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ORIGINAL ARTICLE

Genetic diversity and photobiont associations in selected taxa of the *Tephromela atra* group (Lecanorales, lichenised Ascomycota)

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Abstract Mycobiont and photobiont genetic diversity was investigated in four taxa of the *Tephromela atra* complex, which differ in ecology and substratum preference (from siliceous rocks, limestone to bark), and are differently interpreted by taxonomists. Phylogenetic analyses were performed using mycobiont nuclear ITS, beta tubulin and homologous polyketide synthase gene (PKS) sequences obtained from freshly collected material sampled from the Mediterranean region to the Southern Alps. The silicicolous samples from the Alps form a basal lineage of the entire complex, and despite the morphological similarity, they do not form a monophyletic group with the Mediterranean samples. No resolution was found among the calcicolous and the silicicolous taxa from Mediterranean habitats, which are traditionally segregated at variety or species level. The epiphytic taxon, although nested with the other ecotypes, splits in two well-supported lineages. Among the four taxa, *Tephromela grumosa* is the only morphologically, chemically and genetically distinct taxon. However, it is also nested in the large *T. atra* complex. Phylogenetic analysis of photobionts ITS sequences revealed that thalli from the Mediterranean region are associated with two distinct lineages of *Trebouxia*, but the lineages are not correlated with substrate or mycobiont phenotype. The thalli from the Alps are exclusively associated with *T. simplex*, suggesting a protracted isolation from the other lineages.

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Introduction

In lichen systematics, a "morphological" species concept (Du Rietz 1930; Mayr 1963) is broadly applied. It is generally accepted that those phenotypic differences are of relevance which correlate with variations in ecology, lifestyle, or geographic distribution. Without such correlations, delimitation of lichen species is difficult, and phenotypic variation is then controversially interpreted by lichenologists. Such delimitation problems affect many geographically widespread crustose lichens. Some of these can display an extraordinarily high morphological heterogeneity which is only partly attributable to different ecological conditions. These lichens represent taxonomically unresolved species complexes (Grube and Kroken 2000), which are often burdened with a complicate nomenclature.

The genus *Tephromela* was introduced by Choisy (1929), to allocate a single species of *Lecanora* with dark purple pigmented hymenium and straight conidiospores. Neglected by most authors before, the genus was resurrected by Hafellner (1984) and placed in a new family, Tephromelataceae, within the Lecanorales (Hafellner 1984). After the recent segregation of the phenotypically deviating *T. armeniaca* and *T. aglaea* in the genus *Calvitimela* (Hafellner and Türk 2001), the remaining "core" *Tephromela* species share diagnostic anatomical characters with the type species, *Tephromela atra* (Huds.) Hafellner & Kalb. The species belonging to *Tephromela* s.str., are distinguishable by the poorly developed true exciple, dark violaceous hymenium, *Bacidia*-type ascus with amyloid tholus, un-

branched paraphyses, and straight, chain-forming conidiospores (Hafellner 1984; Purvis et al. 1992). According to recent phylogenetic studies, *Tephromela* is not closely related to Lecanoraceae, but rather forms a monophyletic group with *Mycoblastus* (Mycoblastaceae; Miadlikowska et al. 2006). With the exception of the peculiar shrubby Himalayan *Tephromela siphulodes* (Poelt and Grube 1993), all *Tephromela* species are crustose, and distinguished by more or less clear phenotypic characters or life-style (Rambold 1993; the relationships with *Heppsora*, a poorly understood squamulose relative requires further study; see also Poelt and Grube 1993).

The cosmopolitan *Tephromela atra* is morphologically and ecologically rather polymorphic, and a *par excellence* example for a lichen species complex with a controversial nomenclature. In the present contribution, we study the phylogenetic relationships among four entities of this taxonomically difficult group, which are characterised by distinct substrate preference or reproductive mode, and show slight morphological differences. Two of them occur on siliceous and intermediate rocks, and one on limestone and bark, respectively. The material was collected by the authors in southern Europe on a wide range of substrata from the eu-Mediterranean to the Alpine vegetation belts.

Materials and methods

The taxa

In Europe, fertile thalli without soredia occurring on siliceous rocks are traditionally assigned to *Tephromela atra* s.str., in agreement with the original collection of Hudson (1762). As currently understood, this holarctic taxon has a uniform composition of secondary metabolites, including atranorin, α-collatolic acid, alectoronic acid (Purvis 1992; Hesbacher et al. 1996), shows a wide ecological amplitude, and colonises different substrates, ranging from ultramafic rocks to sandstone and man-made materials such as bricks and cement. In the eu-Mediterranean belt it is restricted to sheltered situations, but elsewhere it preferentially occurs in sun-exposed habitats, although it can also occur in shaded ones.

Thalli occurring on limestone and dolomite are particularly common in open, sunny exposed habitats throughout the Mediterranean region, from sea level to the montane belt (Salvadori and Tretiach 2002). They are absent from the Alps (Nimis and Tretiach 1999), and become progressively rarer northwards, reaching southern Sweden (Fröberg 1989). The thalli occurring on carbonatic rocks are thick, cretaceous, with slightly effigurate margins, and contain two further unidentified secondary metabolites (Huneck et al. 1997). Taxonomically, this eco-morphotype has been interpreted differently, as some authors merged it in *T. atra* s.str. (Fröberg 1989; Wirth 1995; Egea and Alonso 1996; Galun and Mukhtar 1996; Seaward 1996; Llimona and Hladun 2001), or recognised it at form (*T. atra* f. *pachythallina*), variety (*T. atra* var. *calcarea* (Jatta) Clauzade & Cl.Roux: Clauzade and Cl.Roux 1985; Santesson 1993; Litterski and Mayrhofer 1998; Grube et al. 2001; Nimis and Martellos 2003), or at species (*T. cypria* (Körb.) Hafellner: Kalb and Hafellner 1992) level. It will be mentioned throughout the text as "*T. calcarea*".

Thalli growing on bark are not rare in the chestnut and beech belts of southern European mountains, being rarer at lower altitudes (Nimis 1993). They show a clear preference for the first stages of bark colonisation, and are more frequent in humid sites. Taxonomically, they were alternatively segregated from *T. atra* s.str. as var. *torulosa* (Hafellner 1992; Kalb and Hafellner 1992; Suppan et al. 2000; Hafellner and Türk 2001; Nimis and Martellos 2003; Türk et al. 2004), recognised at species level (Motyka 1996), or considered a mere substratum variant of no taxonomic value, as the colonisation of bark would be induced by favourable climatic conditions (Clauzade and Roux 1985; Wei 1991; Purvis et al. 1992; Wirth 1995; Litterski and Mayrhofer 1998; Limona and Hladun 2001; Clerc 2004; Nash et al. 2004). This taxon will be mentioned throughout the text as "*T. torulosa*".

The fourth taxon, closely related to *Tephromela atra* s. str., is *T. grumosa* (Pers.) Hafellner & Roux. Mainly a cool-temperate lichen, found on steeply inclined, acidic siliceous rocks, *T. grumosa* is morphologically and chemically clearly distinct, as the thallus is often completely sorediate. The species is almost always sterile, and differs also by the presence of lichesterinic acid (Purvis 1992), and the absence of the α-collatolic and alectoronic acid of *T. atra* s.str., apart from atranorin. *Tephromela grumosa* is well accepted as a species by the majority of the authors (Clauzade and Roux 1985; Purvis et al.1992; Santesson 1993; Brodo et al. 1994; Wirth 1995; Limona and Hladun 2001; Hafellner and Türk 2001; Nimis and Martellos 2003; Clerc 2004), having also its own specific lichenicolous fungus, *Niesslia robusta* (Tretiach 2002). Only a few authors retain it as a further variety of *T. atra* (Maheu and Gillet 1992; Jüriado 1997). This taxon will be mentioned throughout the text as "*T. grumosa*".

A fifth taxon, *T. pertusarioides* (Degel.) Hafellner & Roux, could not be included in the analysis, as it was not recently collected in the survey area.

Sampling

For this study, two sets of samples were collected. The first consists of 20–30 thalli from four populations of *T. atra* s. str., *T. calcarea, T. torulosa*, and *T. grumosa* co-occurring

in a limited area of 25 km^2 (Mt. Amiata, Central Italy) (Fig. 1 insert a, sites 1–3), and therefore defined as "sympatric". The second set consists in 3 to 10 thalli of each taxon eventually present in 24 further sites (Fig. 1, sites 4–27) scattered throughout Italy (sites 1–18), Greece (sites 19–23), and Austria (sites 24–27), from 300 to 2500 m altitude, i.e. from the Mediterranean to the Alpine vegetation belts, on a wide array of substrata (silicates, limestone, dolomite, bricks, sandstone, bark) (Table 1). Voucher material is deposited in the herbarium of the Department of Biology, University of Trieste (TSB).

Molecular analysis: DNA extraction, PCR-amplification and sequencing

DNA extraction was performed on 20 thalli for each sympatric population of sites 1–3, and 3–10 for each taxon collected in sites 4–27. Saxicolous thalli were scraped off from an area of c. 0.5 cm^2, while only the apothecia and the upper portions were taken from the corticolous thalli to avoid contaminants from the bark below. The DNA isolation protocol of Cubero et al. (1999) was applied for all samples. However, c. 70% of those occurring on limestone were not successfully amplified, notwithstanding several trials in which experimental conditions (DNA elution, $MgCl_2$ concentration, pH of lyses buffer, etc.) were modified. In these cases, the DNAeasy Plant Mini Kit (QIAGEN, Vienna, Austria) and the GenElute™ Plant Genomic DNA Kit (SIGMA, Missouri, USA) was successfully applied to obtain sequence data from additional 10 specimens.

The mycobiont genetic diversity was analysed with sequence data of the ITS region, beta tubulin fragment, and the ketoacyl synthase (KS) domain of a homologous polyketide synthase gene (PKS). The ITS region was amplified with the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), the beta tubulin with primers bt2a and bt2b (Glass and Donaldson 1995), and the KS domain with primers LC1 and LC2c (Bingle et al. 1999). In some cases PCR products of the KS domains were cloned in *E. coli* Xl-1blue using the pGEM-T Easy Vector System (Promega, Madison, USA) following the manufacturer's instructions. The photobiont diversity was analysed using the ITS region by the *Trebouxia*-specific primers ITS1T and ITS4T (Kroken and Taylor 2000).

PCR reactions were prepared for a 30 µl final volume containing 4.05 µl double-distilled water, 3 µl 10× *Taq* polymerase reaction buffer (10 mM Tris pH 8.3), 1.8 µl $MgCl_2$ (25 mM), 3 µl of dNTPs (2.5 mM), 0.15 µl *Taq* DNA polymerase, 1.5 µl for each of the 10 µM primers. PCR amplifications of both the mycobiont and photobiont ITS region were performed under the following conditions: one initial heating step of 2 min at 94°C linked to 30 cycles of 1 min at 94°C, 1 min at 53°C, 2 min at 72°C, and one final extension step of 7 min at 72°C after which the samples were kept at 4°C. The PCR amplification for the beta tubulin genes was performed with 58°C as annealing temperature, while the PCR amplification of the PKS KS

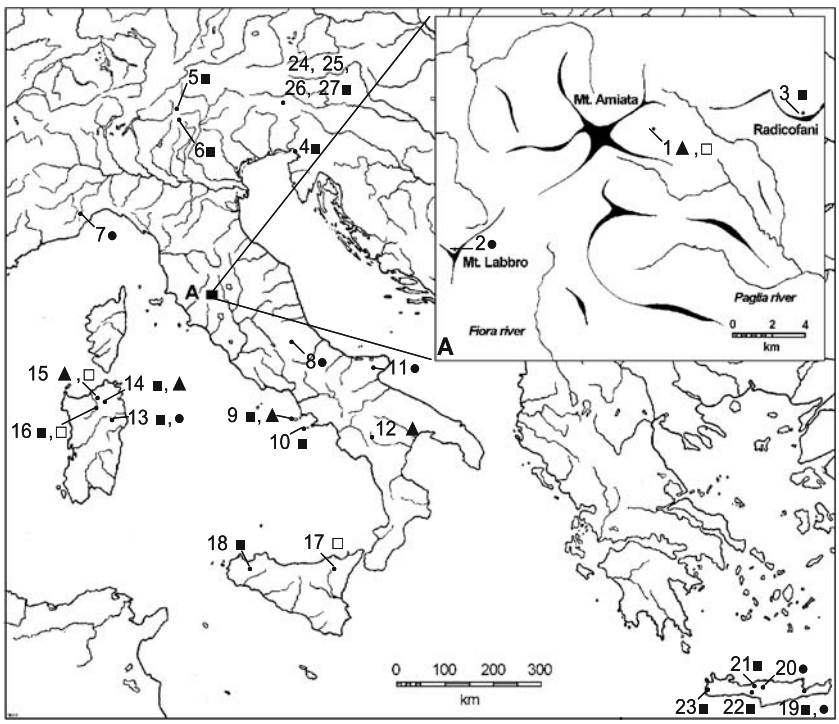

Fig. 1 Map of sampling sites of *Tephromela atra* s.str. (■), *T. calcarea* (●), *T. torulosa* (▲), *T. grumosa* (□). *Insert a*: sympatric populations of Mt. Amiata, central Italy

Table 1 Geographic provenience of specimens. Number of collected and genetically analysed samples are reported for each taxon in each locality

Sample collected (sample analysed)

Locality	*T. atra* s.str	*T. calcarea*	*T. torulosa*	*T. grumosa*
1 Italy, Toscana, Abbadia San Salvatore, Acquapassante, 2004, *Tretiach*.			12 (9)	20 (13)
2 Italy, Toscana, Arcidosso, Mt. Labbro, 2004, *Tretiach*.		16 (11)		
3 Italy, Toscana, Radicofani, 2004, *Tretiach*.	12 (11)			
4 Italy, Friuli-Venezia Giulia, Conconello, 2006, *Tretiach*.	4 (2)			
5 Italy, Trentino Alto-Adige, National Park of Mt. Stelvio, Mt. La Cascata, 2006, *Tretiach & Muggia*.	3 (2)			
6 Italy, Lombardia, Regional Park of Adamello Massiv, P.so Gallinera, 2006, *Tretiach & Muggia*.	1 (1)			
7 Italy, Liguria, Alpi Liguri, loc. Monesi, 2000, *Tretiach & Nimis*.	2 (1)			
8 Italy, Abruzzo, Rocca Calascio, 2004, *Tretiach*.		3 (2)		
9 Italy, Campania, Ischia, Mt. Trippodi, 2006, *Muggia & Tretiach*.	13 (4)		18 (4)	
10 Italy, Campania, Capri, 2006, *Muggia & Tretiach*.	5 (2)			
11 Italy, Puglia, Gargano Promontory, S. Angelo, S.ta Maria di Pulsano, 2004, *Tretiach*.		4 (2)		
12 Italy, Basilicata, Calvello, Cerro Falcone, 2003, *Potenza*.			1 (1)	
13 Italy, Sardegna, Siniscola, Mt. Albo, Punta Cupetti, 2006, *Muggia & Tretiach*.	4 (1)	12 (1)		
14 Italy, Sardegna, Tempio Pausania, P.so Limbara, 2006, *Muggia & Tretiach*.	9 (1)		11 (1)	
15 Italy, Sardegna, Tempio Pausania, Aggius, 2006, *Muggia & Tretiach*.			6 (2)	3 (2)
16 Italy, Sardegna, Marghine Massiv, Ortakis, Villa Piercy, 2006, *Muggia & Tretiach*.	6 (1)			9 (3)
17 Italy, Sicilia, Montalbano Elicona, from Mt. Castellazzo to Roccella, 2005, *Tretiach.*				1 (1)
18 Italy, Sicilia, Nicolosi, to Rif. Sapienza, 2005, *Tretiach*.	5 (1)			
19 Greece, Crete, municipality of Ierapetra, Selakano Forest, 2006, *Tretiach*.	11 (3)	7 (2)		
20 Greece, Crete, municipality of Anoghia, road from Zominthos to Schinakas, 2006, *Tretiach*.		9 (2)		
21 Greece, Crete, municipality of Tylisos, from Kamaraki to Anoghia, 2006, *Tretiach*.	9 (3)			
22 Greece, Crete, municipality of Spili, from Moulines to Aktounda, 2006, *Tretiach*.	10 (2)			
23 Greece, Crete, municipality of Innahorion, Elos, road to Aghios Dikeos, 2006, Tretiach.	3 (2)			
24 Austria, Stiria, Koralpe, Speiksee, 2005, *Muggia & Hafellner.*	1			
25 Austria, Stiria, Peteralm, Peter Kogel, 2005, *Muggia & Hafellner.*	1			
26 Austria, Stiria, Ameringkogel, 2005, *Muggia & Hafellner.*	2			
27 Austria, Carinzia, St. Leonard Alm, 2005, *Muggia & Hafellner.*	2 (1)			

domain required an initial heating step of 2 min at 94°C, linked to 36 cycles of 45 s at 94°C, 45 s annealing conditions alternative with 58–52°C touch-down during the first six cycles and 52°C for the remaining 30 cycles, 1 min 45 s at 72°C, and one final extension step of 7 min at 72°C, after which the samples were kept at 4°C. PCR products were purified using QIAGEN quick spin columns (Qiagen, Vienna, Austria) following the manufacturer's instructions. Both complementary strands were sequenced, and sequences were either run by MWG Biotech-AG Company (Regensburg, Germany) or by ABI 310 (Applera, Vienna, Austria), and assembled in BioEdit (Hall 1999, http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). Specimen and DNA related data are listed with isolation, GenBank accession, and TSB herbarium collection numbers in Table 2.

Alignment and phylogenetic analysis

Four phylogenetic analyses were performed. The first two analyses focused on the ITS sequences belonging to the mycobionts (n=46) and to the photobionts (n=35) of samples belonging to the four sympatric populations of Mt. Amiata (sites 1–3). The sequences of *Tephromela atra* AY541279 and *Trebouxia arboricola* AF389915 were retrieved from GenBank and used as outgroups of fungal and algal datasets, respectively, in these analyses. The second two analyses focused on selected representatives of mycobionts (n=72) and photobionts (n=62) sequences originating from all the 27 sampling sites of Austria, Greece, and Italy. The mycobiont phylogeny was obtained by combining data of the three loci: ITS, beta tubulin, and PKS KS domain. Two new *Lecanora cenisia* ITS sequences (TSB 37464, TSB 37478), and ITS sequences of three representatives of the genus *Lecanora*, *Mycoblastus sanguinarius* (DQ782842), *Calvitimela armeniaca* (AY541278) and *Tephromela atra* (AY541279, DQ534487) were retrieved from GenBank and included in the analysis. Sequences of *Lecanora*, the genus from which *Tephromela* was once segregated, were selected as outgroup. PKS KS sequences were used in the combined

Table 2 Samples of *Tephromela* with GenBank accessions, isolation, and herbarium numbers (top)

Species	ID isolation	ITS micobiont	ITS photobiont	PKS	b tubulin	TSB herbarium N.
Tephromela atra s.str.	A1A	EU558593	EU551473	–	–	38665
	A2A	EU558594	EU551474	EU551551	EU558545	38666
	A4A	EU558595	EU551475	EU551552	–	38668
	A11A	–	EU551476	EU551553	–	37083
	A12	EU558596	–	–	EU558546	37084
	A14	EU558597	–	–	–	37086
	A15	EU558598	–	–	–	37087
	A16	EU558599	EU551477	EU551554	EU558547	37088
	A17	EU558600	EU551478	–	–	37089
	A18	EU558601	EU551479	EF363876	–	37090
	A19	EU558602	EU551480	EU551555	–	38627
	A20	EU558603	EU551481	EU551556	EU558548	37091
	A21A	–	EU551482	–	–	37092
	A22A	EU558691	EU551483	–	–	37093
	L99	EU558642	EU551512	EU551558	EU558559	37094
	L100	EU558643	EU551513	EU551559	–	37110
	L146	EU558644	–	–	–	37096
	L147	–	EU551514	–	–	37097
	L149	–	EU551515	EU551598	–	37099
	L220	EU558646	EU551517	EU551561	EU558560	37116
	L222	EU558647	EU551518	EU551562	EU558561	37118
	L223	EU558648	EU551519	EU551563	EU558562	37119
	L225	EU558649	EU551520	EU551564	EU558563	37121
	L228	EU558650	–	–	EU558564	37124
	L246	EU558655	–	–	–	37137
	L248	EU558656	–	–	EU558569	37133
	L284	EU558661	EU551526	EU551577		37465
	L286	EU558662	EU551527	EU551566	EU558574	37467
	L289	EU558663	EU551528	EU551574	EU558573	37470
	L306	EU558668	EU551534	EU551575	EU558578	37486
	L318	EU558672	–	–	–	37879
	L319	–	EU551538	EU551592	–	37880
	L366	EU558675	EU551541	EU551560	EU558583	37901
	L368	EU558676	EU551542	–	EU558584	37903
	L369	EU558677	EU551543	–	EU558585	37904
	L399	EU558678	–	–	EU558586	33382
	L408	EU558684	EU551544	EU551580	EU558589	37917
	L410	EU558685	EU551545	EU551578	–	37919
	L411	–	–	EU551596	–	37920
	L413	EU558686	EU551546	EU551579	–	37922
	L414	EU558687	EU551547	EU551588	EU558590	37923
	L415	EU558688	–	EU551581	EU558591	37924
	L416	–	EU551548	EU551597	–	37925
	L417	–	EU551549	–	–	37926
	L419	EU558689	–	EU551586	–	37928
	L421	EU558690	EU551550	EU551582	EU558592	37930
T. calcarea	C11	EU558604	EU551484	–	–	37906
	C12	EU558605	–	–	–	37936
	C13	EU558606	EU551485	–	–	37937
	C14	EU558607	–	–	–	37938
	C15	EU558608	EU551486	–	–	37939
	C16	EU558609	–	–	EU558549	37940
	C17	EU558610	–	–	–	38465
	C18	EU558611	EU551487	–	–	37941
	C19	EU558612	EU551488	–	–	37907
	C20	EU558613	EU551489	–	–	37908

Table 2 (continued)

Species	ID isolation	ITS micobiont	ITS photobiont	PKS	b tubulin	TSB herbarium N.
	C21	EU558614	–	–	–	37942
	C22	EU558615	EU551490	–	EU558550	37943
	L90	–	EU551508	EU551594	–	37102
	L91	EU558638	–	EU551595	EU558554	37103
	L93	–	–	–	EU558555	37105
	L94	EU558639	–	EU551557	EU558556	37106
	L95	–	EU551509	EU551593	–	37107
	L96	EU558640	EU551510	EU551576	EU558557	37108
	L272	EU558657	–	–	EU558570	37453
	L273	EU558658	–	–	EU558571	37454
	L275	EU558659	–	–	–	37456
	L280	EU558660	EU551524	–	EU558572	37461
	L400	EU558679	–	–	–	37909
	L401	EU558680	–	–	–	37910
	L403	EU558681	–	–	–	37912
	L405	EU558682	–	–	EU558587	37914
	L406	EU558683	–	–	EU558588	37915
T. torulosa	T1	EU558616	EU551491	EU551599	–	38695
	T2	EU558617	EU551492	EU551600	–	38696
	T3	EU558618	EU551493	EU551601	EU558551	38697
	T4	EU558619	EU551494	–	–	38698
	T5	EU558620	EU551495	–	EU558552	38699
	T13	EU558621	EU551496	–	–	37944
	T15	EU558622	–	–	–	37945
	T16	EU558623	–	–	–	38466
	T17	EU558624	–	–	–	38467
	L150	EU558645	–	–	–	37946
	L229	EU558651	EU551521	EU551584	EU558565	37125
	L230	EU558652	EU551522	EU551585	EU558566	37126
	L234	EU558653	–	–	EU558567	37130
	L235	EU558654	–	–	EU558568	37131
	L292	EU558664	EU551530	EU551602	EU558575	37472
	L296	EU558665	EU551531	EU551587	EU558576	37476
	L297	EU558666	EU551532	–	EU558577	37477
	L301	EU558667	–	EU551583	–	37481
	L303	–	EU551533	–	–	37483
T. grumosa	G2	EU558625	EU551497	EU551569	–	38686
	G3	EU558626	EU551498	–	–	38687
	G4A	–	EU551499	EU551591	–	38688
	G6A	–	EU551500	–	–	38690
	G7A	–	EU551501	EU551589	–	38691
	G8A	–	EU551502	EU551590	–	38692
	G11	EU558627	–	–	–	37071
	G12	EU558628	EU551503	–	–	37070
	G13	EU558629	–	–	–	37072
	G14	EU558630	–	–	–	37073
	G15	EU558631	–	EU551567	EU558553	37075
	G16	EU558632	–	–	–	37074
	G17	EU558633	–	EF363877	–	37076
	G18	EU558634	–	–	–	37077
	G19	–	EU551504	–	–	37078
	G20	EU558635	EU551505	EU551568	–	37079
	G21	EU558636	EU551506	–	–	37080
	G22	EU558637	EU551507	–	–	37081
	L97	EU558641	EU551511	EU551565	EU558558	37082
	L308	EU558669	EU551535	–	EU558579	37488

Table 2 (continued)

Species	ID isolation	ITS micobiont	ITS photobiont	PKS	b tubulin	TSB herbarium N.
	L310	EU558670	EU551536	EU551573	EU558580	37490
	L311	EU558671	EU551537	EU551572	–	37491
	L322	EU558673	EU551539	EU551571	EU558581	37881
	L323	EU558674	EU551540	EU551570	EU558582	37882
Lecanora cenisia	L152	–	EU551516	EU558542	–	37100
	L283	EU558540	EU551525	EU558543	–	37464
	L298	EU558541	EU551529	EU558544	–	37478
L. carpinea		AF070020	–	AY398715	DQ451617	
L. rupicola		AY398707	–	AY398724	DQ451633	
L. swarzii		AY541271	–	AY398728	DQ451612	

analysis since several previous analyses (including lichenised and non-lichenised fungal KS domains) confirmed their homology (not shown). A supplementary haplotype analysis with the TCS program (Clement et al. 2000) was used to show the genetic heterogeneity of this paralog (see Fig. 4, below).

The general analysis of the photobiont ITS included sequences of different *Trebouxia* species retrieved from GenBank (their name and accession number are reported in the phylogeny of Fig. 5, below). The tree was rooted arbitrarily with *Trebouxia higginsiae* AJ249574.

The respective alignments were produced automatically with ClustalW (Hall 1999, http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) as implemented in BioEdit 5.0.6 (Hall 1999) and then manually adjusted. The phylogenetic hypotheses for the four analyses were established using a Bayesian approach as implemented in the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003; Ronquist et al. 2005). The General Time Reversible substitution model (Rodriguez et al. 1990) with estimation of invariant sites and assuming a gamma distribution with four categories (GTR+I+G) was used for likelihood calculations. The optimal nucleotide substitution model was found before with the program MrModeltest 3.7 (written by J.A. A. Nylander and available at http://morphobank.ebc.uu.se/mrbayes/; Posada and Crandall 1998). For other parameters the default setting were used. For all the four phylogenetic analyses the Markov Chain Monte Carlo (MCMC) algorithm was run for 2 million generations, with 6 chains starting from a random tree and using the default temperature of 0.2. Every 100th trees were sampled while the first 200,000 generations were discarded as *burn-in*. The *burn-in* period was determined after testing for stationarity of likelihood values (i.e. by plotting the numbers of generation vs. the log probability and checking for the convergent diagnostic PSRF approaching 1; Ronquist et al. 2005; MrBayes 3.1 Manual). The consensus phylograms based on the mean branch lengths were calculated with the command *sumt* in MrBayes (see MrBayes 3.1 Manual; Ronquist et al. 2005). The phylogenetic trees were drawn using the program TreeView (Page 1996).

In each phylogenetic analysis the ambiguously aligned position were excluded. The MCMC parameters resulted for each Bayesian calculation and the numbers of included, variable and informative nucleotide positions are not reported but are available under request.

Results

Analysis of the sympatric populations: mycobionts

This analysis was performed with the alignment of 46 sequences obtained from 9 to 13 thalli of each substratum-defined taxon collected in the Mt. Amiata area (Fig. 1 insert a). The taxa segregate in two fully supported (100% PP) branches (Fig. 2a): one representing *T. grumosa* (clade I), and the other representing clades of the three ecotypic taxa of *T. atra* (clade II) occurring, respectively, on bark (IIa), limestone (IIb), and trachyandesite (IIc). Three samples from bark (TSB 38467), trachyandesite (TSB 37084), and calcareous rock (TSB 37936), respectively, do not cluster with the other samples of their corresponding ecotype. The first two are basal in the clade II, while the third is nested in the clade IIa of the epiphytic taxon.

Analysis of the sympatric populations: photobionts

The analysis was performed with an alignment of the photobiont ITS sequences obtained from principally the same thalli that were also included in the mycobiont ITS analysis (photobiont sequences could not be obtained for nine thalli). Two highly supported clades are identified, I and II (Fig. 2b). Clade I is composed by two sister clades (one highly supported) in which photobiont from the calcicolous and silicicolous taxa group together. Clade II groups, in a highly supported (99% PP) unresolved branch,

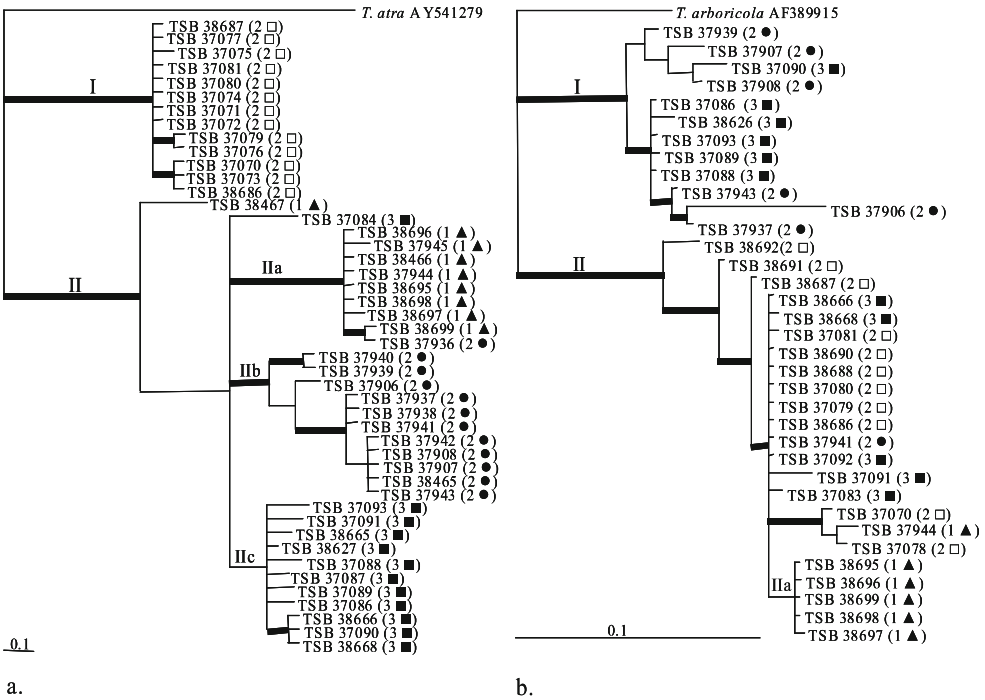

Fig. 2 Phylogenies of the sympatric populations of Mt. Amiata: **a** mycobionts, **b** photobionts. 50% majority-rule consensus trees of ITS sequences data from a B/MCMC sampling procedure. Posterior probabilities are indicated by the thickness of the branches (<90%, 90–94%, >95%), taxa are marked as in Fig. 1

photobiont sequences of *T. grumosa*, the remaining silicicolous *T. atra*, and the epiphytic *T. torulosa*, which form a smaller, moderately supported (87% PP) subclade (clade IIa of Fig. 2b). The phylogenetic hypothesis of the photobionts does not correlate with the substratum-defined taxa of the mycobionts, but the well-defined segregation of the two main clusters suggests the presence of two different photobiont species.

General phylogeny of the mycobionts

The general analysis combining ITS, β-tubulin and PKS KS domain loci includes 16 representatives of the 4 sympatric populations of Fig. 1 (insert), and 56 mycobionts of the same four taxa collected in the 24 other localities of Austria, Greece, and Italy. One to three samples are present for each taxon collected in each locality. With the exception of two single samples (TSB 37901 from the Adamello Massif, and TSB 33382 from the Ligurian Alps), the samples from the Southern Alps group together (clade I Fig. 3); five of them constitute a fully supported but unresolved subclade. The Alpine *T. atra* samples clearly segregate from the *T. grumosa* clade (II in Fig. 3), and from all the other samples, mostly of Mediterranean origin. The rest of the samples are grouped into three major sub branches: the epiphytic specimens split into two fully supported clades (clades III and IV of Fig. 3), while calcicolous and silicicolous specimens, as well as those found on intermediate substrata (sandstone, brick, etc.), cluster together intermixed in the unresolved and moderately supported third one (clade V of Fig. 3). Two samples of *T. atra* from siliceous rocks from insular Greece and Italy (respectively, TSB 37928 from Crete, and TSB 37486 from Sardinia) group together in a branch close to *T. grumosa*, while one sample of *T. calcarea* is nested in the clade IV of *T. torulosa*.

Haplotype analysis of the polyketide synthase genes

Sequences of the ketoacyl synthase domain were obtained for 55 representative mycobionts from almost all the localities of Fig. 1. Preliminary phylogenetic analyses (not shown) including type I PKS genes from other lichenised and not lichenised fungi, and the sequencing of some cloned PCR products, revealed that a single homolog was amplified with the primers used. All the KS sequences differ only in few sites in the coding regions but show more variation in their spliceosomal intron sequences. Six specimens of *Tephromela grumosa* show the same intron sequence, and two further sequences differ only in two nucleotides. Three different intron allels are found in the epiphytic taxon: one differs in eight nucleotides from the other two, which have only a difference of two nucleotides among each other. Intron sequences of the calcicolous and the silicicolous taxa can be divided into four groups, which differ from each other only in one nucleotide. Only the intron sequence of *T. atra* TSB 37094 is clearly different from all others.

The complete KS DNA sequences -including coding and intron parts- were considered in the haplotype analysis. In the haplotype network (Fig. 4), KS sequences of *T.*

Fig. 3 Phylogeny of the mycobionts: combined analysis of ITS, β-tubulin and polyketide synthase KS domain. For further information, see legend to Fig. 2

grumosa group in a well distinguished branch, quite distant from the intermixed KS sequences of the silicicolous and calcicolous taxa. KS domain of the epiphytic taxon segregate in two independent smaller networks which do not correlate with the geographical provenience of the samples.

General phylogeny of the photobionts

The general analysis of the photobionts included 19 representative sequences of the four sympatric mycobiont populations of Mt. Amiata, 43 sequences of samples from the remaining 24 localities of Austria, Greece, and Italy, 2

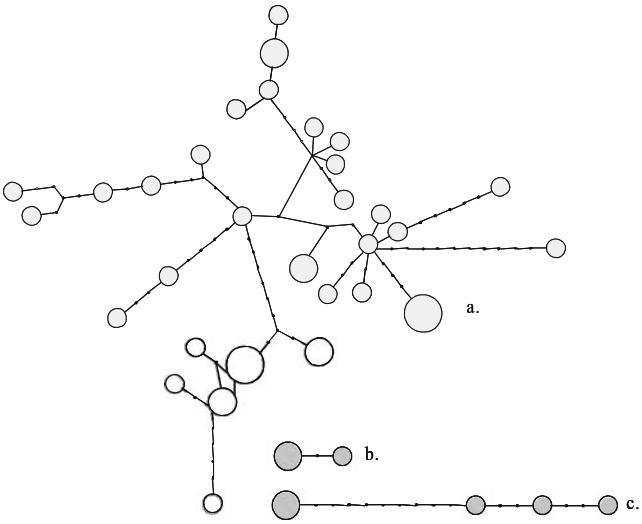

Fig. 4 Haplotype analysis of the polyketide synthase KS domain of the mycobionts: 95% probability networks of epilithic (**a**), and epiphytic (**b**, **c**) taxa. The size of the *circles* are proportional to the number of sampled sequences of the haplotype. *Small dots* represent not sampled haplotypes, the *line* between two haplotype is one mutational step. *Full circles* of (**a**) belong to *Tephromela atra* s.str and *T. calcarea*, *empty circles* belong to *T. grumosa*; (**b, c**): *T. torulosa*

sequences generated for *Lecanora cenisia*, and 38 sequences of *Trebouxia* species retrieved from GenBank, as nothing was known about the phylogenetic position of the photobiont(s) of *Tephromela* species. Six highly supported clades can be identified (Fig. 5). The photobionts of the alpine *Tephromela atra* form a highly supported subclade (clade Ia of Fig. 5) nested with samples of *Trebouxia simplex*, *T. jamesii* and further *Trebouxia* sp. photobionts from different *Lecanora* species. We named this clade (clade I) as *T. simplex sensu lato*, because it refers to a group of taxa, most of which have not been described yet (Hauck et al. 2007). Clades II, III and IV (Fig. 5) represent *T. impressa*, *T. incrustata* and *T. arboricola/ decolorans* respectively, and do not include any photobiont of our *Tephromela*, which cluster in two further highly supported clades (V, VI of Fig. 5). Clade V is sister of *T. arboricola*, while clade VI is well segregated from the others five ones: it groups, in an unresolved topology, the majority of our sequences. Clades V and VI also include selected sequences of the photobionts identified by Blaha et al. (2006) which represent new, undescribed *Trebouxia* species (namely, *Trebouxia* sp. 1: clade V; *Trebouxia* sp. 2: clade VI).

Discussion

The systematic identity of the taxa of *Tephromela atra* occurring on bark, limestone and siliceous rocks, respectively, has for long been a much debated question. Due to the lack of chemical differences (Purvis et al. 1992; Hesbacher et al. 1996), they are regarded as representing the same species by most authors, whereas others distinguished them at different infrageneric level owing to their different ecological requirements and slight morphological differences. On the other hand, there is clear evidence that *T. grumosa* can be considered a morphologically and chemically distinct species: the thallus is almost always sterile and completely sorediate, and contains atranorin and lichesterinic acid as secondary metabolites. Samples assigned to *T. grumosa* group together in the phylogenetic tree, which also contradicts the possibility of sporadic loss of sexuality in a broader concept of *T. atra*. There is little doubt that *T. grumosa* is a reproductively isolated lineage distinct from *Tephromela atra* s.lat. The interpretation of the remaining three taxonomic entities (*T. atra* s.str., *T. calcarea* and *T. torulosa*), which was mainly based on the different substrates, is more difficult.

The restricted ITS analysis of the sympatric populations of the M. Amiata sites identified four well-distinguished clades, yet not all of them fully supported, corresponding to each substratum-characterised taxon. It would be of future interest to investigate whether such supposed local genetic differentiation would also occur in other sympatric populations. However, evidence for an ongoing genetic differentiation is obscured when the sampling is extended to a wider geographic area. Further sampling inevitably introduces a greater genetic variation in the dataset, which could supersede the distinctiveness of clades detected in the sympatric analyses. The four lineages are in fact not resolved as monophyletic lineages agreeing with the substrate type in the multilocus tree of Fig. 3. In this multilocus analysis, *T. atra* from the Alps appears as a basal lineage of the entire complex, whereas *T. torulosa* splits into two lineages, and no resolution is found in *T. calcarea* and *T. atra* s.str. from Mediterranean habitats.

The presence of two distinct groups in *T. torulosa* could indicate that this taxon underwent genetic separation from the other Mediterranean ecotypes. However, it remains unclear whether the two clades formed by *T. torulosa* denote two separate lineages. Motyka (1996) in his posthumous monographic treatment of the genus described 22 species of *Tephromela,* of which 3 represented new epiphytic taxa: *T. depressula*, *T. hibernica*, *T. persordida.* The former differs from *T. torulosa* by a rimose-areolate thallus and the hymenium covered by a hyaline layer. *Tephromela hibernica* and *T. persordida* are mainly distinguished by anatomical characters, such as the large cells of the hyphae of the hypothecium and the irregularly septate paraphyses, respectively. Although Motyka (1996) was recently added to the list of *opera utrique oppressa* (McNeill et al. 2006), the validity of the above mentioned characters for the segregation of infrageneric entities within

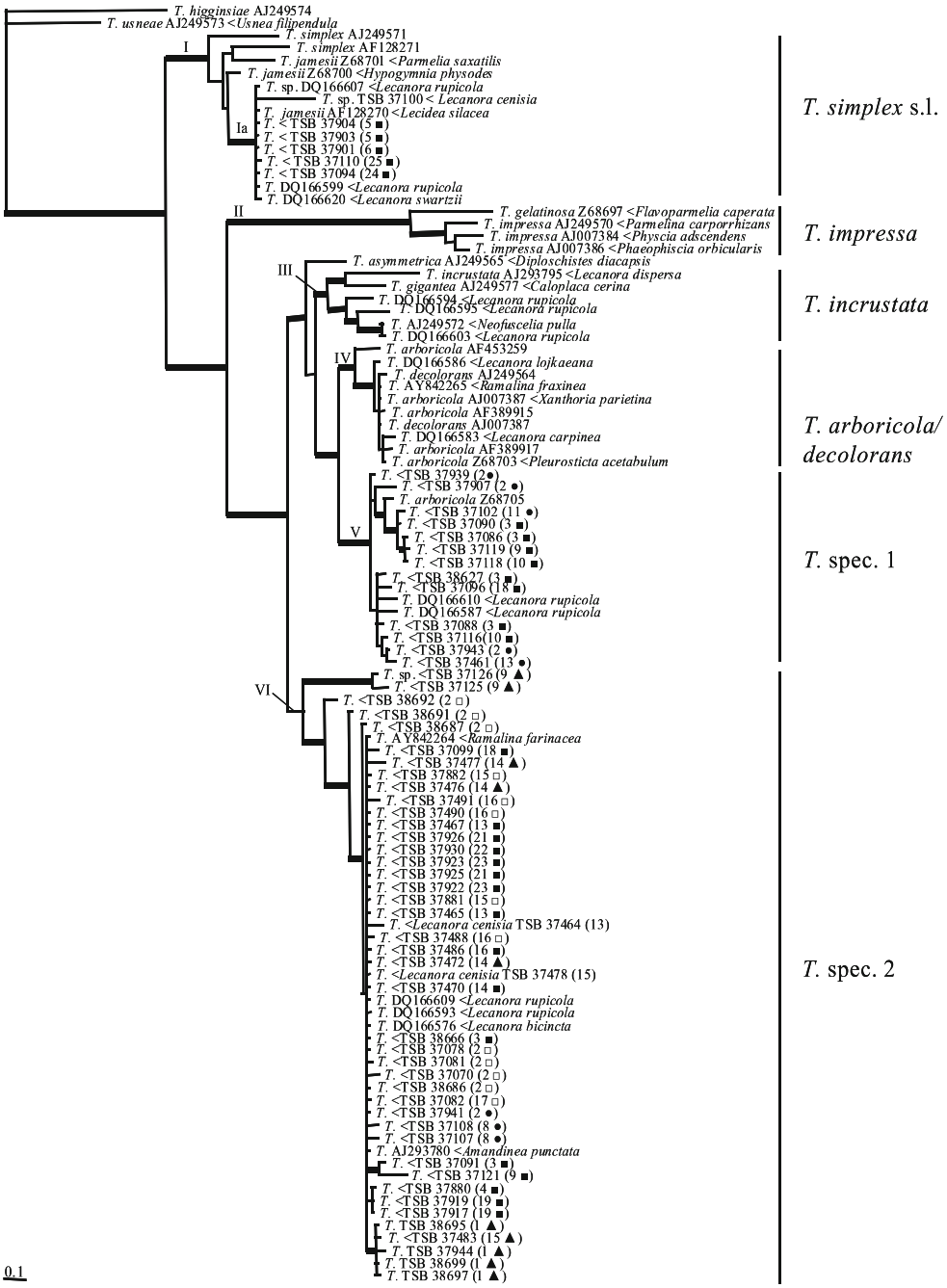

Fig. 5 Phylogeny of the photobionts. Samples selected from the GenBank are reported with their accession number. For further information, see legend to Fig. 2

T. torulosa should still be critically re-evaluated on the basis of Fig. 3.

Two possible hypotheses may explain the low resolution between the Mediterranean *T. calcarea* and *T. atra* s.str. First, this pattern could indicate that no genetic adaptation to the different ecological circumstances is present, and the morphological differences between the two taxa are simply induced by the accumulation of calcium oxalates in limestone occurring thalli, as suggested by Salvadori and Tretiach (2002). Alternatively, the specialisation to the different substrates has not yet led to reciprocally isolated alleles in our investigated loci. Incomplete lineage sorting was demonstrated in *Cavernularia* (Printzen et al. 2003), and in closely related neuropogonoid *Usnea* species (Wirtz et al. 2007), where haplotypes might even be shared among morphologically separable species. The process of lineage sorting may take exceptionally long in lichens (Printzen et al. 2003), which indicates that the results of population genetic studies in these organisms have to be interpreted with great caution, especially when few specimens are analysed. However, the lack of resolution with the mycobiont sequence data and the shared photobionts, the mere morphological and ecological differences between *T. calcarea* and *T. atra* s.str. from Mediterranean habitats,

does not support the distinction of species. Therefore, the form level seems to be appropriate for the taxonomic recognition of the calcicolous ecotype at the present stage, and the correct name is thus *T. atra* f. *pachythallina* Th.Fr. On the other hand, the unique alleles of *T. torulosa* and the distinctiveness of *T. atra* s.str. from the Alps indicate that further, genetically isolated, lineages are likely present in the *Tephromela atra* complex in southern Europe. As no further characters have not been studied in detail so far, at this moment we hesitate to recognise them from the taxonomic point of view.

The phylogenetic analyses of the ITS region of photobionts of *Tephromela atra* s.l. revealed two well-resolved clades which likely represent hitherto undescribed photobiont species. *Trebouxia* sp. 1 and *T.* sp. 2, respectively, have already been found by Helms et al. (2001) in representatives of the Physciaceace, and by Blaha et al. (2006) in *Lecanora rupicola*. Blaha et al. (2006) identified the new photobiont species from crustose thalli growing on acid to sub-neutral to base rich rocks in the Mediterranean region. In our study, they were also identified in thalli occurring on limestone, dolomite, bricks, sandstone, and bark. *Trebouxia* sp. 1 seems to be restricted to saxicolous thalli, and it was recently also identified in a group of calcicolous endolithic *Caloplaca* species (Muggia et al. 2008), but interestingly, it has never been found as photobiont of *T. grumosa*. *Trebouxia* sp. 2 does not display specificity for the substratum, nor for the fungal partner, and was found as photobiont of foliose lichens as well (Helms et al. 2001). The mycobionts of our saxicolous Mediterranean *Tephromela* do not exhibit a high specificity for their photobionts, in agreement with the findings of Blaha and Grube (2006). In some cases, individual thalli of the same population form symbioses with either *Trebouxia* sp. 1 or sp. 2. Nevertheless, in the Mediterranean region a higher selectivity for the photosynthetic partner was found

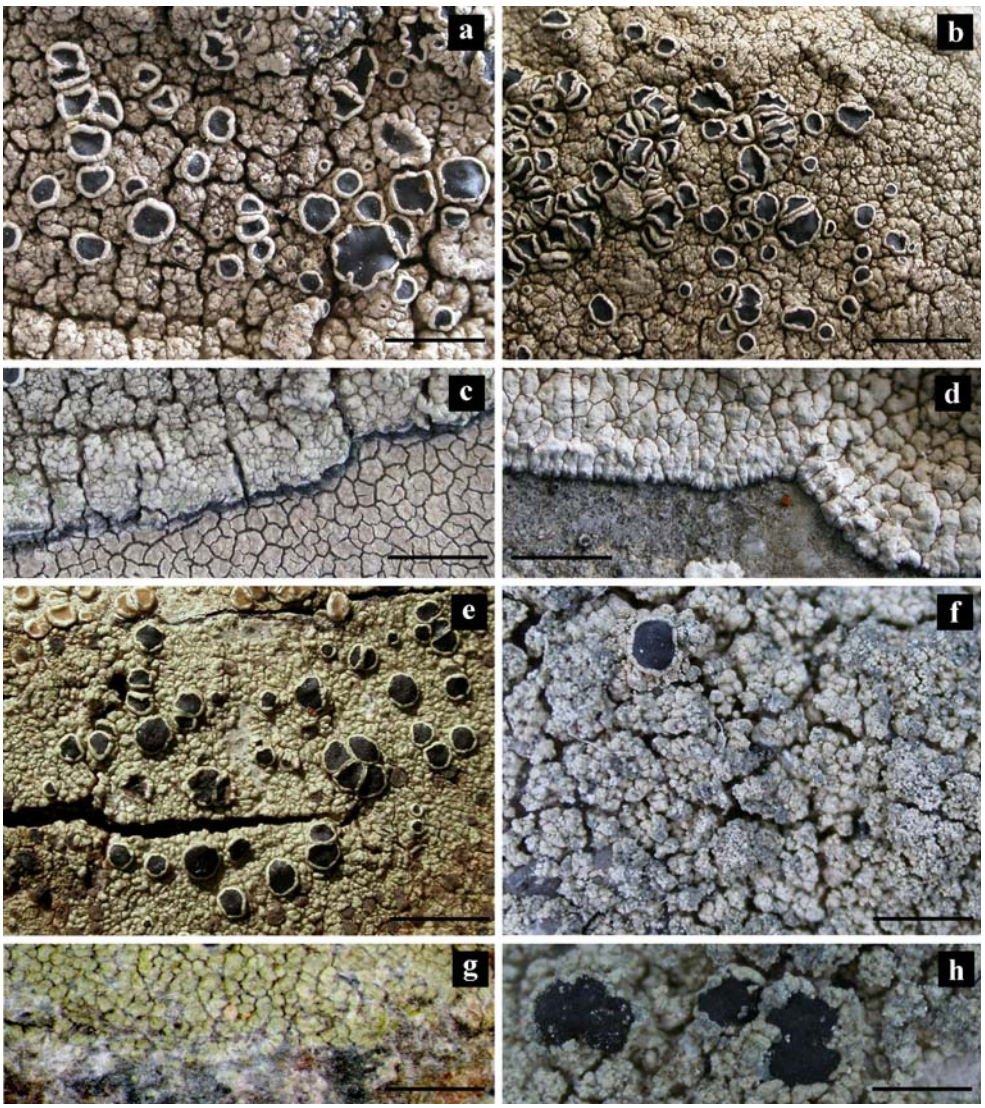

Fig. 6 Habitus of *Tephromela atra* s.str. (**a**, **c**: TSB 38666, *bar*=3 mm, 2.5 mm), *T. calcarea* (**b**: TSB 38684, *bar*= 6 mm; **d**: TSB 38677, *bar*= 4 mm), *T. torulosa* (**e**: TSB 38699; *bar*=2 mm; **g**: TSB 37128, *bar*=2.5 mm), and *T. grumosa* (**f**: TSB 33882, *bar*= 1.5 mm; **h**: TSB 34232, *bar*= 1.0 mm)

in *Fulgensia fulgida*, which associates strictly with *Trebouxia asymmetrica* (Beck et al. 2002).

A single algal lineage has been detected in *T. grumosa*. This can be justified either by its dominant vegetative propagation mode or by a high specificity for a certain photosynthetic partner. The second hypothesis accords well with the fact that, even though the dispersion by soredia (Fig. 6h) ensure *T. grumosa* the propagation of both symbionts together, this lichen reproduces sporadically by sexual means (Fig. 6f), which would allow reassociation with another photobiont lineage. A similar photobiont specificity could also explain the exclusively presence of *Trebouxia* sp. 2 in the epiphytic, esorediate taxon, but it should also be considered that *Trebouxia* sp. 1 may not be available on bark for unknown reasons.

The occurrence of photobionts belonging to *T. simplex* s.l. in alpine *T. atra* correlates with the genetic distinctiveness of the mycobiont sequence data in a separate basal lineage of the *T. atra* complex (Fig. 3). We therefore assume that the Alpine *Tephromela atra* samples are genetically isolated from Mediterranean ones, and that the isolation might be enforced by ongoing association with a climatically adapted photobiont.

Tephromela atra s.l. is a subcosmopolitan species and its morphological heterogeneity is not constrained to the Mediterranean region. This species complex also shows a high morphological diversity in SW North America, SW Australia and Southern Africa, from where new species (Rambold 1989; Elix and Kalb 2006; Wirth 2007) have been described (and will be described; K. Kalb, personal communication). It remains to be studied whether taxa from these parts represent lineages that are genetically distinct from the southern European representatives of the *T. atra* complex. The *T. atra* species complex represents an interesting case, where the joint efforts of molecular and phenotypic studies will reveal new insights in the diversification of lichens.

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