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ORIGINAL PAPER

Diverging diversity patterns in the *Tulasnella* (Basidiomycota, Tulasnellales) mycobionts of *Aneura pinguis* (Marchantiophyta, Metzgeriales) from Europe and Ecuador

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Abstract *Aneura pinguis* (Aneuraceae) is a cosmopolitan thalloid liverwort that shows a specific mycorrhiza-like interaction with basidiomycetes. To date, tropical specimens have not been studied in great depth. Samples of *A. pinguis* were collected from 48 individuals in one plot in South Ecuador and 54 individuals in five European countries. Light and transmission electron microscopy and molecular analyses based on nuclear rDNA coding for the ribosomal large subunit (nucLSU) and from the 5.8s-ITS2 regions were carried out to identify the associated mycobionts and to study their phylogenetic relationships. Microscopic and ultrastructural investigations of the fungal colonisation showed a high congruence between the European and the Ecuadorian sites and confirmed previous results. Tulasnellales are the only mycobionts that could be detected from ultrastructural characters with certainty. Molecular phylogenetic analysis indicated the presence of tulasnelloid fungi from at least 13 distinct clades. The composition of the communities of tulasnelloid fungi in *A. pinguis* differs between Ecuador and Europe. The diversity of tulasnelloid fungal partners was much higher at the Ecuadorian site.

Keywords *Aneura pinguis* · Aneuraceae · Mycobiont · Tulasnellales · Neotropical mountain rainforest · Southern Ecuador · Europe · Diversity

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Introduction

Mycorrhizal interactions seem to have played a key role in terrestrial colonisation by plants (Selosse and Le Tacon 1998; Read et al. 2000; Brundrett 2002; Wang and Qiu 2006; Berbee and Taylor 2007). It is becoming increasingly accepted that liverworts, as the sister group to all other land plants (Wellman et al. 2003; Groth-Malonek et al. 2005; Carafa et al. 2005), are closest to the "inventors" of mycorrhiza-like interactions (Kottke and Nebel 2005; Duckett et al. 2006a). Gametophytes of hepatics form endomycorrhiza-like associations with glomero-, asco-, and basidiomycetes (see Nebel et al. 2004 for a recent overview). However, basidiomycetes—all belonging to the Agaricomycetes—have been detected only in a few families of liverworts (Duckett et al. 2006b). The mycorrhiza-like interaction of the Aneuraceae is unique, because the Aneuraceae are the only thalloid group with basidiomycetous mycobionts and the only liverwort group known to have *Tulasnella* symbionts (see below).

Despite this characterisation (discussed already in Schuster 1992 and noted in Nebel et al. 2004, Fig. 6), the separation of the Metzgeriales in a clade containing the Aneuraceae and Metzgeriaceae from the rest of the simple thalloid liverworts was only recently revealed by molecular methods (Davis 2004; Heinrichs et al. 2005; Forrest et al. 2006), thus overturning monophyly in simple thalloids based solely on morphology (Schuster 1984; Crandall-Stotler and Stotler 2000). In fact, the Metzgeriales are a derived group, probably with a non fungal-dependent ancestor (Kottke and Nebel 2005).

The Aneuraceae are a family of simple-structured and highly polymorphic thalloid liverworts with a worldwide distribution, though they are found more widely in the Southern Hemisphere. Using molecular phylogenetic anal-

ysis, it has been possible to assign *Verdoornia* (Forrest et al. 2006) in addition to the genera *Aneura* (including *Cryptothallus*, Wickett and Goffinet 2008), *Lobatiriccardia*, and *Riccardia* (Frey and Stech 2005) to the Aneuraceae. With a total of more than 100 species, most of them belonging to *Riccardia* (Gradstein 2001), Aneuraceae is the largest family within the simple thalloid liverworts. Fungal colonisation of the Aneuraceae ranges from obligatory to completely lacking. One extreme is represented by *Cryptothallus*, the only fully myco-heterotrophic bryophyte (Ligrone et al. 1993; Bidartondo et al. 2003). The other extreme is marked by *Riccardia*, which has many non- or only facultatively colonised species (Hewson 1970b; Brown and Braggins 1989; Duckett and Ligrone 2008). Despite the presumable autotrophy of *Aneura*, *Lobatiriccardia*, and *Verdoornia*, all published investigations argue for a frequent, and thus probably obligatory, fungal colonisation (Stahl 1949; Hewson 1970a; Ligrone et al. 1993; Duckett et al. 2006b).

Previous investigations revealed a narrow spectrum of fungal partners of Aneuraceae that was obviously clearly restricted to the Tulasnellales, which are basidiomycetes with imperforate and more or less curved parenthesomes, and multilayered cell walls (Ligrone et al. 1993; Read et al. 2000; Nebel et al. 2004; Duckett and Ligrone 2008). Consequently, applying molecular methods enabled the identification of *Tulasnella* species as mycobionts of *Cryptothallus mirabilis* (Bidartondo et al. 2003) and *Aneura pinguis* (Kottke et al. 2003, 2008). *Tulasnella* is a diverse but morphologically well-distinguished cosmopolitan genus, which, according to molecular studies, is closely related to or even nested within the cantharelloid clade of the Agaricomycotina (Moncalvo et al. 2006). *Tulasnella* comprises saprotrophic as well as mycorrhizal species; it is known to include endomycorrhizal partners of the Orchidaceae (Roberts 1999; Taylor and McCormick 2008) and also ectomycorrhizal fungi (Bidartondo et al. 2003).

Because of its cosmopolitan distribution (Szweykowski 1968; Schuster 1992; Damsholt 2002) and tight liaison with mycorrhizal fungi, *A. pinguis* (L.) Dumort. is an interesting taxon for investigating fungal symbionts of Aneuraceae. *Aneura pinguis* shows considerable infraspecific variability and most likely comprises a complex of cryptic species (Schuster 1992; Buczkowska et al. 2006; Wachowiak et al. 2007; Long et al. 2007; Wickett and Goffinet 2008). In view of these taxonomic uncertainties, all taxa are at present best considered as *A. pinguis* s.l. In the Neotropics, *A. pinguis* s.l. is regarded as the only representative of the genus, neglecting dubious records of *Aneura latissima*, *Aneura pseudopinguis*, and *Aneura sessilis* (Gradstein 2001; Gradstein personal communication). The identity of the Ecuadorian *A. pinguis* samples investigated in this study is confirmed by our ongoing molecular phylogenetic studies in Aneuraceae.

We investigated the fungal inventory of *A. pinguis* s.l. from several sites in central Europe, a few locations in other European countries, and one rainforest area in southern Ecuador to shed light on the following questions:

> What is the phylogenetic spectrum of the mycobionts of *A. pinguis* s.l.?
> Are there differences in the mycobiont communities of *A. pinguis* s.l. between Ecuadorian and European samples?

We used ultrastructural as well as molecular methods to identify the *A. pinguis* mycobionts and analysed the results taking ecological and geographical aspects into consideration.

Materials and methods

Sample collection

Samples were taken during 2004 and 2005. All tropical samples were collected at the Reserva Biológica San Francisco (RBSF) situated on the eastern slope of the Cordillera El Consuelo in South Ecuador (03°58′S, 79°04′ W) in a tropical mountain rainforest area of about 12 ha between 1,850 and 2,200 m a.s.l. The forest is exceptionally rich in tree species, ericads, and orchids (Homeier and Werner 2008), and also in liverworts and mosses (Nöske et al. 2003; Parolly et al. 2004). The liverwort plants in our study grew in two types of virgin mountain rainforest. The locations Quebrada 2 (Q2; nine individuals collected), Quebrada 3 (Q3; four individuals), and Quebrada 5 (Q5; five individuals) are ravines in the lower mountain belt covered by forests with closed canopy of about 30 m height. The locations Transecto 1 (T1; three individuals) and Transecto 2 (T2; five individuals) belong to less inclined slopes of the upper mountain belt characterised by forests with a lower and more open canopy (for details, see Homeier et al. 2008). Information about the proveniences of the extratropical samples from five European countries is given in Table 2. The altitudinal gradient comprises sites from 220 to 1,600 m a.s.l. Most samples were collected in southern Germany (13 locations); one sampling site was situated in northern Germany. Other samples were obtained from two neighbouring sites each in Ireland and Sweden and from single sites in Austria and Switzerland. Up to five individuals were collected depending on population size. All samples are stored at the herbarium of the State Museum of Natural History in Stuttgart.

Most of our tropical samples are from damp rotten wood, the main substrate of *A. pinguis* in the investigated tropical mountain rainforest, but two collections were made

from a wet concrete wall and one from soil. In Europe, the main colonised substrates are wet soil and calcareous tufa. Accordingly, our European *Aneura* samples were collected from very calcareous and wet habitats at 12 out of a total of 20 localities (Table 2). These habitats comprise calcareous tufa rocks (seven localities), calcareous fens (four localities), and a wet limestone rock. The remaining eight localities represent damp to wet, modest acid to modest alkaline-rich environments such as siliceous rocks (two localities), wet embankments (two localities), springs, and fens (four localities). Both tropical and European samples comprise large and small, male and female plants. The liverwort plants were separated from the substrate and cleaned under tap water. After light microscopic verification of hyphal colonisation, a 0.5–1-cm long piece of the thallus from living and healthy plants was dried and stored on silica gel in 1.5 ml reaction tubes for DNA isolation. Each second sample was fixed in 2.5% glutaraldehyde-paraformaldehyde in 0.2 M KH_2PO_4/Na_2HPO_4 buffer, pH 7.2 (Karnovsky 1965).

Light and transmission electron microscopy

In order to test the current evidence that only a narrow spectrum of fungal symbionts are found in the Aneuraceae, we conducted light microscopic studies for all samples and transmission electron microscopy (TEM) for 15 samples of *A. pinguis* (11 tropical individuals and four European). For light microscopic investigation, transversal sections were made by hand from the middle part of each liverwort individual using a razor blade not later than 1 day after collection. Sections were stained in 0.05% methyl blue solution (Merck C.I. 42780, Darmstadt, Germany) in lactic acid for 5 min on microscope slides. The samples were examined in lactic acid under a Leitz Laborlux microscope. For TEM investigation, the glutaraldehyde-fixed samples were washed six times in Sorensen buffer and fixed in 1% osmium tetroxide for 1 h in the dark. Samples were dehydrated in an acetone series and embedded flat in Spurr's resin (low viscosity, longer pot-life formulation; Spurr 1969). After hardening at 70°C for 3 days, semithin sections were cut, stained with neofuchsin crystal-violet, and mounted in Entellan (Merck, Darmstadt, Germany). Samples with apparently vital hyphae, originating from different plant individuals, were selected for ultrathin cutting. Sections were mounted on Formvar-coated copper grids and stained with 1% uranyl acetate (1 h) and lead citrate (12 min). Sections were examined using a Zeiss TEM 902.

DNA extraction, PCR, cloning, and sequencing

DNA was extracted from the dried samples using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the amplification by the polymerase chain reaction (PCR), we chose a fragment of nuclear rDNA comprising the 5′ domain of the gene coding for the ribosomal large subunit (nucLSU), the internal transcribed spacer 2 (ITS2), and the 5.8S ribosomal subunit to get sequences from both conserved and more variable regions. Furthermore, these regions are those addressed by most other molecular studies on *Tulasnella*. In preliminary experiments, we used combinations of universal fungal primers ITS1F/NL4, ITS1F/LR5, and ITS1F/TW14 (Vilgalys and Hester 1990; Gardes and Bruns 1993; O'Donnell 1993; http://plantbio.berkeley.edu/~bruns/tour/primers.html) to amplify genomic DNA. Many of the sequences obtained did not belong to the putative mycorrhizal fungi of the Aneuraceae as revealed by our ultrastructural investigations. Hence, we chose the tulasnelloid-specific primer 5.8S-Tul (Suárez et al. 2006) for subsequent experiments using the combinations 5.8S-Tul/NL4 and 5.8S-Tul/TW14.

The PCR reaction volume was 50 µl with concentrations of 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM of each of the primer using 1 U of Taq polymerase and 1 µl of the mostly undiluted DNA extract. The PCR reaction was optimised by adding 0.2 µl of 1% bovine serum albumine (Sigma-Aldrich, Munich, Germany) to the reaction volume. PCR products were purified using a QIAquick protocol (Qiagen). Direct sequencing of PCR products was performed using the PCR primers as sequencing primers. The PCR products of nine tulasnelloid fungal samples were cloned into competent cells with a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Inserts were reamplified from clones using the M13 primers by picking two to six bacterial clones with a toothpick and placing them directly into the PCR reaction mixture. One to three reamplified inserts were sequenced from each cloned PCR product. Cycle sequencing was conducted using the ABI PRISM Dye-Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Electrophoresis and data sampling were performed on an automated sequencer (ABI 3100; Applied Biosystems); both strands of DNA were sequenced. Sequence editing was performed using SEQUENCHER software, version 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

The sequences obtained were verified to be *Tulasnella* sequences using BLAST sequence similarity searches (Altschul et al. 1997) against the National Center for Biotechnology Information database (GenBank; http://www.ncbi.nlm.nih.gov). GenBank accession numbers of the sequences obtained are given in Table 1.

Phylogenetic analysis

To construct an overview of all the different and currently available tulasnelloid nucLSU sequences in one phyloge-

Table 1 Assignment of the tulasnelloid nuclear rDNA sequences (5.8S-ITS2 and nucLSU) from Ecuadorian and European samples of *Aneura pinguis* s.l. to phylogenetic clades (Figs. 3 and 4)

Locality of origin	Plant number	Accession number of mycobionts	Figure 3	Figure 4		Date of collection	Voucher number
			Clade number	Clade Europe 1	Clade Europe 2		
Ecuador							
Quebrada 2	Q2/2c	EF429157	1			10.07.2004	MPE04202
Quebrada 2	Q2/3a	EF429161	3			11.07.2004	MPE04013
Quebrada 2	Q2/3b	EF429171	12			11.07.2004	MPE04014
Quebrada 2	Q2/4a	EF429158	1			11.07.2004	MPE04148
Quebrada 2	Q2/4d	EF429172	12			11.07.2004	MPE04149
Quebrada 2	Q2canal2	EF429160	3			10.07.2004	MPE04142
Quebrada 2	Q2canal1b	EF429159	3			10.07.2004	MPE04147
Quebrada 3	Q3/1a	EF429168	10			22.07.2004	MPE04011
Quebrada 3	Q3/2b	EF429169	10			22.07.2004	MPE04012
Quebrada 5	Q5/2a	EF429155, EF429164	1, 5			22.07.2004	MPE04002
Quebrada 5	Q5/2b	EF429173	12			22.07.2004	MPE04010
Quebrada 5	Q5/3a	EF429163	5			22.07.2004	MPE04145
Quebrada 5	Q5/4a	EF429165	6			22.07.2004	MPE04146
Transecto 1	T1/1a	EF429162, EF429166	4, 9			26.06.2004	MPE04143
Transecto 1	T1/2a	EF429156	1			26.06.2004	MPE04144
Transecto 2	T2/2a	EF429170	11			26.06.2004	MPE04185
Transecto 2	T2/3a	EF429167	9			26.06.2004	MPE04187
Europe							
Austria	AUSa	EF363107		x		13.08.2005	MN051096
Austria	AUSb	EF363108		x		13.08.2005	MN051096
Austria	AUSc	EF374106		x		13.08.2005	MN051096
Ireland	IR1a	EF363113			x	31.03.2005	MP05190
Ireland	IR1b	EF374107			x	31.03.2005	MP05190
Ireland	IR1c	EF374108			x	31.03.2005	MP05190
Ireland	IR1d	EF374109			x	31.03.2005	MP05190
Ireland	IR2a	EF363105		x		31.03.2005	MP05191
Ireland	IR2b	EF374103		x		31.03.2005	MP05191
Sweden	SV1a	EF363106		x		03.08.2005	MP05764
Sweden	SV1b	EF374104		x		03.08.2005	MP05764
Sweden	SV2	EF363115			x	02.08.2005	MP05765
Switzerland	SWI	EF363119			x	14.07.2005	MP05767
Germany/Allgäu	GA1	EF363114			x	13.05.2005	MP05769
Germany/Allgäu	GA2a	EF363118			x	13.05.2005	MP05770
Germany/Allgäu	GA2b	EF374113	13			13.05.2005	MP05770
Germany/Allgäu	GA3a	EF363117			x	08.06.2005	MP05771
Germany/Allgäu	GA3b	EF374112			x	08.06.2005	MP05771
Germany/Allgäu	GA3c	EF374114	13			08.06.2005	MP05771
Germany/Allgäu	GA4a	EF363110			x	08.06.2005	MP05772
Germany/Allgäu	GA4b	EF363112			x	08.06.2005	MP05772
Germany/Allgäu	GA5a	EF374115	13			12.06.2005	MP05773
Germany/Allgäu	GA5b	EF374116	13			12.06.2005	MP05773
Germany/Allgäu	GA6a	EF363111			x	08.07.2005	MP05774

Table 1 (continued)

Locality of origin	Plant number	Accession number of mycobionts	Figure 3	Figure 4		Date of collection	Voucher number
			Clade number	Clade Europe 1	Clade Europe 2		
Germany/Allgäu	GA6b	EF374117	13			08.07.2005	MP05774
Germany/Allgäu	GA7a	EF363109		x		08.10.2004	MP05766
Germany/Allgäu	GA7b	EF374105		x		08.10.2004	MP05766
Germany/Black Forest	GB1a	EF363099		x		24.09.2005	MN051089
Germany/Black Forest	GB1b	EF374099		x		24.09.2005	MN051089
Germany/Black Forest	GB2a	EF363104		x		09.07.2005	MN051087
Germany/Black Forest	GB2b	EF374102		x		09.07.2005	MN051087
Germany/Black Forest	GB3a	EF363100		x		29.10.2005	MP05606
Germany/Black Forest	GB3b	EF363103		x		29.10.2005	MP05606
Germany/Black Forest	GB3c	EF374100		x		29.10.2005	MP05606
Germany/Keuper highland	GK1a	EF363116			x	14.07.2005	MN051085
Germany/Keuper highland	GK1b	EF374110			x	14.07.2005	MN051085
Germany/Keuper highland	GK1c	EF374111			x	14.07.2005	MN051085
Germany/Keuper highland	GK2	EF363098		x		10.01.2006	MP06189
Germany/Chiemsee	GCH	EF363102		x		14.07.2005	MP05768
Germany/Weserbergland	GWEa	EF363101		x		14.03.2004	MP03186
Germany/Weserbergland	GWEb	EF374101		x		14.03.2004	MP03186

GenBank accession numbers for the mycobionts and voucher information are given. For more information about European plants, see Table 2

netic tree, we used the Taxonomy Browser and BLAST to search for homologous tulasnelloid sequences in GenBank. After deleting identical sequences, we obtained a set of 56 sequences from GenBank, to which we added the 48 sequences from this study (excluding identical sequences), yielding a final dataset of 104 sequences. Because of the heterogeneity of the tulasnelloid sequences, we had to exclude highly divergent portions of the sequences for phylogenetic analysis. We assembled an automatic alignment of 395 bp from the 5′ terminal domain of the nucLSU using MAFFT version 5.7 with the E-INS-I strategy (Katoh et al. 2005). A nucLSU sequence of *Multiclavula mucida* was included in the sequence sampling as an outgroup sequence. To estimate phylogenetic relationships, the alignment was analysed using heuristic maximum likelihood as implemented in PHYML (Guindon and Gascuel 2003), with a general time-reversible model of nucleotide substitution, and assuming a percentage of invariant sites and gamma-distributed substitution rates at the remaining sites (GTR+I+G), approximating the gamma distribution with four discrete rate categories, and starting from a BIONJ tree (Gascuel 1997). All model parameters were estimated using maximum likelihood. Branch support was inferred from 1,000 replicates of non-parametric bootstrapping (Felsenstein 1985), with model parameters estimated via maximum likelihood individually for each bootstrapped alignment.

Additionally, we performed a Bayesian Markov chain Monte Carlo (MCMC) analysis using MrBayes 3.1 (Ronquist and Huelsenbeck 2003). We ran two independent MCMC analyses, each involving four incrementally heated chains over ten million generations, using the GTR+I+G model of nucleotide substitution and starting from random trees. Model parameters were not fixed but sampled during MCMC. Trees were sampled every 100 generations, resulting in an overall sampling of 100,000 trees per run, from which the first 40,000 trees of each run were discarded (burn in). The remaining 60,000 trees sampled in each run were pooled and used to compute a majority-rule consensus tree to get estimates for the posterior probabilities. Stationarity of the process was controlled using the Tracer software (Rambaut and Drummond 2004).

In addition, we ran phylogenetic analyses exclusively for our own European *Tulasnella* sequences, which showed a high homogeneity. We aligned a portion of 820 bp in the same manner as explained above, including the 3′ end of the 5.8S and the ITS2 region and performed ML and MCMC analyses. Deviating from the parameters detailed above, we used two million generations in MCMC, storing 20,000 trees in each chain, from which the first 4,000 were discarded before calculation of a pooled majority-rule consensus.

Results

Light and transmission electron microscopy of the mycorrhiza-like interaction

Light microscopic studies showed high uniformity of the fungal colonisation in every specimen from every site. The hyphae ascended via a few rhizoids to the rhizoid base and colonised the neighbouring parenchyma cells where the formation of more or less compact coils occurred (Fig. 1a, c). The epidermal cells never showed colonisation by fungal hyphae (Fig. 1a, b). Collapsed hyphal balls could be observed in parenchyma cells in ca. 30% of the specimens (Fig. 1b, d). Vital hyphae were more or less regularly septate, never displaying clamps (Fig. 1c). Significant morphological differences between the fungi from Europe and Ecuador were not found. As well as our light microscopic studies, our ultrastructural investigations showed the same features in the mycobionts of *A. pinguis* from Ecuador and Europe. Septate hyphae with imperforate parenthesomes were detected in all 15 samples from both regions. The parenthesomes were mainly dish-shaped (Fig. 2b). A conspicuous feature seen in half of the specimens was a widening of the fungal cell walls (multilayered cell walls) at apparently random locations. Obviously, fibrillar material was accumulated between the separated wall layers (Fig. 2a).

Molecular identification and phylogenetic analysis of the mycobionts

The combination of universal primers yielded PCR products which, after sequencing, were preliminarily identified

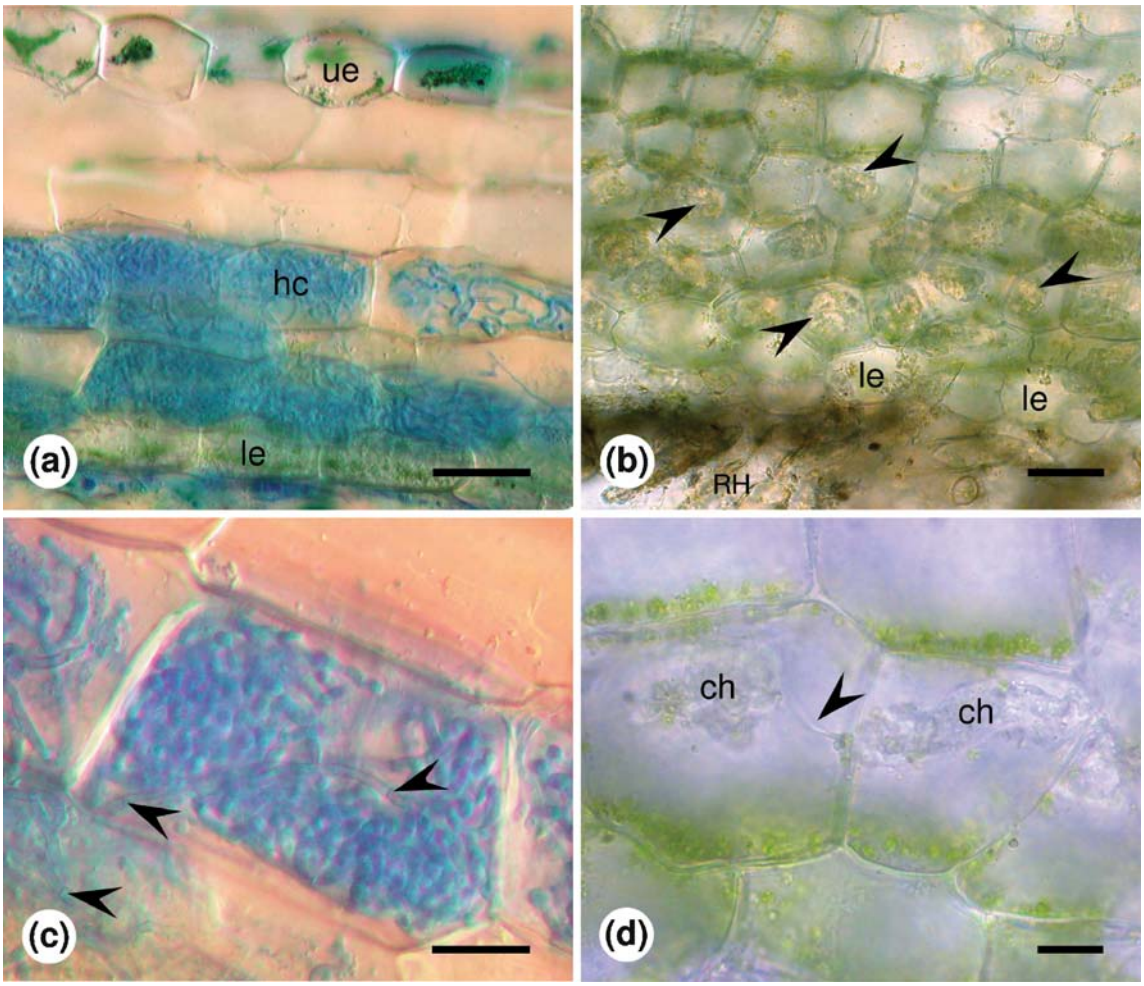

Fig. 1 Light microscopic features of the colonisation of fresh tropical material of *Aneura pinguis* s.l. by putative tulasnelloid mycobionts. Hyphae were stained with aniline blue in (**a**) and (**c**). **a** Cross-section with parenchyma confined by upper (ue) and lower (le) epidermis. Lower parenchyma cells are filled in with more or less dense hyphal coils (hc), while upper parenchyma cells are free of hyphal colonisation. Bar, 50 µm. **b** Cross-section with lower epidermis (le) and some rhizoids (RH) partially visible. Only the lower parenchyma cells show lumps of collapsed hyphae (*arrowheads*). Bar, 50 µm. **c** Parenchyma cell filled with hyphal coils; *arrowheads* mark hyphal septae without clamps. Bar, 20 µm. **d** Two parenchyma cells each with collapsed hyphal pelotons (ch), which are linked by one hypha (*arrowhead*). Bar, 20 µm

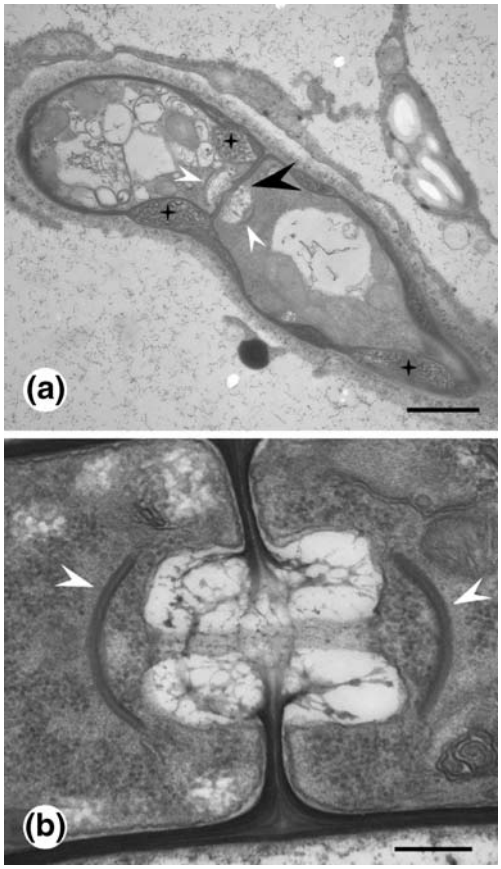

Fig. 2 Transmission electron micrographs of the fungal colonisation of tropical samples of *Aneura pinguis*. **a** Intracellular hypha with a dolipore (*black arrowhead*) with imperforate parenthesomes (*white arrowheads*). The fungal cell wall shows abundant formation of multilayered cell walls (*asterisks*). Bar, 2 µm. **b** Close-up of a median section of the dolipore from an intracellular hypha. The parenthesomes (*arrowheads*) are slightly dish-shaped. Bar, 0.3 µm

by BLAST searches as closest to the ascomycetous taxa *Aleuria*, *Chalara*, *Crinula*, *Oidium*, *Schaereria*, and *Tubeufia*, and the basidiomycetous *Antrodiella* and *Sebacina*. *Tulasnella* sequences were obtained only six times. Applying the selective primer combinations 5.8S-Tul/NL4 and 5.8S-Tul/TW14 resulted in tulasnelloid sequences for the majority (85%) of samples as revealed by BLAST searches. Finally, every individual with successful sequencing (n= 66) yielded tulasnelloid sequences. A total of 74 sequences could be assigned to *Tulasnella* by BLAST searches. Excluding sequences of inferior quality, we were able to use 66 of these sequences for molecular phylogenetic analyses (41 from Europe and 25 from Ecuador). For the overview tree (Fig. 3), we used 48 sequences (25 from Ecuador and three from Europe from this study; 20 from Ecuador from a previous study from the same site, Kottke et al. 2008). For the presentation of the European sequences (Fig. 4), we used 22 sequences (excluding identical sequences).

Broad *Tulasnella sequence spectrum*

The comprehensive analysis of tulasnelloid nucLSU sequences (Fig. 3) shows a distribution of the tulasnelloid sequences in three groups. Two smaller groups (clades A and B) take a basal position. The first (well supported by both branch support values) contains the sequence of *Tulasnella cystidiophora*. The *Tulasnella* clade B is only weakly supported and presently composed exclusively of sequences of *Cypripedium* mycobionts. The remaining numerous sequences form a well-supported group (*Tulasnella* clade C in Fig. 3). Sequences from all liverwort mycobionts are arranged in thirteen clades (Fig. 3, numbers 1–13), which belong to *Tulasnella* clades A and C. Only two of the clades with liverwort mycobionts are contained in *Tulasnella* clade A, thus eleven belong to the *Tulasnella* clade C. Fungal sequences from Ecuadorian and European liverworts were clearly separated phylogenetically. Fungal sequences from colonised Ecuadorian and European liverworts cluster together in one clade in only one instance (clade 13). Sequences from the specimens found at the RBSF are arranged in eleven clades and are absent only in clades 7 and 8; those from European samples are members of clades 7, 8, and 13. Members of the *Tulasnella* clades A and C were detected in *A. pinguis* s.l. in both sampling areas, Ecuador and Europe. None of the Ecuadorian and only two of the European sequences (clades 8 and 13) sampled in this project are identical to any sequence in GenBank, even on the short part of the nucLSU regarded here. The only available sequence from a mycobiont of *A. pinguis* (AY298949) is a nucLSU sequence completely identical to the nucLSU sequence of our *A. pinguis* sample GA1 (clade 8).

Systematic placement of the sampled sequences is uncertain, since close affinities to named sequences in GenBank are lacking. In many cases, the hepatic mycobionts form their own clades with high support (clades 1, 3, 4, 5, 9, 10, and 11). Sometimes they are clearly separated from adjacent named sequences (clade 2 and 8). However, two sequences (clade 7 and 8) are members of a well-supported clade containing differing sequences assigned to *Tulasnella calospora*. The available *Tulasnella* sequences from Ecuadorian liverworts were distinct from those from Ecuadorian orchids. Clades of sequences from liverwort fungi were generally clearly separated from those published in GenBank, which have mainly been obtained from orchids. However, there is one exceptional case of identity of a fungal sequence in the *Tulasnella* clade A from an orchid (*Dactylorhiza majalis*) and the liverwort *A. pinguis* (clade 13). Additionally, within the clade containing several *T. calospora* sequences, a close relationship between fungal sequences from liverworts (clades 7 and 8) and orchids is apparent.


from Aneura pinguis Q2/2a DQ368695
from Aneura pinguis T2/2c DQ368696
from Riccardia smaragdina T2/1a DQ368697
from Aneura pinguis Q5/2a
from Riccardia smaragdina T2/1b DQ368698
from Aneura pinguis T1/2a
from Aneura pinguis Q2/2c
from Aneura pinguis Q2/4a
1
T. asymmetrica (culture from Thelymitra epipactoides OM) DQ388047
T. asymmetrica (culture from Thelymitra epipactoides OM) AY152406
from Aneura pinguis T2/1b DQ368694
2
from Stelis hallii (OM) DQ178066
from Stelis hallii (OM) DQ178051
from Pleurothallis lilijae (OM) DQ178067
Tulasnella pruinosa AF518662
Tulasnella pruinosa AY243522
Tulasnella violaea AY293216
T. asymmetrica (culture from Thelymitra luteocilium OM) DQ388046
from Aneura pinguis Q2/canal2
from Aneura pinguis Q2/canal1b
from Aneura pinguis Q2/canal1a DQ368699
from Aneura pinguis Q5/1c DQ368700
from Aneura pinguis Q2/3a
3
from Aneura pinguis T1/1a
from Riccardia smaragdina T2/2a DQ368701
4
from Aneura pinguis Q5/3a
from Aneura pinguis Q5/2a
from Aneura pinguis Q2/2c DQ368702
5
from Aneura pinguis Q5/4a
6
from Pleurothallis lilijae (OM) DQ178049
from Stelis concinna (OM) DQ178031
from Stelis superbiens (OM) DQ178036
T. calospora (culture from Thelymitra sp. OM) DQ388044
from Aneura pinguis GCH
7 (cp. clade Europe 1, Fig. 4)
from Aneura pinguis AY298949
from Aneura pinguis GA1
8 (cp. clade Europe 2, Fig. 4)
Tulasnella calospora AY243521
Tulasnella calospora (culture from OM) DQ388045
T. calospora (culture from Acianthus exsertus OM) DQ388041
from Stelis hallii (OM) DQ178044
from Stelis superbiens (OM) DQ178044
Epulorhiza anaticula AY243520
from Aneura pinguis T1/1a
from Aneura pinguis T2/3a
9
from Stelis hallii (OM) DQ178060
from Stelis superbiens (OM) DQ178064
from Stelis superbiens (OM) DQ178062
from Stelis superbiens (OM) DQ178048
from Pleurothallis lilijae (OM) DQ178049
from Aneura pinguis Q2/3a DQ368692
from Aneura pinguis Q2/2b DQ368693
from Aneura pinguis Q3/1a
from Aneura pinguis Q3/2b
10
from Cryptothallus mirabilis AY192482
from Cryptothallus mirabilis AY192445
Tulasnella irregularis AY243519
from Aneura pinguis Q2/1a DQ368704
from Aneura pinguis Q2/4a DQ368705
from Aneura pinguis Q2/2d DQ368706
from Aneura pinguis Q2/8a DQ368707
from Riccardia sp. Q2/1a DQ368708
from Aneura pinguis T2/1c DQ36871
from Aneura pinguis T2/2a
from Aneura pinguis Q2/7b DQ368712
from Aneura pinguis Q2/2e DQ368709
from Aneura pinguis Q2/6b DQ368710
from Aneura pinguis Q2/4b DQ368713
from Aneura pinguis Q5/2a DQ368703
from Aneura pinguis Q2/7a DQ368714
from Aneura pinguis Q2/4c DQ368715
11
Tulasnella violacea DQ520097
Tulasnella sp. AJ406436
Tulasnella obscura AJ406435
Tulasnella sp. (culture from Stelis concinna OM) DQ178029
Tulasnella clade C
from Cypripedium montanum (OM) AY578214
from Cypripedium parviflorum (OM) AY578213
from Cypripedium montanum (OM) AY578215
from Cypripedium guttatum (OM) AY578204
from Cypripedium fasciculatum (OM) AY578239
from Cypripedium parviflorum (OM) AY578211
from Cypripedium montanum (OM) AY578208
from Cypripedium fasciculatum (OM) AY578200
from Cypripedium fasciculatum (OM) AY578237
from Cypripedium fasciculatum (OM) AY578190
from Cypripedium fasciculatum (OM) AY578194
from Cypripedium parviflorum (OM) AY578206
from Cypripedium fasciculatum (OM) AY578189
from Cypripedium fasciculatum (OM) AY578188
from Cypripedium fasciculatum (OM) AY578236
Tulasnella clade B
from Cypripedium californicum (OM) AY578226
from Aneura pinguis Q5/2b
from Aneura pinguis Q2/8c DQ368716
from Aneura pinguis Q2/4d
from Aneura pinguis Q2/3b
12
from Cypripedium montanum (OM) AY578220
from Cypripedium montanum (OM) AY578219
from Cypripedium californicum (OM) AY578218
from Cypripedium parviflorum (OM) AY578225
from Cypripedium candidum (OM) AY578221
from Cypripedium candidum (OM) AY578222
from Dactylorhiza majalis (OM) AY634130
from Aneura pinguis GA2
from Riccardia sp. Q2/1b DQ368717
13
Tulasnella calospora AY152407
Tulasnella cystidiophora AY585831
Multiclavula mucida AF287875
Tulasnella clade A
0.05 substitutions/site

◀ **Fig. 3** Phylogenetic relationships of the detected tulasnelloid mycobionts of *Aneura pinguis*: maximum likelihood (ML) analysis of an alignment of a representative sequence spectrum of partial nuclear DNA sequences coding for the large ribosomal subunit (nucLSU rDNA). The tree was rooted with *Multiclavula mucida*. *Numbers on branches* designate ML-bootstrap values / MCMC estimates of posterior probabilities (only values exceeding 50% are shown). Sequences from the liverwort mycobionts from Ecuador and Europe are restricted to the designated clades, which are discussed in the text. Sequences from European samples are marked with a *square*. *OM*, orchid mycorrhiza

Four individuals of Ecuadorian *A. pinguis* s.l. showed simultaneous colonisation by tulasnelloid fungi that belong to different clades. Mycobionts of *A. pinguis* T1/1a are represented in clades 4 and 9; mycobionts of *A. pinguis* T2/1b are represented in clades 1 and 2; mycobionts of *A. pinguis* Q2/2c, in clades 1 and 5; and mycobionts of *A. pinguis* Q2/3a, in clades 3 and 10. In one case, mycobionts from one specimen are represented in three clades (*A. pinguis* Q5/2a, clades 1, 5, and 11), demonstrating the presence of three colonising fungal species in a single host individual.

Assessment of European sequences

Sequences of European mycobionts belong either to the *T. calospora* group (clades 7 and 8) or to one clade (clade 13) in the *Tulasnella* clade A (Fig. 3). All five European sequences obtained from *Tulasnella* clade A (one is shown

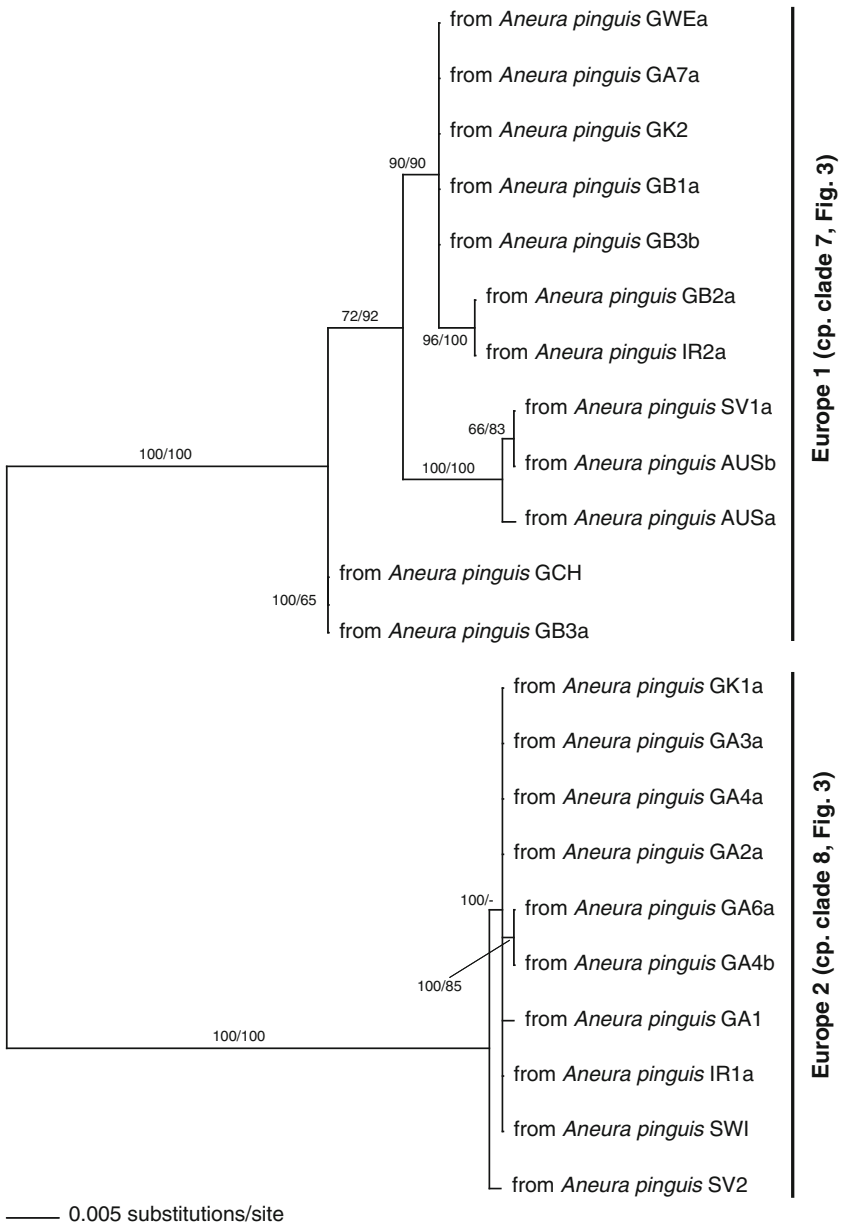

Fig. 4 Phylogenetic relationships of the detected European tulasnelloid mycobionts of *Aneura pinguis* belonging to clades 7 and 8 of Fig. 3: maximum likelihood (ML) analysis of an alignment of partial 5.8S–ITS2–partial nucLSU rDNA sequences. The tree was midpoint-rooted. *Numbers on branches* designate ML-bootstrap values / MCMC estimates of posterior probabilities (only values exceeding 50% are shown). Designated clades are discussed in the text

in Fig. 3) came from the region Allgäu in Southern Germany and show a similarity of 99% (not shown). Because of their heterogeneity, it was not possible to integrate ITS sequences of *Tulasnella* clades A and C in one phylogenetic analysis. Thus, we restricted the presentation of the European ITS sequences to the broad *T. calospora* group already mentioned above. The phylogenetic tree comprises all different sequences as well as identical sequences if they originated from different sampling regions (Fig. 4). Restricted to the nucLSU region considered in Fig. 3, all sequences of clade 7 and 8, respectively, are identical and are thus represented by only one example in Fig. 3. The investigated ITS sequences, however, are well divided into two groups, here referred to as Europe 1 and Europe 2 (Fig. 4). A total of 20 sequences (12 are shown in Fig. 4) belong to Europe 1 and come from ten different sample sites. Europe 2 consists of a total of 16 sequences (ten are shown in Fig. 4) from nine sites. The subdivision of the two clades is not reflected by geographical origin of the specimens. In each of the two clades, sequences from four different countries are represented. Europe 1 is more heterogeneous than Europe 2 and may be grouped into four well-supported subgroups. Colonisation by fungi belonging to more than one clade was not detected in the individuals of European *A. pinguis*, even when members of different clades were present at one locality (Table 1: sites GA2, GA3, and GA6).

Discussion

First records of Tulasnellales as mycobionts in Aneuraceae in the tropics were presented by us in a previous study on the diversity of mycorrhization in an Ecuadorian mountain rainforest (Kottke et al. 2008). Here, we present complementary data of Ecuadorian Aneuraceae mycobionts and compare the mycobiont spectrum of Ecuadorian samples to that of samples from Central Europe, Ireland, and Sweden. Our ultrastructural observations are in line with previous studies (Ligrone et al. 1993; Read et al. 2000; Duckett and Ligrone 2008) and support the importance of Tulasnellales as mycobionts of Aneuraceae. However, the presence of other fungal mycobionts cannot be excluded: firstly, since we obtained rDNA sequences from other fungi in preliminary PCR experiments using unspecific primers; and secondly, since hyphae with more or less uncurved parenthesomes could be detected in our TEM study in a few sections. All these samples showed tulasnelloid ultrastructural characters when further sections were consulted, and we were able to get tulasnelloid sequences from all samples with the help of *Tulasnella*-specific primers.

This supports the view that if not exclusively occupied, thalli were at least predominantly occupied by tulasnelloid symbionts, in spite of the wide geographical range and ecological flexibility of Aneuraceae. Nevertheless, significant genetic diversity is hidden behind this apparent uniformity, as revealed by our molecular investigations. The diversity of tulasnelloid mycobionts in *A. pinguis* s.l. approximately equals the diversity of tulasnelloids detected in the pleurothallid orchids at the Ecuadorian site (Fig. 4; cp. Suárez et al. 2006). Previous investigations on the glomeromycetous (Russell and Bulman 2005: *Marchantia*), the ascomycetous (Chambers et al. 1999; Upson et al. 2007: *Cephaloziella*; Pressel et al. 2008: *Pachyschistochila*), and the basidiomycetous symbionts (Kottke et al. 2003: *Calypogeia* and *Lophozia*) of liverworts suggested a lower diversity.

The remarkable differences in the fungal diversity pattern of *A. pinguis* s.l. between Europe and Ecuador provoke questions as to the possible reasons for this observation. Diversity was not correlated with the number of investigated individuals in Ecuador and Europe, which was nearly the same. Recent investigations also showed considerable diversity of mycorrhizal Tulasnellales in the northern hemisphere (Shefferson et al. 2007) as well as in the tropics (Suárez et al. 2006); potential mycobionts for Aneuraceae should therefore principally be available in both regions. The substrate type has no essential influence on fungal composition since Ecuadorian samples from soil and concrete show colonisation by several fungi, and members of the same fungal clade could be found in samples from different substrates. Nevertheless, the substrates of the liverworts are quite different between the two investigation areas; rotten wood (the preferred substrate of tropical specimens of *A. pinguis* s.l.) might be a more suitable substrate for facultatively mycorrhizal tulasnelloids than soil and rock (the preferred substrate of European specimens). In consideration of the high genetic diversity of *A. pinguis* s.l. in Europe (Wachowiak et al. 2007; Wickett and Goffinet 2008)—the same may hold for this taxon in the tropics—and our geographically and ecologically wide sampling, the restriction of the *Tulasnella* mycobionts to three out of 13 clades is astonishing. In contrast, in Ecuador, simultaneous colonisation by up to three *Tulasnella* mycobionts belonging to different clades could be detected in a single liverwort individual. Thus, the genetic variability of the host is probably not a crucial factor for the diversity of the mycobionts.

Previous investigations suggested that differences in altitude or ecological conditions may influence the mycobiont composition of Aneuraceae, thus assuming a broad spectrum of mycobiont species because of the heterogeneity of colonised habitats (Stahl 1949; Ligrone et al. 1993; Duckett et al. 2004). However, our study shows that ecological heterogeneity is not a prerequisite for a high mycobiont diversity in the studied tropical region. Ecolo-

gical factors might nevertheless have some influence, at least for European *A. pinguis* s.l. Here, the distribution of tulasnelloid sequences of the clades Europe 1 and 2 (Fig. 4; corresponding to clades 7 and 8 in Fig. 3) seems to be correlated with habitat conditions. As inferred from associated species, all habitats of members of Europe 2 are characterised by very wet and calcareous conditions (Table 2). The habitats of members of Europe 1 show less specific conditions and are more varied in terms of soil humidity and acidity.

The differences in mycobiont diversity between European and tropical members of *A. pinguis* s.l. may also have been influenced by the enormous difference in long-term development of both areas. Considering the past million years, the conditions at the Ecuadorian sites should have been unstable enough for several speciation processes but

Table 2 Location and ecological characterisation of the European sites with investigated samples of *Aneura pinguis* s.l.

Position of locality, altitude	Locality ID	Habitat	Associated bryophytes
Austria			
Vorarlberg, Montafon, between Vergalden and Gargellen, 1,500 m	AUS	Calcareous fen	*Bryum pseudotriquetrum*, *Campylium stellatum*, *Cratoneuron commutatum*, and *Rhizomnium punctatum*
Ireland			
Roadside SW of Killarney, 250 m	IR1	Calcareous tufa	*Cratoneuron filicinum*
Roadside SW of Killarney, 200 m	IR2	Base rich siliceous rock	*Amphidium mougeotii*, *Entosthodon attenuatus*, and *Tortella tortuosa*
Sweden			
Härjedalen, Tännas, west part of mire 1 km WSW of Mt. Hem-Kröket, 620 m	SV1	Calcareous fen	*Amblyodon dealbatus*, *Bryum pseudotriquetrum*, *Campylium stellatum*, *Cratoneuron falcatum*, and *Meesia uliginosa*
Härjedalen, Tännas, 200 m north of Lake Mejsatjärnen, 800 m	SV2	Calcareous fen	*Cratoneuron commutatum* and *Campylium stellatum*
Switzerland			
Canton Waadt, St. Georgen, L´eau-pendante, 1,250 m	SWI	Wet limestone rock	*Cratoneuron commutatum* and *Eucladium verticillatum*
Germany, Allgäu			
Markt Rettenberg, Bayersried, below Schönlings, 760 m	GA1	Calcareous tufa	*Cratoneuron commutatum*, *Eucladium verticillatum*, and *Pellia endiviifolia*
Seeg, near bridge over the Vobach, 785 m	GA2	Calcareous tufa	*Cratoneuron commutatum*, *Eucladium verticillatum*, and *Preissia quadrata*
Rottach reservoir, south side, 840 m	GA3	Calcareous tufa	*Cratoneuron commutatum* and *Bryum pseudotriquetrum*
Sonthofen, cascade of Hinang, 900 m	GA4	Calcareous tufa	*Cratoneuron commutatum*
Unterschmayenbach, ravine of Rotwasser, 950 m	GA5	Calcareous fen	*Cratoneuron commutatum*
Between Oberjoch and Schattwald, 1,200 m	GA6	Calcareous tufa	*Cratoneuron commutatum*
Wertacher Hörnle N Hindelang, 1,600 m	GA7	Base rich spring	*Bryum pseudotriquetrum*, *Fissidens adianthoides*, and *Hypnum lindbergii*
Germany, Black Forest			
Rotmurgtal NW Obertal, 650 m	GB1	Wet embankment	*Chiloscyphus pallescens* and *Rhizomnium punctatum*
Hinter Heubach NE St. Roman, 612 m	GB2	Spring fen	*Bryum pseudotriquetrum* and *Pellia endiviifolia*
Wildschapbach, 510 m	GB3	Base rich fen	*Fissidens adianthoides*, *Riccardia multifida*, and *Trichocolea tomentella*
Germany, Swabian Keuper highland			
NNE Harbach, 305 m	GK1	Calcareous tufa	*Campylium stellatum*, *Cratoneuron commutatum*, and *Eucladium verticillatum*
Heidenklinge W Stuttgart, 360 m	GK2	Wet embankment	*Dicranella varia*, *Eurhynchium hians*, and *Pellia endiviifolia*
Germany, others			
Chiemsee	GCH	Reed swamp	*Cratoneuron filicinum* and *Pellia endiviifolia*
Weserbergland, Hooptal east of Negenborn, 220 m	GWE	Wet sandstone rock	*Brachythecium rutabulum*, *Rhizomnium punctatum*, and *Sanionia uncinata*

generally stable enough for the preservation of both symbiotic partners and the symbiotic interaction itself (cp. McKenna and Farrell 2006). On the other hand, adaptation processes of the mycorrhiza-like interaction presumably were negatively influenced at the European sites by Pleistocene disturbances.

Aneuraceae and Tulasnellales both are diverse groups of organisms with insufficiently known biodiversity and phylogenetic relationships (cp. Duckett and Ligrone 2008; Moncalvo et al. 2006). Many more studies, particularly molecular studies, are needed to test more detailed hypotheses on ecological and biogeographical aspects of their interaction.

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