 nrDNA region are represented by black arrows. Primer names in the table are followed by their oligonucleotide sequences, their position relative to *Pleopsidium chlorophanum* GenBank accession no. AY316151 (Haugen et al. 2004), and their respective sources.

secondary structure. The 70-group I intron data set was subjected to Bayesian and NJ with ML distance (NJ-ML) analyses, whereas the 63-intron sequence data set was subjected to Bayesian and weighted MP + A analyses (as described above, except that the P8 region containing the HEG was entirely removed from these analyses).

Results and Discussion

Screening for the Presence/Absence of S943 Group I Introns and Intron-Encoded HEGs

A total of 48 S943 group I intron sequences were found in our study (Table 1). In 22 of these introns, an inferred full-length HEG was recovered that encodes a putative HE of 256 amino acids. In two additional introns we found a potentially full-length HEG for which we sequenced only ~60 nt at the 3′ end of the ORF due to sequencing problems (''inc'' in Table 1). Two introns contain HE-pseudogenes, with one missing a single nucleotide (thus generating a frame shift mutation and premature stop codons) and one lacking the last 520 nt. Finally, 22 of the group I intron sequences (including two with incomplete sequencing: ''inc'' in Table 1) contain no HEG. In four cases we found both HEG^- and HEG^+ group I introns within a single DNA isolate: *P. flavum* 11, *P. chlorophanum* 41 and 137, and *P. gobiense* 19 (columns 4 and 5 in Table 1). We also found 13 cases where, within a single DNA isolate, PCR amplification products showed the presence of group I introns at position S943 coexisting with products lacking an S943 intron (columns 3 and 4 in Table 1). Of the 27 other Acarosporaceae members screened, 15 had a group I intron at position S943 but none encoded for an HEG (Table 1).

Description and Distribution of the Homing Endonuclease Gene in Pleopsidium

Our study revealed that HEGs are concentrated in Europe, where 81% of the specimens that were screened showed the presence of an HEG within the S943 group I intron (Fig. 2). This geographical distribution of HEGs is independent of species barriers, e.g., both *P. chlorophanum* and *P. flavum* from Europe show the presence of an HEG, whereas the same species from other parts of the world generally lack the HEG (Table 1). We found two distinct types of HEGs, which we called Type 1 and Type 2 for convenience. They are believed to have the same origin, as they both are located at the same insertion site of the P8 extension of the S943 group I intron, are in an antisense orientation, are 771 nt in length, and align perfectly with each other (no gaps). In addition, the two types of HEG share a most common ancestor based on an alignment of 30 His-Cys HEGs of various origins (including *Pleopsidium* Type 1 HEG; Haugen et al. 2004) to which we added the Type 2 HEG and analyzed using phylogenetic methods (results not shown). However, their nucleotide sequences differ by 22.6%, and their amino acid sequences by 16.4%. The Type 1 HEG was first

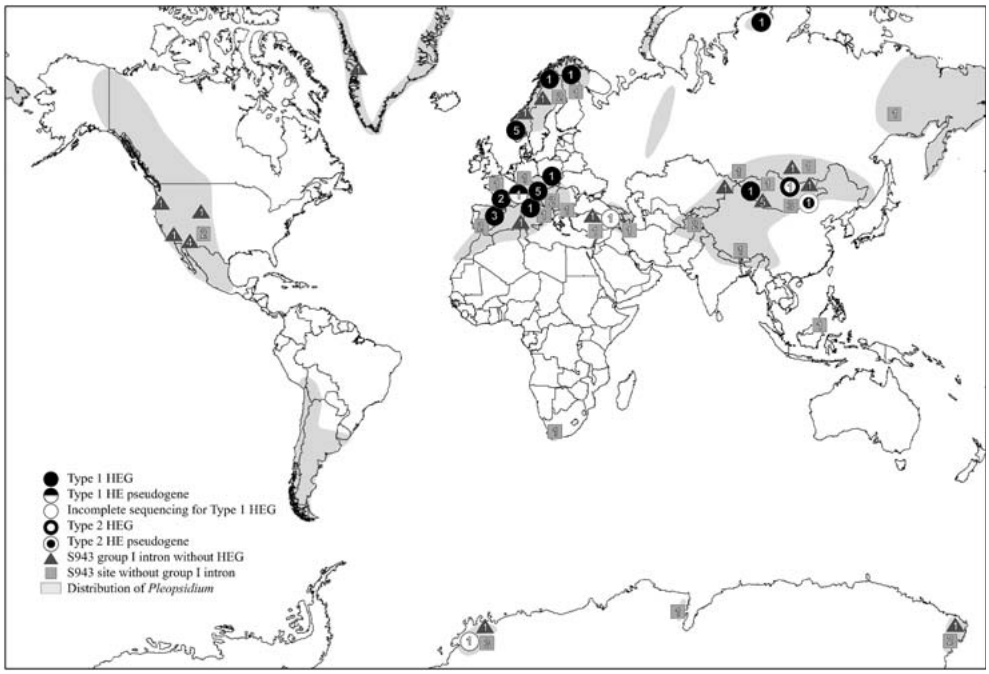


Fig. 2. Geographical distribution of *Pleopsidium* specimens found without group I introns, as well as with HEG^{-} and HEG^{+} group I introns. Localities for all specimens screened for the presence of the S943 group I intron are shown. Numbers within symbols correspond to the number of times a specific S943 status was found in a given area. Areas shaded in light gray correspond to geographical localities where *Pleopsidium* was recorded according to the literature (Magnusson 1929; Hafellner 1993; Wirth 1995; Obermayer 1996; Thomson 1997), herbarium databases (Arizona State University Phoenix, ASU [http://seinet.asu.edu/collections/selection.jsp]; University of Trieste, TSB [http://www.univ.trieste.it/cgi-bin/g/bot/leggi]; Swedish Museum of Natural History, S [http://www2.nrm.se/kbo/saml/lavkoll.html.en]), and specimens gathered for this study.

reported from the *Pleopsidium chlorophanum* 129 specimen from Austria (Table 1) by Haugen et al. (2004) and referred to as I-*Pch*I, according to the current nomenclature (Roberts et al. 2003). The complete sequence of the Type 2 HEG was found in *Pleopsidium gobiense* 145 from the Töv region of Mongolia (Table 1, Fig. 2), during the course of the present study. Twenty-four of the 26 HEGs recovered are of Type 1 and only 2, found exclusively in Mongolia, are of Type 2 (Fig. 2). Because *Pleopsidium* Type 1 HEGs are found at a high frequency in specimens from Europe, and the gene sequence is highly conserved (99.6%–100%), Type 1 HE is expected to be active. However, the activity of Type 2 HE remains in question, as it is found only in low abundance throughout the world, and one of the two Type 2 HEGs was found to be radically truncated.

Mobility of HEGs Revealed by Pleopsidium *Phylogeny*

The genus *Pleopsidium* is divided into three main groups, each of which is significantly supported by all four types of analyses (Fig. 3). Type 1 HEGs are found in each of the three main *Pleopsidium* clades and group together within clades (with or without support). Type 2 HEGs are found exclusively in two specimens (*P. gobiense* 145 and *P. chlorophanum* 150) from Clade 3. These two taxa do not share a most recent common ancestor according to the ITS + *RPB*2 data set analyses but do form a monophyletic group based on the ITS phylogeny alone (without support in the 39-individual data set and with high support in the 59-individual data set; results not shown).

To test whether the sporadic distribution of HEGs among *Pleopsidium* species/specimens was due to horizontal transfer or to inheritance from a common ancestor with several successive losses, we reconstructed the ancestral character states for the presence/absence of HEGs at given nodes of both the 39-individual ITS + *RPB*2 and the 59-individual ITS host phylogenies, using various settings (see Reconstruction of Ancestral States on Host Phylogenies, under Materials and Methods). In summary, Type 1 HEGs have been acquired three times through horizontal transfer during the evolution of *Pleopsidium*, once in each of the three main *Pleopsidium* clades, which also correspond to one horizontal transfer acquisition in each species *P. chlorophanum*, *P. flavum*, and *P. gobiense* (dark-gray arrows A, B, and C in Fig. 3). Once acquired, the HEG is vertically inherited from one generation to the next (in Clades 1 and 2) or can be lost (*P. chlorophanum* 115 and 123 in Clade 1 and *P. gobiense* 40 in Clade 3). The acquisition of the Type 1 HEG at internode A is in accord

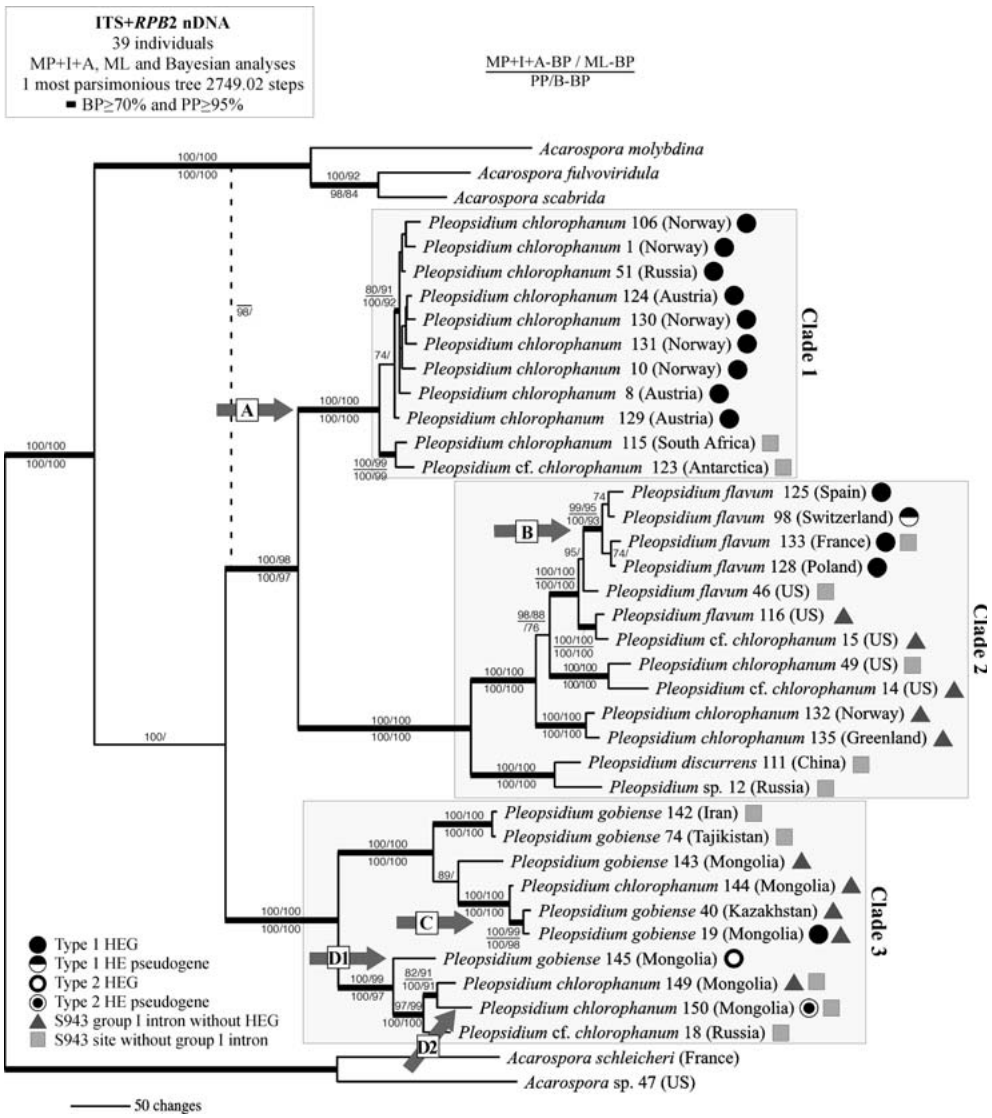


Fig. 3. Host tree based on the ITS and *RPB2* combined data set analysis for 34 species of *Pleopsidium* and 5 species of *Acarospora*, of which 2 are used as outgroups. The phylogram represents one single most parsimonious tree from the weighted MP analysis, including INAASE and arc characters (MP + I + A). Due to space limitations, only significantly supported values are presented here, i.e., bootstrap proportions (BP) ≥ 70% and posterior probability (PP) ≥ 95%. The thicker lines in the phylogram represent internal branches for which all four types of support were shown to be significant. The gray boxes delimit the three main *Pleopsidium* clades. The dashed line indicates an alternative branching pattern that is significantly supported (PP = 98%) where the three-taxon *Acarospora* clade originated after *Pleopsidium* clade 3. Symbols following each country of origin are the same as in Fig. 2. Dark-gray arrows represent possible origins of the acquisition of HEGs based on ancestral character state reconstruction. D1 and D2 arrows show two independent acquisition of the Type 2 HEG on the combined ITS + *RPB2* 39-individual data set, while the 59-individual ITS data set, where *P. chlorophanum* 145 and 150 significantly group together, supports a single acquisition of that HEG (supported by 100% of the trees tested that had the corresponding node).

with the potential presence of an HEG in *P. chlorophanum* 137 from Antarctica, a specimen likely to group with *P. chlorophanum* 115 and 123, but for which neither ITS nor *RPB2* could be sequenced due to limited material available. In Clade 3, the Type 2 HEG seems to have been acquired twice through horizontal transfer based on the 39-individual combined data set, i.e., once in each species of *Pleopsidium* where it has been found. However, when considering the 59-individual ITS data set, the Type 2 HEG was acquired only once in the common ancestor of *P. gobiense* 145 and *P. chlorophanum* 150 (nonsignificant result).

Evolution of the S943 Group I Intron and Its Encoded HEG

In order to determine the evolutionary history of the S943 group I intron, we reconstructed a phylogeny for two different types of group I introns (IE and IC1) found at six insertion sites of the SSU nrDNA (Fig. 4A). In general, group I introns share a most

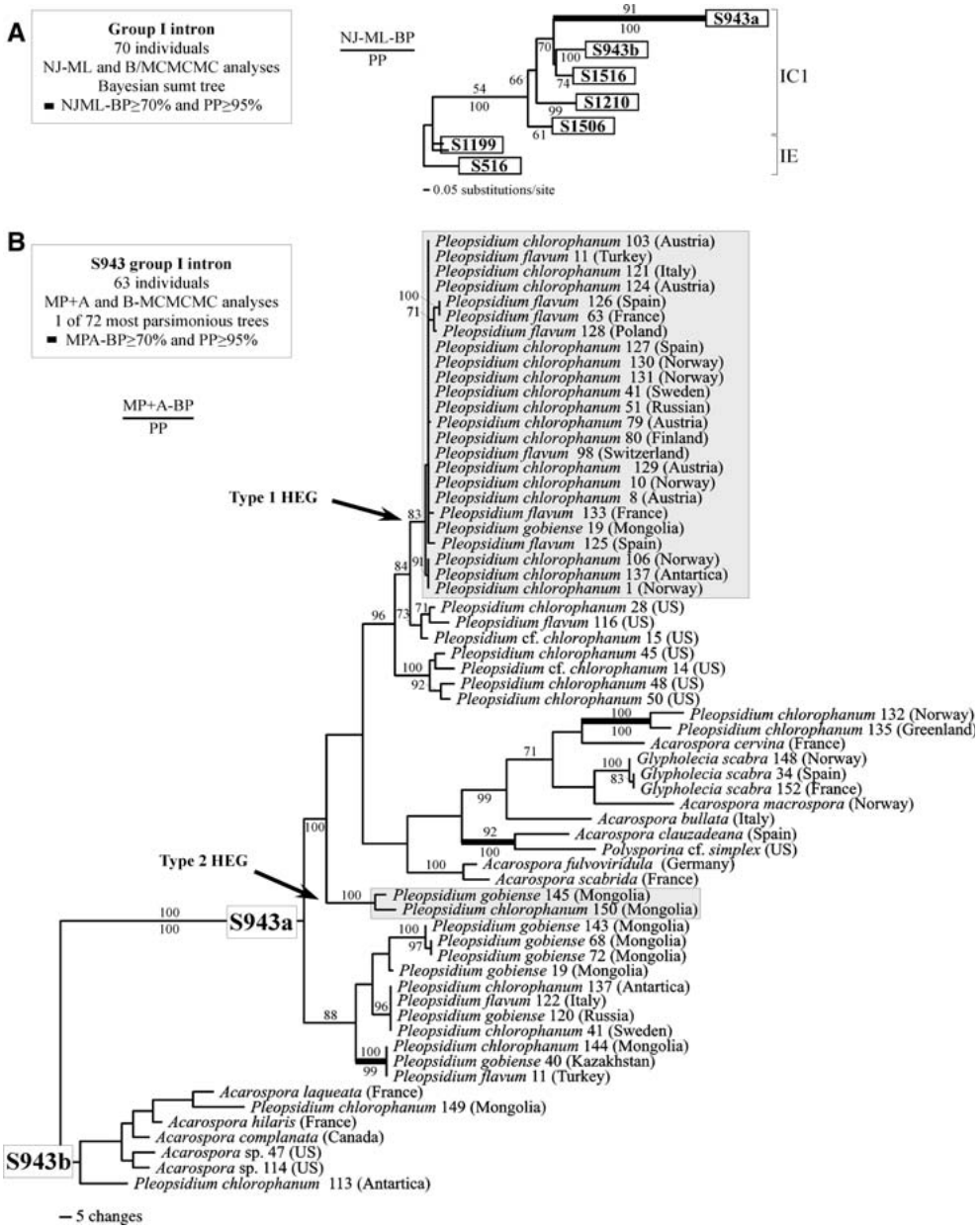


Fig. 4. Phylogenetic reconstruction of group I intron relationships. **A** Schematic representation of a B-MCMCMC sumt tree for 70 group I introns (IE and IC1) found at six insertion sites of the SSU nrDNA. Values above internodes are NJ-ML-BP and values below are PP. The thicker lines in the phylogram represent internal branches for which both types of support were shown to be significant. **B** One of the 72 most parsimonious trees for 63 S943 group I introns found in members of the Acarosporaceae. Values above the internodes are MP + A-BP and values below the internodes are PP. Thicker lines in the phylogram represent internal branches for which both types of support were shown to be significant. All group I introns containing the Type 1 HEG or the Type 2 HEG (each type forms a monophyletic group) are indicated by black arrows and delimited by a gray box.

recent common ancestor according to their type (IE vs. IC1) and their insertion sites (Wang et al. 2003; Haugen et al. 2004), even if they reside in distantly related organisms (reviewed by Haugen et al. 2005a). However, according to our data, the S943 group I introns found in the Acarosporaceae form two distinct groups. The S943a group diverged extensively from the other closely related group I introns, denoting an accelerated rate of nucleotide substitution. The S943b group I introns might share a most recent common ancestor with the S1516 group I introns. However, the monophyly of the S1516-S943b clade is not supported, and it is still possible that both S943 groups share a most recent common ancestry rather than having two independent insertions at the same splicing site.

The majority of the *Pleopsidium* S943 group I introns are of the S943a type (46 versus 2 in S943b; Fig. 4B). The topology within the S943a clade does not follow a species pattern but, rather, reflects the presence of the HEG in the intron. All S943 introns that encode the Type 1 HEG share a most common

ancestor, as do those encoding the Type 2 HEG (Fig. 4B). This incongruency between the intron and the host tree is characteristic for the horizontal movement of introns through homing. Furthermore, these phylogenetic results (Figs. 3 and 4B) associated with the highly conserved sequences of both the HEG+ intron and the HEG show that the introns and the HEG move together rather than in an independent manner. However, introns containing the Type 1 HEG and those containing the Type 2 HEG are not closely related, and their DNA sequences differ substantially, as is the case for the open reading frames they encode.

As mentioned earlier, the two types of HEGs are very likely to have the same origin, a belief further reinforced by the fact that the group I introns encoding these HEGs share a common ancestor. One possible explanation for the sequence divergence observed between the two types of intron-HEG elements could be that both were acquired from the same donor, but at different periods of time, thus allowing for divergence. Alternatively, both elements could have originated as a single acquisition within an ancestral host but later diverged as a consequence of geographical isolation and speciation of the host lineage (Fig. 3). In the latter case, the intron-HEG element would have been lost in several lineages of *Pleopsidium* and possible other members of the Acarosporaceae. This second hypothesis is not supported by our reconstruction of the evolution of the HEG within the phylogenetic context of the host (Fig. 3).

Sporadic Distribution of Intron-HEG Elements Within Pleopsidium

The sporadic distribution of the intron-HEG elements in worldwide samples of *Pleopsidium* can be explained by a combination of horizontal transfers among hosts (arrows A, B, C, and D in Fig. 3), and vertical inheritance with successive losses. If both types of intron-HEG elements have the same origin, acquisition of the intron-HEG element within the genus is most likely to have occurred twice by horizontal transfer from an unknown donor. Furthermore, because both ancestral intron-lacking taxa and taxa containing the Type 1 HEG^+ intron coexist within a clade of the host phylogeny, it is clear that after acquisition of the intron-Type 1 HEG element from the unknown donor, this element was then horizontally transferred from one species of *Pleopsidium* to another. This is also likely the case for the Type 2 HEG^+ intron.

Once acquired, the intron-HEG element was most likely transmitted through vertical inheritance within and between populations (Fig. 3). We also recovered a potentially degenerated HEG (*Pleopsidium chlorophanum* 98, Clade 2; Fig. 3) and intron-HEG losses (*P. chlorophanum* 115 and 123, Clade 1 [Fig. 3]; *P. flavum* 83, Clade 2, in the 59-individual ITS data set [results not shown]) within clades of taxa containing HEG. This pattern of *Pleopsidium* HEG evolution, especially as shown by Clade 2 (in both the 39- and the 59-individual data sets), is in accord with the cyclical model of HEG evolution proposed by Goddard and Burt (1999). How much loss contributes to the sporadic distribution of the HEG compared to horizontal transfers is not yet known, nor is the frequency of these phenomena.

Another potential explanation for the sporadic intron-HEG element distribution in *Pleopsidium* could involve the preexistence of group I introns at the S943 site, thus preventing the HE from generating a double-strand break within the nrDNA and its insertion into the nuclear genome. This could be the case for *Pleopsidium chlorophanum* 132 from Norway. This specimen of *Pleopsidium* was found less than a kilometer away from two other populations of *P. chlorophanum* (130 and 131), both of which contained the HEG. The host phylogeny in Fig. 3 shows *P. chlorophanum* 132 to be more closely related to *P. chlorophanum* 135 from Greenland than to any other *P. chlorophanum* from Norway, as is also the case for its S943 group I intron (Fig. 4B). If *P. chlorophanum* 132 originated from Greenland with a group I intron already at position S943, it could possibly be unaffected by the HEG from nearby *Pleopsidium* populations.

Horizontal Transfer of Intron-HEG Elements

How introns are spread from one organism to another is still unknown. However, homing events seem to depend on the frequency at which HEG^+ and HEG^- chromosomes come into contact, a phenomenon that varies widely among host organisms (Burt and Koufopanou 2004). Shared niche or physical contact between the intron donor and the intron recipient has been invoked to play an important role in the transfer of HEG-containing introns between distantly related species. For example, vectoring agents such as viruses, bacteria, and aphids and organisms involved in parasitism or symbiosis are expected to promote horizontal transfer of genetic materials (Vaughn et al. 1995; Bhattacharya et al. 1996; Cho et al. 1998; Nishida et al. 1998; Holst-Jensen et al. 1999; Sandegren and Sjöberg 2004).

One of the most probable explanations for gene transfer between closely related species of fungi resides in interspecific hybridization by mean of sexual or parasexual processes (reviewed by Schardl and Craven 2003). Interspecific hybridization has been shown to be a common mechanism in the evolution of some fungal lineages (Craven et al. 2001; Moon et al. 2002; Gentile et al. 2005) and could be an important evolutionary

process in most fungal phyla (reviewed by Schardl and Craven 2003). During sexual hybridization, the gametangia of two fungal species with complementary mating types fuse to form a dikaryotic cell. The dikaryotic state, where the nuclei of both species are in physical contact, is favorable for quick integration of the intron-HEG element into an intron-less chromosome, either by the action of the HE on empty sites or by recombination. If meiotic spores are produced from the dikaryotic state, the intron-HEG element quickly spreads within a population because 70%–99% of the progeny inherits that HEG, rather than the expected Mendelian inheritance rate of 50% (Burt and Koufopanou 2004). During parasexual cycles, vegetative hyphae of two fungal species (with or without complementary mating types) fuse and allow the migration of nuclei (heterokaryosis) and cytoplasm to various degrees between both hyphae (Burnett 2003; Glass et al. 2004), thus also potentially favoring the insertion of the HEG-intron element in the intron-less genome.

Hybridization is largely influenced by time since divergence (reviewed by Mallet 2005). Two species that have accumulated numerous genetic differences are less likely to hybridize, as various mechanisms will prohibit the fusion between the sexual or vegetative cells. However, if breeding barriers are strong enough to drive speciation but are not yet insurmountable, hybridization can take place between these newly divergent species. Even if only a few genes are passed on through introgression, this would be enough for the HEG to transfer into an intron-less chromosome.

Finally, the spread of intron-HEG elements between different host organisms can be maximized when the HE recognition site lies within conserved genes, as those involved in replication and transcription (Edgell et al. 2000; Burt and Koufopanou 2004). For instance, the gene coding for the nuclear ribosomal small subunit, where *Pleopsidium* intron-HEG element was found, is virtually conserved across kingdoms.

Conclusions and Future Studies

The present study on the evolution of the group I intron-encoded HEG found within the genus *Pleopsidium* is the first study done on a worldwide scale. The results are clearly in favor of a cyclical model of evolution (Goddard and Burt 1999) involving both acquisition of the intron-HEG element through horizontal transfer (from an unknown donor and intrageneric) and its vertical inheritance within a population followed by degeneration and possible loss. However, reinvasion has not been observed in our data set. It is also clear that geographical proximity plays an important part in intron-HEG element mobility in *Pleopsidium*, because most of the HEGs were concentrated in European specimens.

Some issues encountered during the present study remain to be clarified to extend our understanding of intron-HEG evolution within *Pleopsidium* and lichens in general. One of them concerns the heterogeneity of S943 intron presence/absence within a single DNA isolate of some *Pleopsidium* specimens (Table 1 and Screening for the Presence/Absence of S943 Group I Introns and Intron-Encoded HEGs, under Results and Discussion). It is not yet clear if these findings are due to heterogeneity among rDNA repeats (Simon et al. 2005) or to the presence of several individuals within a lichen thallus (Jahns 1972; Schuster et al. 1985; Murtagh et al. 2000). This issue will be addressed in a later study by initiating axenic cultures issued from single spore isolates. Cross hybridization between species lacking and species encoding the intron-HEG element will also be essential to understand the mechanisms involved in the horizontal transfer of these elements in *Pleopsidium*. Finally, determination of endonuclease activity (i.e., ability to support homing) for the *Pleopsidium* HEGs could provide insights into the present geographic distribution of these two types of HEG.

Acknowledgments. We thank Tassilo Feuerer, Ester Gaya, Josef Hafellner, Christine Keller, Jolanta Miadlikowska, Claude Roux, Christophe Scheidegger, Peter Scholz, and Mohammad Sohrabi, as well as the curators of the Herbaria of B, BG, C, CANL, DUKE, GZU, M, O, and TSB, for providing dry material used in this study. We are grateful to Dawn Simon for her help in the laboratory, Claude Roux for identification of specimens, Frank Kauff and Cymon Cox for their help in computer work, Molly McMullen for editing, Mike Skakuj for advice in producing the distribution map, and members of the Lutzoni laboratory as well as two anonymous reviewers for comments on the manuscript. We gratefully acknowledge support from two grants from the National Science Foundation, USA (Doctoral Dissertation Improvement Grant DEB-0105194 to F.L. and V.R. and Grant MCB 01-10252 to D.B.), and a Biology Grant-In-Aid from Duke University, three A. W. Mellon Training Grants from Duke University, two Grants-in-Aid of Research from Sigma-Xi, and a Graduate Fellowship from the Mycological Society of America (all to V.R.).

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