Evolution and host specificity in the ectomycorrhizal genus *Leccinum*

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Summary

- Species of the ectomycorrhizal genus *Leccinum* are generally considered to be host specialists. We determined the phylogenetic relationships between species of *Leccinum* from Europe and North America based on second internal transcribed spacer (ITS2) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).
- We plotted host associations onto the phylogenies using maximum likelihood and parsimony approaches.
- Resolution of the phylogeny was greater with *Gapdh* vs ITS2, plus the *Gapdh* and ITS phylogenies were highly incongruent. In *Leccinum* the coding region of *Gapdh* evolved clocklike, allowing the application of a molecular clock for the reconstruction of host specificity. Almost all species of *Leccinum* are highly host tree specific, except *Leccinum aurantiacum*, which associates with a broad range of host trees. Maximum likelihood reconstructions of the ancestral host associations show that this taxon evolved from a specialist.
- Our results indicate episodes of rapid speciation coinciding with or immediately following host switches. We propose a model where host niche contraction through geographic isolation and host niche expansion through ecologically equivalent hosts drive cycles of speciation. The role of host race formation and incipient speciation is discussed.

Key words: *Leccinum*, host specificity, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), internal transcribed spacer (ITS), specialist, generalist, speciation.

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Introduction

Mycorrhizal fungi and, to a lesser extent, mycorrhizal plants, display different degrees of host specificity. What the evolutionary advantage of specialization is for either of the symbiotic partners is still unclear. Bruns *et al.* (2002) hypothesized the advantage for the fungus of specializing on a (phylogenetically) narrow range of hosts is found in a greater physiological compatibility of the fungus to its host. This increased physiological compatibility would then enable a specialist fungus to obtain more carbohydrates from the plant host than generalist competitors do. The advantage for the plant host to associate with specialist mycorrhizal fungi is less clear, because the costs of associating with a specialist are greater than that of associating with a generalist. From the plant's perspective, specialization may lead to decreased functional compatibility. Finlay (1989) reported that *Suillus grevillei* and *Suillus cavipes* – two associates with larch – were able to form ectomycorrhizas with pine, but hardly any nutrients were transferred to the plant host. Alternatively, associations with specialized fungi would reduce the chances of indirectly helping competing plant species (Molina *et al.*, 1992), as generalist fungi can connect individuals of hosts from the same or different species and are able to translocate carbon between hosts (Simard *et al.*, 1997). However, the significance of carbon translocation by generalist fungi is unclear. Robinson & Fitter (1999) suggested that in the case of AM the carbon stays in the generalist fungus, instead of being translocated from host to host, while in the case of ectomycorrhiza (EM) the evidence is still equivocal.

While the processes that select for or against specialization in EM symbiosis are still unknown, the fact that EM fungi display different levels of specialization is well known. Some species

Table 1 Host specificity and general distribution on the northern hemisphere of the main clades found in phylogenetic research of Binder & Besl (2000) and Den Bakker *et al*. (2004)

Clade	Subclades	Host	Distribution
Luteoscabrum		Mainly Fagaceae and Betulaceae (subfamily Coryloideae)	Temperate and subtropical regions
Leccinum	*Leccinum*	1. Salicaceae (*Populus*), Betulaceae (*Betula*) rarely Fagaceae	Temperate and sub-boreal regions
		2. Ericaceae (subfamily Arbutoideae)	California and Costa Rica
	Scabra	Betulaceae (*Betula*)	Mainly subboreal regions

of EM fungi are associated with a phylogenetically broad range of hosts (generalists), such as *Amanita muscaria* (Trappe, 1962), while others are specialized to a phylogenetically narrow range of hosts, for example species of the genera *Rhizopogon* or *Suillus*, which are almost exclusively associated with Pinaceae (Massicotte *et al*., 1994; Molina & Trappe, 1994; Kretzer *et al*., 1996). The evolutionary history of specificity of EM fungi towards their plant host has received little attention, despite host specificity being, without doubt, a key element in understanding the present day distribution and diversity of EM fungi.

Leccinum S.F. Gray (Boletaceae, Boletales) is a genus of ectomycorrhizal fungi associated with a wide range of hosts (Table 1). The genus occurs mainly in the temperate and boreal regions of the northern hemisphere with some secondary expansion to the neotropics (Halling & Mueller, 2003). Reports of species of *Leccinum* occurring in Africa (Heinemann, 1964) probably refer to species that are better classified in the genus *Tylopilus*. Their relatively large size and distinctive appearance make their gross distribution well known. Most species of this genus are considered specialists (Singer, 1986). Consequently, host association is used as a distinctive character in keys. In addition to the fact that it is not always easy to determine which host plant the fruitbody is associated with (especially if more than one possible candidate host is present), the possibility that species are generalists and associated with more than one host species seems to be ruled out. (In this paper we will deal with the European species *L. aurantiacum* a generalist according to Den Bakker and Noordeloos (unpubl. data); Table 2.) Other authors consider this species to consist out of two specialists, *Leccinum quercinum*, when associated with Fagaceae and *Leccinum populinum*, when associated with *Populus* (Korhonen, 1995). A molecular phylogeny allows an evaluation of the status of these species and an investigation of the evolutionary history of host specificity from the fungal perspective.

Previous phylogenetic studies (Binder & Besl, 2000; Den Bakker *et al*., 2004) with conventional nuclear ribosomal markers (28S, ITS) have left the relationships within the sections *Scabra*/*Leccinum* clade relatively unresolved. In this study, sequences of the second internal transcribed spacer (ITS2) will be used in combination with about 1200 bp of the single copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, EC 1.2.1.12), in the hope of obtaining better resolution. The latter gene has proven to be phylogenetically informative for various groups of Ascomycetes (Berbee *et al*., 1999; Yun *et al*., 1999; Câmara *et al*., 2002), especially for studies at the species level. The gene has so far not been used for phylogenetic studies in Basidiomycetes.

In this paper a molecular phylogenetic study of a representative sample of species of sections *Leccinum* and *Scabra* is provided. These phylogenies will be used to assess the level of host specificity of the individual lineages/species found and to reconstruct the evolutionary history of host specificity.

Materials and Methods

Taxon sampling

Data of all collections used in this study are summarized in Table 3. A priori designation (through identification by the first author) of the collections to morphospecies and nomenclature are according to Den Bakker & Noordeloos (unpubl. data) for the European material and according to Smith and Thiers (1971), Thiers (1975) and Halling and Mueller (2003) for the American material. In some cases fresh material was placed in cetyltrimethylammonium bromide (CTAB) (100 mM

Table 2 Taxonomic changes of European taxa according to Den Bakker and Noordeloos (unpubl.)

New names	Synonyms	Host range
Leccinum leucopodium	*Leccinum aurantiacum sensu* Pilat	*Populus*
Leccinum aurantiacum	*Leccinum quercinum*	*Populus*, *Betula*, *Quercus* (*Fagus*, *Castanea* and *Tillia* reported*)
	Leccinum populinum	
Leccinum versipelle	*Leccinum cerinum*	*Betula*, occasionally *Arctostaphylos*?
	Leccinum callitrichum	
	Leccinum roseotinctum	

*Lannoy & Estades (1995), Korhonen (1995).

Table 3 Samples used in analyses, including voucher number, geographic origin, host and GenBank accession numbers. Numbers behind the geographical origin of some of the accessions refer to numbers used in Figs 2 and 3

Species designation	Voucher collection	Geographical origin	Host	GenBank accession number	
				ITS2	*Gapdh*
Outgroups					
Leccinum crocipodium	rw1659	Sommauthe/Beaumont-en-Argonne, Ardennes, France	*Carpinus*	AF454589	AY538783
Leccinum carpini	hdb065	Breukelen, Utrecht, The Netherlands	*Carpinus/Corylus*	AF454588	AY538785
Leccinum talamancae	halling8001	San Gerardo, Dota, San José, Costa Rica	*Quercus*	AY544779	AY538783
Fumosa clade					
Leccinum duriusculum	wtoo1	Wassenaar, Zuid Holland, The Netherlands	*Populus*	AF454576	AY538787
Leccinum nigellum	4676P	Vibraye, France	*Populus*		AY538815
Leccinum uliginosum	hdb330	Whitefish Falls, Ontario, Canada	*Populus*	AY538825	AY538786
Subsection *Leccinum*					
L. aurantiacum sensu lato					
L. aurantiacum	van Brummelen	Foret de Belleme, Orne, France (2)	*Betula*	AY538853	
L. aurantiacum	van Brummelen	Ige, Orne, France (3)	*Populus*	AY538857	AY538823
L. aurantiacum	hdb003	AWD, Noord Holland, The Netherlands, (3)	*Populus*	AY538856	
L. aurantiacum	Hills2001219	Windsor Great Park, Berkshire, England	*Betula*	AY538854	AY538819
L. leucopodium	rw1656	Sommauthe/Beaumont-en-Argonne, Ardennes, France	*?*	AF454469	AY538795
L. leucopodium	hdb93	Sogndal, Sogn og Fjordane, Norway	*Populus*		AY538817
L. sp. 1	halling6580	Twobridge Swamp, Franklin County, NY, USA	*Populus*	AY538836	
L. sp. 2	tdb304	USA	*?*	AY538835	
L. sp. 3	arora 00–53	Along Dempster Highway, Yukon Territory, Canada	*Populus*	AY538841	AY538824
L. sp. 4	hdb317	Manitoulin Island, Ontario, Canada	*Populus*		AY538821
L. brunneum	hdt49122	Cascade, Valley County, ID, USA	*Populus*	AY538850	
L. insigne	hdb320	Manitoulin Island, Ontario, Canada	*Populus*	AY538851	AY538822
L. insigne	hdt50455	Vicinity North Adams, MA, USA	*Betula*	AY538842	
L. aurantiacum	mk11850	Vantaa, Nylandia, Finland	*Populus*	AY538861	AY538797
L. aurantiacum	hdb94	Sogndal, Sogn og Fjordane, Norway	*Populus*	AY538860	AY538817
L. aurantiacum	hdb286	Leusden, Gelderland, The Netherlands (2)	*Quercus*	AY538855	
L. aurantiacum	van Brummelen	Foret de Cessey, Doubs, France (1)	*Quercus*	AY538852	AY538796
L. aurantiacum	rw1683	Oignies-enThiérarche, Belgium	*Quercus*	AY538859	
L. aurantiacum	hdb102	Roden, Drenthe, The Netherlands (1)	*Quercus*	AY538858	AY538816
L. *versipelle sensu* lato					
L. atrostipitatum 1	halling3131	Togue Pond Road, Piscataquis County, ME, USA	*Betula/Populus*	AY538834	
L. atrostipitatum 2	halling3081	Baxter State Park, Piscataquis County, ME, USA	*Betula*	AY538833	
L. atrostipitatum	27-8-84/3	Nouveau Quebec, Quebec, Canada	*Betula*	AY538832	AY538802
L. versipelle	2270P	Aumont-Aubrac, Lozère, France	*Betula*	AY538829	AY538818
L. versipelle	hdb070	Kall, Jämtland, Sweden (1)	*Betula*	AY538827	AY538801
L. versipelle	hdb285	Leusderheide, Gelderland, The Netherlands	*Betula*	AY538831	AY538799
L. versipelle	OF64036	Lærdal, Sogn og Fjordane, Norway	*Arctostaphylos*	AF454574	AY538798
L. versipelle	men95702	Utsjoki, Inarilapland, Finland (1)	*Betula*	AY538828	AY538800
L. versipelle	mk11452	Kilpisjarvi, Enontekio Lappi, Finland (2)	*Betula*	AY538826	AY538802
L. versipelle	hdb74	Kall, Jämtland, Sweden (2)	*Betula*	AF454575	

Table 3 *continued*

Species designation	Voucher collection	Geographical origin	Host	GenBank accession number	
				ITS2	*Gapdh*
L. versipelle	hdb57	Borgsjö, Jämtland, Sweden (3)	*Betula*	AY538830	
Pinaceae associates					
L. piceinum	MEN2048	Obertiliach, Lienz, Austria	*Picea*	AF454579	AY538794
L. vulpinum	hdb92	Sogndal, Sogn og Fjordane, Norway	*Pinus*	AF454580	AY538792
Ericaceae associates					
L. arbuticola	arora 00–293	Boonville, Mendocino County, CA, USA	*Arbutus*	AY538837	AY538789
L. manzanitae	LG464	Santa Cruz Island, CA, USA,	*Arctostaphylos*	AY538838	AY538789
L. manzanitae	Ecv2404	California, USA	*Arctostaphylos*		AY538790
L. monticola	halling8288	Cerro de la Muerte, Dota, San José, Costa Rica	*Comarostaphylos*	AY538839	AY538788
L. monticola	halling8325	Costa Rica	*Comarostaphylos*	AY538840	AY538820
Section *Scabra*					
Leccinum scabrum	hdb048	Hoogeveen, Drenthe, The Netherlands	*Betula*	AF454585	AY538813
L. scabrum	hdb301	Midhurst, Ontario, Canada	*Betula*	AY538849	AY538814
Leccinum holopus	hdb329	Manitoulin Island, Ontario, Canada	*Betula*	AY538844	AY538808
Leccinum holopus	hdb40	Nieuwkoop, Zuid Holland, The Netherlands	*Betula*	AF454561	AY538807
Leccinum brunneogriseolum	hdb39	Schiermonnikoog, Friesland, The Netherlands	*Betula*	AF454560	AY538806
Leccinum cf *snellii*	halling6914	Indian Creek, Swain County, NC, USA	*Betula*	AY538845	AY538811
Leccinum cf *snellii*	halling4472	Raquette Lake, Hamilton County, NY, USA	*Betula*	AY538846	AY538812
Leccinum schistophilum	MK11145	Vantaa, Nylandia, Finland	*Betula*	AY538847	AY538809
Leccinum schistophilum	hdb121	Orne, Foret Dominial du Perche, France	*Betula*	AY538848	AY538810
Leccinum variicolor	hdb051	Erica, Drenthe, The Netherlands	*Betula*	AF454572	AY538804
Leccinum snellii	hdb327	Manitoulin Island, Ontario, Canada	Betula	AY538843	AY538805

Table 4 Primer sequences used for GPD, and corresponding primer position in Fig. 1

Primer name	5′–3′	Fig. 1
GPD forward general	cgg ccg tat cgt cct ccg taa tgc	1
GPD reverse general	gag ta(at) cc(gc) cat tcg tta tcg tac c	2
Primer internal forward GPD *Leccinum*	cga agg tct cat gag cac tat cca	5
Primer internal reverse GPD *Leccinum*	tgg ata gtg ctc atg aga cct tcg	6
GPD0623F*	ttg cca agg tcg tca acg	3
GPD1035R*	gtg taa gca acg ata ccc ttc ag	4

Data from R. Kjøller (pers comm.)

Tris-Cl, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 2% CTAB, pH 8.0) in the field for further processing, otherwise dried herbarium material was used. We have sampled all known host associations within sections *Leccinum* and *Scabra*. Care was also taken to obtain (if possible) both American and European representatives of a known host association. Voucher specimens are deposited in L, GENT, H, O, P, NY and SFSU (herbarium abbreviations according to Holmgren *et al*., 1990).

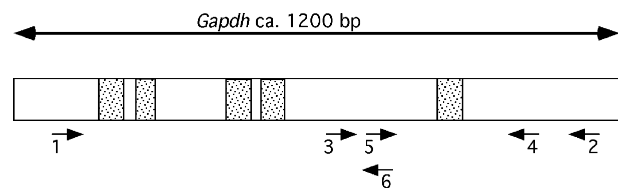

Fig. 1 Primer positions of *Gapdh*. Numbers refer to the primer sequences given in Table 4. Dotted areas indicate the position of introns.

Host designation

In most cases the labels that accompanied the herbarium material noted the host(s). In two cases no host tree species information was provided. In one case two potential hosts were indicated. In one case the host could not be designated unambiguously in the field. Here, ectomycorrhizal root tips were collected under the fruit body. The host was then identified by means of DNA sequencing. The DNA was extracted from the root tips of the presumed host with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the protocol supplied by the manufacturer. The EM was identified using *Gapdh* primers (see below) and compared with the above-ground fruit body. The identity of the root tips was determined using the plastid trnL–trnF sequence. Amplification of this region used the primers TabE and TabF (Taberlet *et al*., 1991). For this PCR we used the same conditions as used for the amplification of *Gapdh* (see below). We did a BLAST (GenBank) search on the plastid sequence to compare it with known sequences.

DNA extraction, polymerase chain reaction (PCR) and sequencing of fungal material

The DNA of a small number of accessions was extracted by means of a modified CTAB procedure, as described by Den Bakker *et al.* (2004). DNA of all other accessions was obtained from either CTAB-preserved or herbarium material using the DNeasy Plant Mini Kit (Qiagen) following the protocol supplied by the manufacturer.

Internal transcribed spacer 2 was amplified using the primers ITS3 and ITS4 (White *et al*., 1990). The PCR reactions for amplification of ITS2 followed Den Bakker *et al.* (2004). Initially, a small section of *Gapdh* (*c.* 400 bp) was amplified by using the primers GPD0623F (all the primer sequences used for amplification of *Gapdh* are listed in Table 4, relative positions in Fig. 1) and GPD1035R (designed by Rasmus Kjøller, University of Copenhagen, Denmark). Because amplification failed for a number of species we designed an alternative forward primer, GPDlecF. With this primer, its reverse complement GPDlecR and two general primers GPDforward and GPDreverse (designed by slight modification of the primers published by Kreuzinger *et al*., 1996) we managed to amplify *c.* 1100 bp of the *Gapdh* gene in two pieces: a *c.* 600 bp piece (using primers GPDforward and GPDlecR) and a 500 bp piece (using primers GPDlecF and GPDreverse).

To amplify the desired regions we used 2 µl of genomic DNA in a 25-µl reaction mixture. The mixture contained 1× PCR buffer (Qiagen), 2.5 nmol dNTPs, 4 pmol of both the forward and reverse primer, 2 ng bovine serum albumin (BSA), 3.75 mM $MgCl_2$, and 1 unit *Taq* polymerase. Cycling parameters were: initial denaturing at 95°C for 2 min followed by 34 cycles of 30 s at 95°C, 30 s at 54°C and 30 s at 72°C, with a final extension of 2 min at 72°C. The PCR products were electrophoresed in a 1.25% agarose gel in 1× Tris-borate-ethylenediaminetetraacetic acid (TBE) (pH 8.3) buffer, stained with ethidium bromide to confirm a single product and cleaned following the Qiaquick PCR Cleanup protocol (Qiagen). In cases where multiple bands were encountered, PCR products of the right length were extracted from the agarose gel following the QIAquick Gel Extraction Kit (Qiagen).

The purified PCR products were directly sequenced using the amplification primers. Samples were sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) using standard dye-terminator chemistry following the manufacturer's protocols.

Phylogenetic analyses

The *Gapdh* sequences were aligned with CLUSTAL X (Thompson *et al.*, 1997) and refined by eye. The ITS2 sequences were aligned with the online version of Partial Order Alignment (Lee *et al.*, 2002, http://www.bioinformatics.ucla.edu/poa/POA_Online/Align.html) and subsequently refined by eye. Large sections of the ITS2 sequences of *Leccinum talamancae*, *Leccinum crocipodium* and *Leccinum carpini* could not be aligned with confidence to the ingroup taxa and were left out of the alignment.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted using PAUP*4.0b10 (Swofford, 2002). In all analyses gaps were treated as missing data. MP and ML phylogenies were obtained using the heuristic search option, 10 random sequence additions and tree bisection and reconnection (TBR) branch swapping. Maxtrees was set to 20 000 trees. In the MP analyses characters were treated as unordered and unweighted. For the ML analyses the program MODELTEST version 3.06 (Posada & Crandall, 1998) was used to find the model of sequence evolution least rejected given the data set. The model and its parameters were chosen based on the outcomes of a hierarchical likelihood ratio test ($\alpha = 0.01$) as implemented in the software. Initially, *Boletus edulis s.l.*, *Boletus subglabripes* and *Tylopilus chromapes* (considered by some authors as *Leccinum chromapes*) were used as outgroups. The use of these outgroups significantly lowered the resolution of the topology of the ingroup and ITS2 sequences and intron regions of *Gapdh* were hard to align without ambiguity. Analyses of *Gapdh* with these outgroups, however, showed the Costa Rican endemic *L. talamancae* to have a well-supported sister group relationship with the other accessions of *Leccinum. Leccinum talamancae* was therefore used as the outgroup for the MP, ML and Bayesian analyses presented in this paper.

Bayesian and bootstrap analyses

Bayesian analyses were performed using MRBAYES v3.0b4 (Huelsenbeck & Ronquist, 2001) In order to perform a Bayesian analysis of the *Gapdh* data set the data were divided into eight partitions: The coding region was divided in three partitions representing the different coding positions. The noncoding region consisted of five introns, each treated as a separate partition. The program MRMODELTEST (J.J.A. Nylander, available from the internet: http://www.ebc.uu.se/systzoo/staff/nylander.html) was used to select (based on the implemented hierarchical likelihood ratio test ($\alpha = 0.01$)) the least rejected model of sequence evolution for each individual partition. Likelihood and prior settings were changed in MRBAYES to meet with the settings necessary to apply the models found for each partition. The analysis was initiated with a random starting tree and was run for 5×10^6 generations, keeping one tree every 1000 generations. The first 10^6 generations (burn-in) were discarded and the remaining 4000 trees (representing 4×10^6 generations) were used to calculate a 50% majority rule tree and to determine the posterior probabilities for the individual branches. The ITS2 data set was not partitioned. MRMODELTEST was used to find the least rejected model of sequence evolution and likelihood and prior settings were changed according to the model found. The ITS2 analysis was conducted under the same settings as the *Gapdh* set. In order to check whether both analyses converged to the same optimum, we repeated the analyses several times with 1×10^6 generations.

Nonparametric bootstrapping (Felsenstein, 1985) was performed to determine the levels of support for the internal nodes. We performed 1000 bootstrap replicates. The MP parameters were the same as in the heuristic search except the branch swapping option was set to search for 10 s for each replicate and the sequence addition procedure was set to simple.

Molecular clock analysis

To test if the *Gapdh* sequences in *Leccinum* evolve clockwise, we used a likelihood ratio test to test for rate constant evolution (Huelsenbeck & Rannala, 1997). This likelihood ratio test determines whether there are significant differences between the likelihood scores of trees where the branch lengths are unconstrained compared with a tree with the same topology where the branch lengths are constrained so that the terminal ends are contemporaneous. BEAST version 1.0.3 (Drummond & Rambaut, 2003) was used to calculate the posterior probabilities of the clades found when a molecular clock could be assumed.

Compatibility tests and topology tests

The compatibility of the different datasets was tested a priori with the partition homogeneity test (Farris *et al.*, 1995) as implemented in PAUP*. A total of 10 000 replicates were performed and maxtrees was set to 100. In order to test if the topologies of the different analysis and the different datasets were significantly different we used the likelihood based Shimodaira–Hasegawa (SH) test as implemented in PAUP*, using the RELL option and 10 000 bootstrap replicates to calculate the test distribution. This test is more robust to violations of the model of sequence evolution than other likelihood-based topology tests (Buckley, 2002).

Reconstruction of the evolution of host associations

To trace the history of host associations we used a likelihood reconstruction method (the STOCHCHAR package, Maddison & Maddison, 2003b) as implemented in MESQUITE version 0.966 (Maddison& Maddison, 2003a). The one-parameter Markov k-state model (Lewis, 2001) was chosen to estimate the ancestral states, using the default settings. Differences in likelihood of two possible ancestral states were considered

significant when they exceeded a cut-off point of two log units (Pagel, 1999). The different host associations were coded as one multistate character. A well-resolved and well-supported topology was chosen to trace the history of host associations. Additional to the likelihood reconstruction a parsimony-based reconstruction was performed as implemented in MACCLADE 4.0.5 (Maddison & Maddison, 2002).

Results

Host designation by molecular methods

One collection *Leccinum* sp. 4 from Ontario, Canada was found near a *Pinus banksiana* tree. A BLAST search of the trnL-F sequence from root tips collected under the fruit body and colonized by the mycorrhiza of that species, showed a close match to Balanophoraceae, a family that belongs to the Malphigiales. The genus *Populus* (Salicaceae) also belongs to this order. *Populus* trees were present in the area and we concluded these must have been the host trees and not a pine.

Gapdh phylogeny

For 26 accessions both the first *c.* 600 bp and the second *c.* 500 bp of the *Gapdh* gene were sequenced. For one accession only the first 600 bp was sequenced, for 14 other accessions only the second 500 bp. The position of the five introns was congruent with that of *B. edulis*, as shown by Kreuzinger *et al.* (1996). The data set comprised 41 accessions, 1160 characters and 213 potentially phylogenetically informative characters.

Using MODELTEST, the general time-reversible model was chosen for the ML analysis, with variable sites assumed to follow a gamma distribution (shape set to 0.5222), nucleotide frequencies set to A 0.2417, C 0.2661, G 0.2260, T 0.2662 and substitution rates set to 1 (AC), 2.7316 (AG), 1 (AT), 1 (CG), and 4.5152 (CT). The models used for the individual partitions in the Bayesian analysis can be found in Table 5. Trees obtained by MP (> 20 000 MP trees, 500 steps, CI = 0.796, RI = 0.895), maximum likelihood (three trees, –Ln L 4452.98) and Bayesian analyses of the *Gapdh* data did not differ significantly from each other. The Bayesian inference topology is depicted in Fig. 2 and shows that *Leccinum* can be subdivided into four very well supported groups. (1) *L. carpini* and *L. crocipodium* (clade H) show a well-supported sister group relation with the rest of *Leccinum* examined. The remaining accessions form three well to moderately supported clades: (2) a clade (the *Scabra* clade) formed by species that are all associated with *Betula*; (3) a clade comprising *L. duriusculum*, *L. nigellum* and *L. uliginosum* (the *Fumosa* clade), accessions that are all associated with *Populus*; and (4) a clade which will be referred to as the *Leccinum* clade and is composed of species which are associated with *Populus*, *Betula*, Arbutoideae, Pinaceae and Fagaceae. The relation between the *Leccinum*, *Scabra* and *Fumosa* clades remains unresolved.

The *Leccinum* clade is very strongly supported (100% Bootstrap Support (BS), 100% Posterior Probability (PP)). Within this clade we can recognize five well to highly supported clades: (i) clade E formed by the two collections of *L. monticola* (associated with *Comarostaphylis*, Arbutoideae); (ii) clade D formed by *L. vulpinum* and *L. piceinum* (associated with Pinaceae); (iii) clade C formed by *L. manzanitae* and *L. arbuticola* (associated with Arbutoideae); (iv) clade B containing the North American *L. atrostipitatum* and the European *L. versipelle* accessions (except for one accession, all associated with *Betula*); and (v) a clade A comprising the European *L. aurantiacum*, *L. insigne*, *L. leucopodium* and some North American samples morphologically similar to *L. aurantiacum* (associated with a diversity of hosts). None of the relationships between these five clades receive any significant support. Clade A is composed of two well-supported subclades, one comprising *L. aurantiacum* (with a diversity of broad leaved hosts) and a moderately supported clade with four accessions under *Populus* and one accession of which the host plant associate was not recorded.

Molecular clock *Gapdh*

It has been shown for *Drosophila* that the protein coding sequences of *Gapdh* evolves clocklike at the nucleotide level (Ayala *et al.*, 1996). To calculate if *Gapdh* in *Leccinum* also evolved clocklike we used a data set containing 25 taxa. A pairwise relative-rate test as implemented in the HYPHY package 0.95beta (S.L. Kosakovsky Pond and S.V. Muse available from the authors at *http://www.hyphy.org*) showed that the

Table 5 Models of sequence evolution used for the individual partitions in the Bayesian analysis of *Gapdh* sequence data

Codon/intron	Model
Codon 1	Felsenstein 81 model (Felsenstein, 1981), variable sites assumed to follow a gamma distribution.
Codon 2	Felsenstein 81 model (Felsenstein, 1981), variable sites assumed to follow a gamma distribution.
Codon 3	General time reversible model (Rodríguez *et al.*, 1990), variable sites assumed to follow a gamma distribution.
Intron 1	Kimura 2-parameter model (Kimura, 1980).
Intron 2	Symetrical model (Zarkikh, 1994).
Intron 3	Kimura 2-parameter model (Kimura, 1980).
Intron 4	Hasegawa–Kishino–Yano model (Hasegawa *et al.*, 1985).
Intron 5	Kimura 2-parameter model (Kimura, 1980), variable sites assumed to follow a gamma distribution.

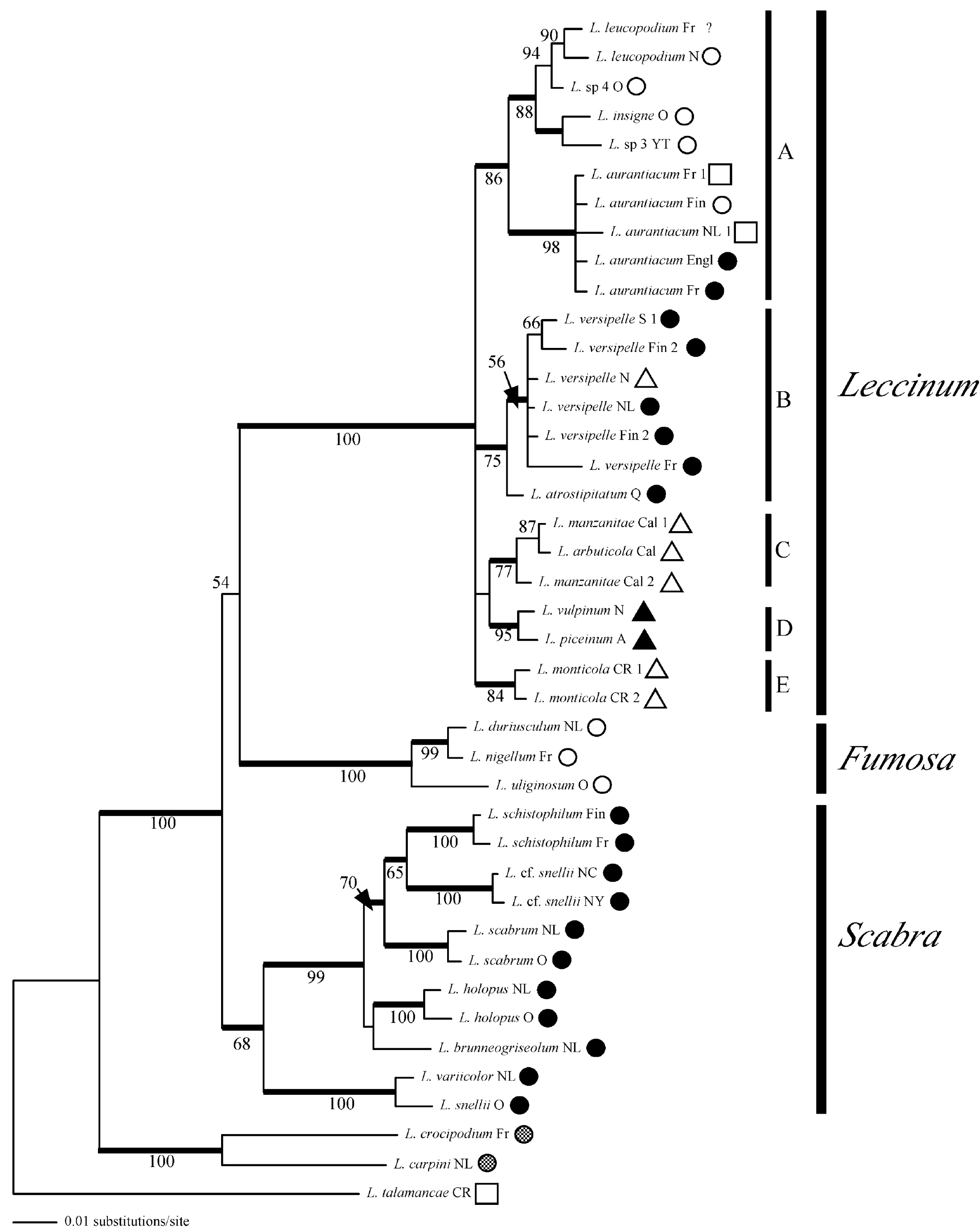

Fig. 2 Tree based on the outcome of a Bayesian analysis of the *Gapdh* data. Thickened branches receive posterior probabilities of 95% or more. The values below the branches are bootstrap support values based on maximum parsimony analysis. Bootstrap support values < 50% are not indicated. Squares, Fagaceae; closed circles, *Betula*; open circles, *Populus*; tinted circles, *Corylus/Carpinus*; open triangles, Ericaceae; closed triangles, Pinaceae.

mutation rate of *L. duriusculum* significantly differed from most other taxa and therefore this taxon was removed form the clock analyses. When only the protein coding sequences were used, the hypothesis of a constant rate could not be rejected (–Ln constrained 2779.9693, –Ln unconstrained 2763.794, 2Δ = 32.35, df = 22, P = 0.07). When *L. talamancae*, *L. crocipodium* and *L. carpini* were excluded, a molecular clock could be assumed for the complete *Gapdh* sequences (–Ln constrained 3373.395, –Ln unconstrained 3359.998, 2Δ = 26.794, df = 19, P = 0.11). The topology of the tree based on the complete *Gapdh* gene sequences differed from the trees based only on the coding part of *Gapdh* by the fact that all the Arbutoideae-associated species are placed together with the Pinaceae associated species. The trees resulting from the ML analysis

of the complete *Gapdh* sequences contradict the monophyly of this group, the Californian Arbutoideae-associated *L. manzanitae* is placed basal to all other species in the *Leccinum* clade, while the European Pinaceae-associated species form a separate clade with the Costa Rican Arbutoideae-associated species. The Bayesian analysis shows there is no significant support for this separate placement of *L. manzanitae* and therefore the topology cannot be considered incongruent with the one inferred from the complete sequences.

ITS2 phylogeny

The data set comprised 50 accessions of 536 characters of which 60 characters were potentially phylogenetically informative. Six accessions (*L. versipelle* Norway, *L.* cf. *aurantiacum* Canada, *L. insigne* Massachusetts, *L. manzanitae* California and both accessions of *L. monticola* from Costa Rica) shared a 40 bp deletion.

The MP analysis yielded more than 20 000 most parsimonious trees (154 steps, CI = 0.805, RI = 0.918). The ML analysis yielded 8 trees (–Ln = 1638.17), one of these trees is shown in Fig. 3. The MP, ML and Bayesian inference topologies did not differ significantly, though the Bayesian analysis showed somewhat less resolution. *L. talamancae* (the outgroup), *L. crocipodium*, and *L. carpini* are sister to the remaining *Leccinum* samples (69% BS). The other accessions fall into three main clades: (1) a weakly supported clade formed by the *Populus*-associated *L. duriusculum* and *L. uliginosum* (the *Fumosa* clade); (2) a highly supported clade containing most accessions of the *Scabra* clade (except *L. variicolor* and *L. snellii*) and a part of the *Leccinum* clade as found in the *Gapdh* analysis. Within the *Scabra* clade resolution shows three well-supported clades: (i) uniting *L. holopus* and *L. brunneogriseolum*; (ii) formed by accessions of *L. schistophilum*; and (iii) uniting *L.* cf. *snellii* and *L. scabrum*. The third major clade contains *L. variicolor* and the larger part of accessions of the *Leccinum* clade. However, this clade receives bootstrap support and posterior probability lower than 50%.

Compatibility of ITS2 and *Gapdh*

The partition homogeneity test showed that the phylogenetic signal of the two data sets (*Gapdh* and ITS2) are highly incongruent ($P < 0.001$). The SH test showed that the topology of the trees obtained from the different data sets yielded significantly different ($P < 0.001$) likelihood scores when tested with either the *gapdh* dataset or the ITS2 dataset.

Reconstruction of the evolution of host associations

The ML trees with a molecular clock enforced of the *Gapdh* data were used to make a likelihood reconstruction of the ancestral character states (Figs 4 and 5). *Leccinum versipelle* was treated as an associate of *Betula*, although one accession was associated with *Arctostaphylos uva-ursi*. This is the only report of this species with this host and therefore we consider this an exception. Because one taxon (*L. aurantiacum*) was a generalist, we had to overcome the problem that MESQUITE cannot handle polymorphisms. Therefore, we compared reconstructions where the host association of *L. aurantiacum* was coded in different ways: (1) *Betula*; (2) *Populus*; (3) Fagaceae plus Coryloideae in the reconstruction based on the tree in Figs 4; (4) Fagaceae in the reconstruction based on the tree in Fig. 5. The reconstruction where *Populus* was coded as the mycorrhizal associate of *L. aurantiacum* received the highest likelihood score (see Table 5) in the reconstruction based on the coding sequences of *Gapdh* as well as in the reconstruction based on the complete *Gapdh* sequences. For the remainder of the discussion of the results we will mainly discuss the results of the reconstructions based on the complete *Gapdh* sequences, because this tree shows more resolution and most relationships are better supported. *Betula* received the highest likelihood score for being the mycorrhizal associate of the most recent common ancestor (MRCA) of taxa of the *Scabra*, *Leccinum*, and *Fumosa* clade, irrespective of the coding of the mycorrhizal association of *L. aurantiacum* and the tree used. The different coding of the mycorrhizal association of *L. aurantiacum* did affect the reconstructions of ancestral host associations of the two basal nodes (nodes 1 and 2 in Fig. 5) of the *Leccinum* clade and the host association of the MRCA of the species of node 3. When *L. aurantiacum* was coded to be a Fagaceae associate, Arbutoideae received the highest likelihood score for being the associate of the MRCA of the *Leccinum* clade (nodes 1 and 2), and *Populus* the associate of the MRCA of *L. aurantiacum* and *L. leucopodium*, *L. insigne* and *Leccinum* sp. 3 and 4 (node 3). Coding of the mycorrhizal association of *L. aurantiacum* as either *Betula* or *Populus* resulted in a likelihood score of the ancestral states of nodes one, two and three in favour of all being either *Betula* or *Populus*, respectively (Table 6).

Table 6 Differences in results of likelihood reconstructions of ancestral host associations when the association of *Leccinum aurantiacum* is coded as being either one of the observed associated host species. The different nodes refer to the nodes with the same number in Fig. 5

Host association *L. aurantiacum*	Estimated marginal probability (–log likelihood)	Nodes 1 and 2	Node 3
Fagaceae	19.37	Arbutoideae	*Populus*
Betula	16.79	*Betula*	*Betula**
Populus	15.85	*Populus*	*Populus**

*Significantly higher likelihood score for ancestral state of given host as compared to likelihood scores of other ancestral host states.

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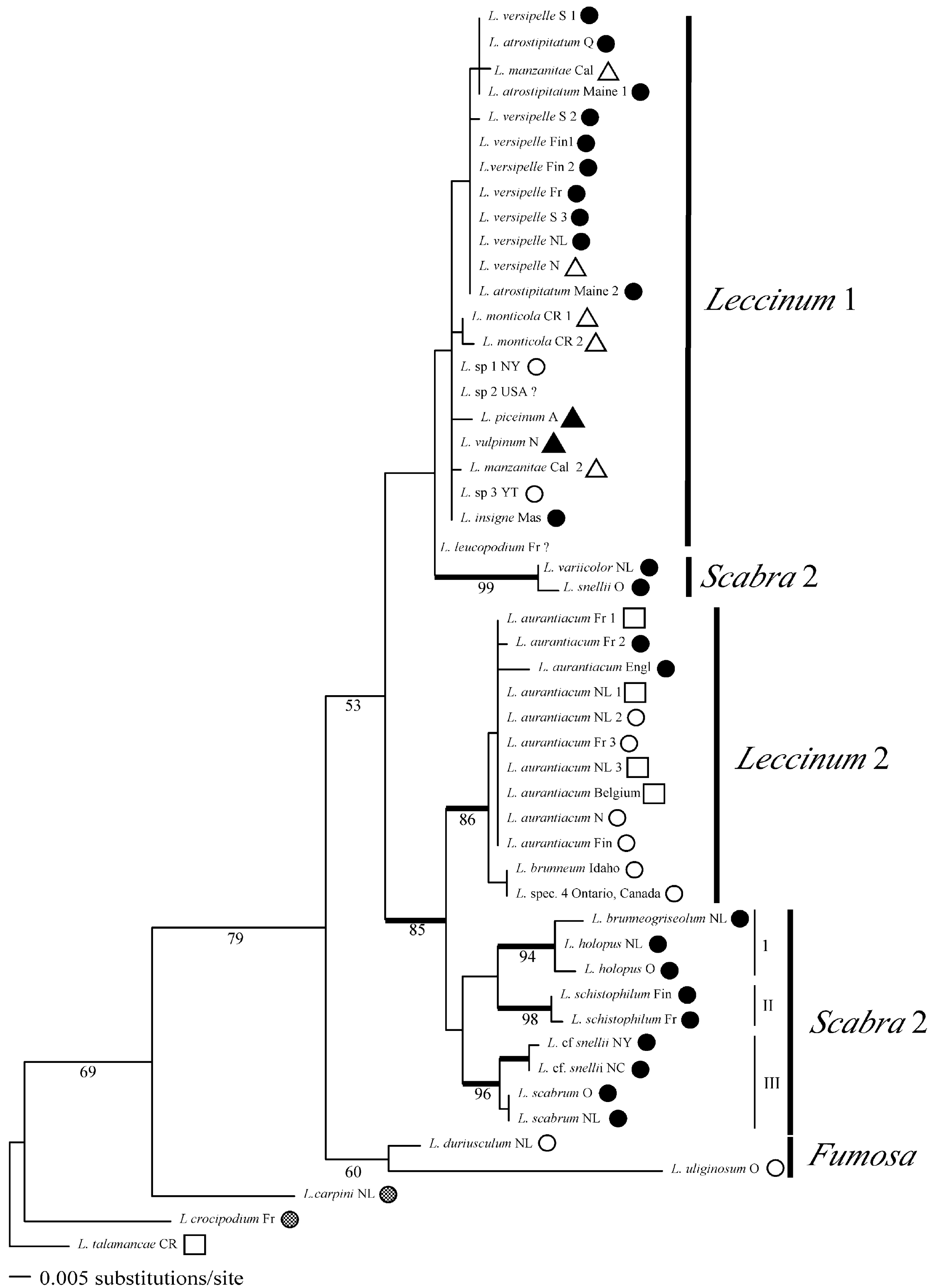

Fig. 3 One of eight maximum likelihood trees based on ITS2 sequences. Thickened branches receive posterior probabilities of 95% or more. Values below clades indicate maximum parsimony bootstrap values. Values < 50% are not indicated. Squares, Fagaceae; closed circles, *Betula*; open circles, *Populus*; tinted circles, *Corylus/Carpinus*; open triangles, Ericaceae; closed triangles, Pinaceae.

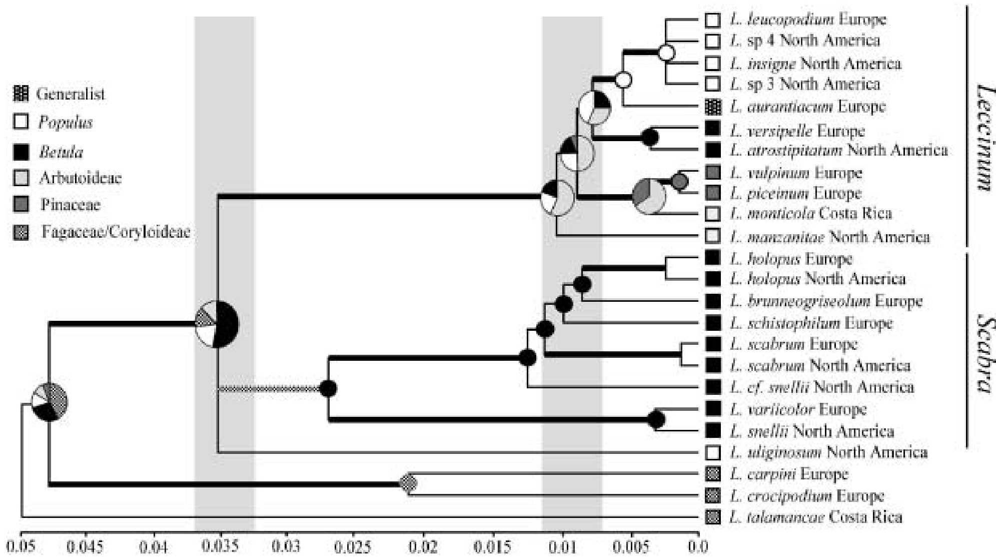

Fig. 4 Maximum likelihood tree with molecular clock enforced based on only the coding sequences of *Gapdh*. Thickened branches receive posterior probabilities of 95% or more in Bayesian analysis. Hatched branches receive posterior probabilities of between 90% and 95%. The axis below the tree gives the estimated number of substitutions per site. The likelihood reconstruction of ancestral host associations pictured here is the one where *Populus* was used as host for *Leccinum aurantiacum* s.s. Pie chart diagrams indicate proportional likelihood scores of nodes that could not be reconstructed unambiguously. Superimposed grey areas indicate episodes of rapid speciation.

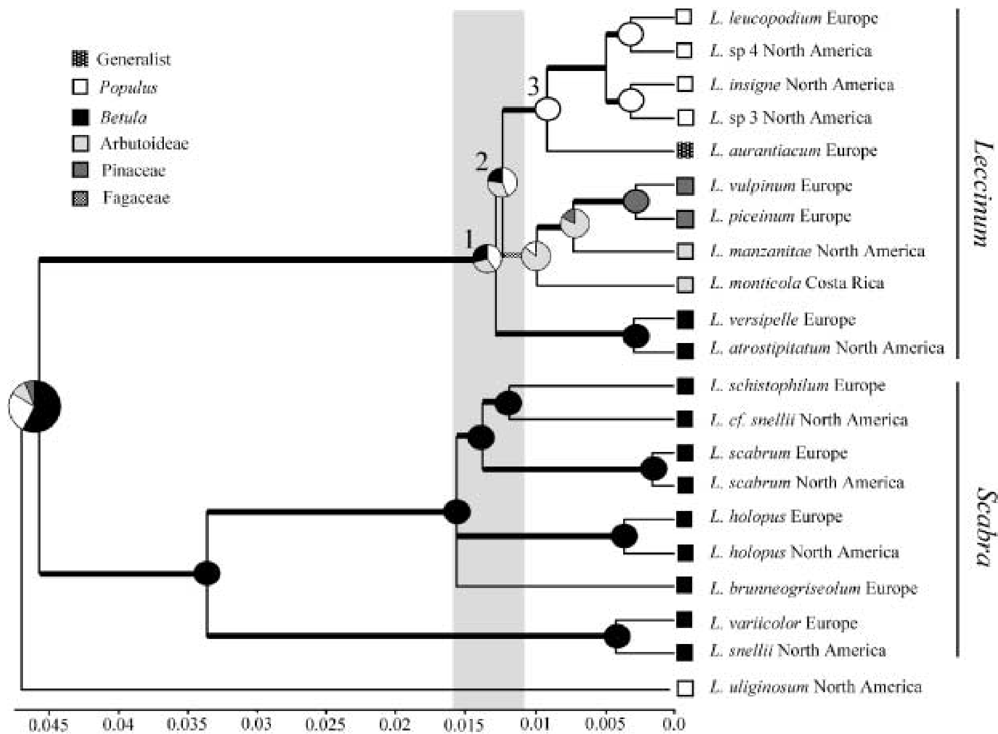

Fig. 5 Maximum likelihood tree with molecular clock enforced based on total *Gapdh* sequences. Thickened branches receive posterior probabilities of 95% or more in Bayesian analysis. Hatched branches receive posterior probabilities of between 90% and 95%. The axis below the tree gives the estimated number of substitutions per site. Numbers near nodes refer to the maximum likelihood reconstructions in Table 5. The likelihood reconstruction of ancestral host associations pictured here is the one were *Populus* was used as host for *Leccinum aurantiacum* s.s. Pie chart diagrams indicate proportional likelihood scores of nodes that could not be reconstructed unambiguously. Superimposed grey areas indicate episodes of rapid speciation.

An additional parsimony reconstruction was performed based on the same trees as the ML reconstruction, with the exception that branches with a length close to zero were collapsed. This resulted in an unresolved relationship between the *Scabra*, *Leccinum* and *Fumosa* clades and the merging of nodes 1 and 2 (data not shown). The resulting polytomy were considered to be soft polytomies. An advantage of parsimonies reconstruction methods is that polymorphisms are allowed. Therefore, the associations could be coded according to genus or (sub)family (Fagaceae, *Populus*, *Betula*, Arbutoideae, Pinaceae, Coryloideae). In the parsimony reconstruction *L. crocipodium* was coded as being associated both with Fagaceae and Coryloideae, and *L. aurantiacum* as being associated with Fagaceae, *Populus* and *Betula*. The parsimony reconstruction showed the association of the MRCA of the *Fumosa*, *Leccinum* and *Scabra* clade could not be reconstructed unambiguously, as all hosts, except Pinaceae and Arbutoideae, were equally possible as the associate of this MRCA. The MRCA of the *Leccinum* clade was associated with *Betula* and/or *Populus* as was the MRCA of node 3.

The ML and parsimony reconstructions gave complementary information about ancestral mycorrhizal associations in *Leccinum*. Where parsimony showed an ambiguous reconstruction for the association of the MRCA of the *Fumosa*, *Leccinum* and *Scabra* clades, the ML reconstruction indicated that *Populus* and *Betula* were most likely the ancestral host. With both reconstruction methods Pinaceae or Arbutoideae can be ruled out as the ancestral host. Both reconstruction methods pointed toward *Populus* and/or *Betula* being the host of the MRCA of the *Leccinum* clade. This indicated that the contemporary Pinaceae and Arbutoideae associates evolved out of an ancestor that was associated with *Populus* and/or *Betula*. The second conclusion that can be drawn from these reconstructions is that the ability of *L. aurantiacum* to form mycorrhiza with Fagaceae is newly derived, and indicates a recent broadening of its host range.

Discussion

Host specificity

Species of *Leccinum* are generally considered to be highly host specific (i.e. specialized on a phylogenetically restricted range of hosts). Our results show this to be generally true but with one major exception. *Leccinum aurantiacum* is associated with a broad range of hosts, found with Fagaceae (*Quercus* and *Fagus*), *Betula* and *Populus*. There are further records of associations with *Tilia* (Tiliaceae). Interestingly, the reconstruction of the ancestral host association provided clear evidence that this generalist evolved from an ancestor that was associated with a narrower host range, most likely *Betula* and/or *Populus*. It is not possible with the genes that we investigated to determine whether *L. aurantiacum* still behaves as a panmictic population or whether evidence exists of subsequent host race formation. Further investigations to address that question based on other molecular markers would be very useful. Schluter (2000) showed through compiling diverse phylogenetic studies that more often than expected generalists can evolve from specialists. His compilation and our observations on *L. aurantiacum* show that the generally held concept that ecological specialization must lead to more increased specialization may not always be valid.

Although within the *Leccinum* clade a generalist evolved from a more specialized ancestor when it concerns host specificity, a trend towards increased edaphic specialization is observed in the *Scabra* clade. This clade has a long history of association with *Betula*. Although all found on one host, in The Netherlands, in various locations, several species of this clade co-occur, showing edaphic niche differentiation: *Leccinum scabrum* on dry acidic soils, *L. holopus* in humid acidic areas and *L. schistophilum* on slightly calcareous, humid areas (Den Bakker, unpubl. obs.).

Incongruence of ITS2 and *Gapdh*

The ITS2 sequences and phylogeny showed two peculiarities. First, the presence of a shared 40 bp deletion in six accessions (*L. versipelle* Norway, *Leccinum* sp. 3 Canada, *L. insigne* Massachusetts, *L. manzanitae*, California and both accessions of *L. monticola* from Costa Rica). With the exception of *L. monticola*, closely related species or even sequences from different individuals of the same species (for example *L. versipelle*, clade 3 in Fig. 2) did not show this deletion. Most likely this represents an ancestral polymorphism, which is the best explanation for the exactly identical position of the deletion.

Another peculiarity of the ITS2 gene tree is the well supported (BS 85%, PP 98%) placement of the European *L. aurantiacum* and the North American *Leccinum* sp. 4 and *L. brunneum* (*Leccinum* clade 2), together with most species of the *Scabra* clade, except *L. variicolor* and *L. snellii*. In the *Gapdh* gene tree *L. aurantiacum* forms a monophyletic group with *L. leucopodium*, *L. insigne* and *Leccinum* sp. 4. Comparison of two loci in the ITS2 alignment (Table 7) shows the length of a single nucleotide 'A' repeat and sequence identity of these two loci are congruent with clades B, C, D and E in the *Gapdh* gene tree. In clade A in the *Gapdh* gene tree. however, we found several

Table 7 Clade and accession specific nucleotide patterns found on two different loci in ITS2

Clade in Fig. 2	Position 211	Position 335
Clade A		
Leccinum leucopodium	GCAA	$AC_{(3)}$
Leccinum sp. 4	$A_{(6)}$	TCATT
Leccinum insigne and *Leccinum* sp. 3	$A_{(6)}$	$AC_{(3)}$
Leccinum aurantiacum	GCAA	TCATT
Clade B	$A_{(5)}$	$TC_{(3)}$
Clades C and D	$A_{(10/9)}$	$AC_{(3/4)}$
Clade E	$A_{(8)}$	ACTC

different sequences at the ITS2 loci. An explanation for this phenomenon would be that we are dealing here with paralogous copies of either gene. However, paralogous copies of *Gapdh* appear to be rare and are (to date) only found in photosynthetic plants (Figge *et al*., 1999). By contrast, paralogy in ITS is often encountered in plants and is associated with phenomena such as ancient introgression, hybridization and polyploidy (Álvarez & Wendel, 2003). The taxonomy of the North American species of the group of *L. insigne* and *aurantiacum*-like species is notoriously difficult and processes such as hybridization might account for these difficulties. More data are needed on this group.

Host switches and speciation

The reconstruction of the ancestral host associations shows two major host switching events (Figs 4 and 5). First, a switch by the MRCA of the *Fumosa* and *Leccinum* clade from *Betula* to *Populus*. Second, a switch by the MRCA of the *Leccinum* clade from *Populus* to *Betula* and to Arbutoideae. Remarkably, these host switches are associated with or followed by episodes of rapid speciation, as indicated by the unresolved polytomies and short branch lengths in the clock trees. The same phenomenon of extensive speciation (adaptive radiation) after host switches has been noted in *Suillus* (Kretzer *et al*., 1996), *Hebeloma* (Aanen *et al*., 2000) and also in *Pisolithus*, where all four species of lineage B are associated with eucalypts and acacias, and the three species of lineage AII are associated with pines (Martin *et al*., 2002). The fact that the second episode of rapid speciation in the *Leccinum* clade seems to coincide with an episode of rapid speciation in the *Scabra* clade makes us think that the cause of this rapid speciation must be found outside host specificity, since there is no host shift taking place in the *Scabra* clade. We therefore think that genetic isolation of allopatric populations during times of glaciation in the Quaternary may account for this pattern. A possible scenario to explain the pattern of host shifts in the *Leccinum* clade could be genetic isolation of allopatric populations, leading to a narrowing of the host range as a consequence of a decrease in the number of potential host tree species in areas influenced by drastic climatic changes. Narrowing of the host range could also be driven by ecological specialization. Evidence for this scenario is found that most host switches took place between host communities of ecologically equivalent species instead of phylogenetic groups within genera or families. The switch from Coryloideae plus Fagaceae to *Populus* and *Betula* could then be explained by a separation of ancestral populations of warmer and colder climates, since Coryloideae and Fagaceae represent thermophilous hosts and *Populus* and *Betula* are typical representatives of sub-boreal vegetation types. The importance of ecology as a factor promoting niche expansion is also consistent with the observation that the species associated with Pinaceae and Arbutoideae share a common ancestor and have evolved from *Populus* and *Betula*. In the current distribution area of *L. manzanitae* and *L. monticola* (associates of Arbutoideae), the coastal forests of California and the highlands of Costa Rica, respectively, *Betula* and *Populus* are virtually absent. Possibly a host-switch occurred by the extinction or decrease of the distribution area of *Betula* and *Populus* that originally overlapped that of *Arctostaphylos* in the Californian floral region. A subsequent switch (or niche expansion) to an association with Pinaceae is likely, since *Pinus* and *Pseudotsuga* can co-occur with *Arbutus* and *Arctostaphylos* and share the same mycorrhiza (Molina *et al*., 1997; Horton *et al*., 1999). A similar host niche expansion from eucalypts to acacias may have occurred in *Pisolithus* lineage B (Martin *et al*., 2002).

If host specificity (or at least host niche contraction) is a side-effect of geographic isolation and allopatric speciation, this strongly suggests episodes of relaxed specificity in periods in which several hosts can be exploited, otherwise the disappearance of the one specific host will mean the extinction of the associated specialist fungi. Relaxation of specificity could also occur in marginal areas, for example as in the case of niche expansion from eucalypts to *Kunzea* (Myrtaceae–Leptospermoideae) in geothermal areas in New Zealand (Moyersoen *et al*., 2003).

In conclusion, species within the genus *Leccinum* are generally host specific as widely assumed. However, *L. aurantiacum* associates with a broad range of ectomycorrhizal broad-leaved trees. This shift from a *Populus*-associated specialist to a generalist probably took place recently in the evolutionary history of the genus and shows that, in contrast to the theory that evolution of a symbiont leads to increased specialization, the opposite can occur. This has taxonomic and evolutionary implications. Taxonomically the ability to grow on a new host cannot be taken a priori as evidence that a new *Leccinum* species has evolved. Phylogenetic studies can serve as a starting point for further research on the evolutionary biology of host specificity in mycorrhizal fungi. Cycles of niche contraction (switches from generalists to specialists) and niche expansion (from specialists to generalists) are essential to explain speciation and the evolution of host specificity in mycorrhizal fungi.

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