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THE ' MEDITERRANEAN ' *RAMALINA PANIZZEI* NORTH OF THE ALPS: MORPHOLOGICAL, CHEMICAL AND rDNA SEQUENCE DATA

Urs GRONER* and Scott LaGRECA‡

Abstract: *Ramalina panizzei* De Not. is reported from Switzerland and north of the Alps for the first time. Recent collections and thalli found amongst specimens of *R. fastigiata* (Pers.) Ach. are described; the species is obviously not restricted to the Mediterranean. The confusion in several herbaria around this and related corticolous species, particularly *R. subgeniculata* Nyl. and *R. fastigiata*, can be traced back to imprecise original and subsequent diagnoses, all of which lack a clear species delimitation. Similarities and differences of these species are discussed. In addition, sequences from the rDNA ITS regions were determined for two individuals of *R. panizzei* and two of *R. fastigiata*, including one of each from a site where both species grow intermixed. Kimura 2-parameter genetic-distance estimates indicate that *R. panizzei* and *R. fastigiata* are as different from each other as either is from the reference species *R. siliquosa* (Hudson) A. L. Sm. *s.l.* A broad-based taxonomic revision of involved species is not possible due to the limited number of analyses, but the results demonstrate the potential for using DNA sequence data to investigate species-level questions in lichens. Based on morphology, chemistry, and DNA sequence data, *R. panizzei* is retained as a distinct species.

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Introduction

Ramalina panizzei De Not. belongs in section *Fistularia* (Vain.) Räsänen, characterized by a hollow thallus. Nylander (1870) placed it in the *R. pusilla* group with more or less smooth, fistulose or subfistulose and perforated thalli. The distribution of the species was summarized as: ' Regio mediterranea, corticola ' by Zahlbruckner (1930: 501). Subsequently the species was only rarely reported and current keys to the European lichen flora such as Poelt (1969) and Clauzade & Roux (1985) consider *R. panizzei* to be a rare Mediterranean species. A poor knowledge of the species and, as Nimis & Poelt (1987) pointed out, an unclear species circumscription, are the reasons for the confusion surrounding *R. subgeniculata* Nyl., *R. fastigiata* (Pers.) Ach. and *R. panizzei* in several European collections.

Ramalina sp. A in Groner (1990) with fistulose, robust, perforate and fenestrate thalli, has been identified as *Ramalina panizzei*. This determination was suggested by H. Krog, who had previously examined the type material. The occurrence of *R. panizzei* north of the Alps in Switzerland, distant from the Mediterranean, is incompatible with previous knowledge and prompted a closer look at the species. This paper gives information on the new localities, and some of the taxonomic problems connected with this and similar corticolous *Ramalina* species are discussed.

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DNA sequence data for *R. fastigiata* and *R. panizzei* are also presented and discussed with regard to species delimitation. These data were collected from the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA), which have been shown to possess variability that is useful at the species level in many groups of organisms (Hillis & Dixon 1991). While a number of large-scale, molecular phylogenetic studies of lichenized ascomycetes have been initiated at the ordinal and higher levels (DePriest & Gargas 1996; Gargas *et al.* 1995; Lutzoni *et al.* 1996; Tehler 1995), the current paper is one of the first attempts to use DNA sequence data to resolve species questions in these organisms.

Materials and Methods

Morphology, chemistry and ecology of *Ramalina panizzei* are based mainly on the thalli collected in Switzerland (see *Specimens examined* and Table 1). The type and other Italian specimens (TSB) were not available, but photographs of the type have been seen; there is not much left of the original collection (Bartsch, *in litt.*). ‘ *Ramalina panizzei* ’ from H-NYL together with specimens from BERN, G and MARSSJ were examined. In addition, collections of fertile shrubby species of *Ramalina* (especially *R. fastigiata* and *R. subgeniculata*) from the herbaria BERN, G, Z and ZT were searched for misidentified *R. panizzei*. For these specimens, microcrystal tests (Hale 1979) were used on small thallus fragments to exclude specimens with divaricatic (*R. subgeniculata*) or evernic acid (*R. fastigiata*). All collections of *R. panizzei* were analysed by thin-layer chromatography (TLC) (Culberson & Ammann 1979; Culberson & Johnson 1982). A total of 43 thalli containing sekikaic acid is included in the morphological and chemical study.

Ramalina panizzei De Not.

Giorn. bot. ital. II, 1, I: 211 (1846); type: Italy, Liguria, near San Remo, *Panizzi* (TSB) [TLC: homosekikaic and sekikaic acids; Nimis, *in litt.*; Bartsch 1992].

Specimens examined: **France:** *Haute-Savoie*: Sommet du Voirons, 1881, *J. Rome* (G).—**Switzerland:** *Bern*: Bellelay, 1960, *E. Frey* 23.418 (BERN); 1993, *U. Groner* 1480 (hb. Groner); *Graubünden*: Engadin, *C. Egli* (Z); *Schwyz*: Muotatal, Bödmerenwald, 1985, *U. Groner* 53 and 115; 1988, *U. Groner* 614; *Vaud*: Vallon de Naye, 1991, *U. Groner* 1147; 1995, *U. Groner* 1761 (all hb. Groner).—**Romania:** *Karpaten*: Rodna burback, 1910, *Z.*, hb. Frey 15.905 (BERN).

DNA sequence data: collection of specimens

Two specimens of *R. fastigiata* and two of *R. panizzei* were collected in Switzerland, one of each from a pure population and from a locality where the two species grow intermixed on *Acer pseudoplatanus* (Table 1). Two specimens of *R. siliquosa* (Huds.) A. L. Sm. *s.l.*, collected by W. L. and C. F. Culberson in Wales, were chosen as reference species. Secondary product chemistry of all specimens was determined by TLC by the collectors.

Preparation of Genomic DNA

Lyophilized pieces of individual *R. fastigiata* and *R. panizzei* thalli weighing 15–25 mg were ground in fine sand in 1·5-ml Eppendorf tubes, and the total genomic nuclear DNA was isolated using the DTAB/CTAB procedure of Armaleo & Clerc (1995). The guanidinium procedure (Method I) of Armaleo & Clerc (1991) was used for extraction of individual thalli of *R. siliquosa*. Prior to DNA extraction, lyophilized thallus pieces of *R. siliquosa* weighing 35–60 mg were extracted four times (twice at room temperature, then twice on a slide warming tray) in 2 ml acetone, for 5 min each time, to remove lichen secondary products.

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TABLE 1. *The species, collection locality, chemistry, method of preservation, and specimen information for each of the six* Ramalina *samples sequenced for this study*

Sample name	Species	Locality	Chemistry (major products)	Method of preservation	Specimen and GenBank no.
R.fas.a	*R. fastigiata*	Switzerland: *Schaffhausen*, Beringen, Lieblosental. *Acer campestre*	Evernic acid	Freezer	*Groner* 1760 (hb. Groner, DUKE) U84582
R.fas.b	*R. fastigiata*	Switzerland: *Bern*, Bellelay, Tourbière La Sagne. *Acer pseudoplatanus*	Evernic acid	Silica gel	*Groner* 1479 (hb. Groner, DUKE) U84583
R.pan.a	*R. panizzei*	Switzerland: *Vaud*, Veytaux, Naye, Preise au Maidzo. *Acer pseudoplatanus*	Sekikaic acid	Freezer	*Groner* 1761 (hb. Groner, DUKE) U84584
R.pan.b	*R. panizzei*	[same as R.fas.b]	Sekikaic acid	Silica gel	*Groner* 1480 (hb. Groner, DUKE) U84585
R.sil.st	*R. siliquosa s.l.*	United Kingdom: *Wales*, Anglesey, Holy Island, Trearddur Bay. Maritime cliff	Stictic acid	Freezer	*W. L. Culberson* 13 087 (DUKE) U84586
R.sil.nst	*R. siliquosa s.l.*	[same as R.sil.st]	Norstictic acid, stictic acid	Freezer	*W. L. Culberson* 13 100 (DUKE) U84587

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rDNA fragment amplification and preparation

The primer pair BMB-CR (5′-GTACACACCGCCCGTCG-3′) (Lane *et al.* 1985) and LR1 (5′-GGTTGGTTTCTTTTCCT-3′) (Vilgalys & Hester 1990) was used for polymerase chain reaction (PCR) amplification of a *c.* 750 base pair (bp) double-stranded fragment of rDNA including both internal transcribed spacer regions (ITS-1 and ITS-2) and the entire 5·8 S rRNA gene. BMB-CR was used as the 5′ flanking primer because preliminary tests indicated that, compared with the primers SLG-1 (see below), SR6R, and ITS-1, it yielded the highest amount of desired lichen fungus PCR product and the least amount of extraneous (e.g. algal) products at the high annealing temperature used (LaGreca 1997). Amplifications were performed using Amplitaq DNA polymerase (Perkin-Elmer Cetus) with buffer conditions recommended by the manufacturer and the following parameters: 30 cycles of 1-min denaturation at 94°C, 45-s annealing at 60°C, and 2-min extension at 72°C, followed by 1 cycle of 5-min extension at 72°C. Two microlitres of PCR product were electrophoresed on an agarose gel to verify product size, and the remaining product was then purified using Magic PCR Preps DNA Purification System (Promega Corp.). Two microlitres of purified product were then electrophoresed on an agarose gel with various amounts of 10 ng μl^{-1} lambda genomic DNA standard in order to determine the concentration of each template DNA.

DNA Sequencing and Sequence Analysis

Sequencing of all template DNAs was performed using an Applied Biosystems Inc. Model 373 DNA Sequencing System and the following primers: SLG-1 (5′-TTGCGCAACCTGC GGAAGGAT-3′), 5·8 SR (5′-TCGATGAAGAACGCAGCG-3′), 5·8 S (5′-CGCTGCGTTC TTCATCG-3′), and LR1. SLG-1 binds near the 3′ end of the 18 S rRNA gene, and its 5′ end is designed to overlap the 3′ end of a Group I intron found at position 1516 (using *Escherichia coli* as the reference sequence; Gutell 1993) in other *Ramalina* sequences (LaGreca 1997). This primer worked well for sequencing the *R. fastigiata*, *R. panizzei*, and *R. siliquosa* samples included in this study, despite the fact that these samples lack this intron.

Chromatograms were analysed using the computer programme Sequencher (Gene Codes, Inc.). Sequences were aligned by eye. Average nucleotide composition and pairwise Kimura 2-parameter genetic-distance estimates were calculated using the programme MEGA version 1·0 (Kumar *et al.* 1993). Positions with gaps and missing data were excluded when calculating genetic-distance estimates.

Results

Morphology

Characteristics: see Table 2 and Figs 1 & 2. *Ramalina panizzei* is a polymorphic species, often recalling or looking like *R. fastigiata*: slender, small tufted morphs with mostly apical apothecia have been found, together with rigid, irregularly and sparingly branched thalli. Intermediates of these morphotypes are common.

Chemistry

Sekikaic acid, homosekikaic acid; 4′-*O*-methylnorhomosekikaic acid, probably 4′-*O*-demethylsekikaic and/or 4′-*O*-methylnorsekikaic acids and other related substances. Medulla $UV_{254\,nm}$+yellowish-white to bright green, according to Bartsch (1992). TLC patterns of the specimens are very similar and show, with regard to the relative proportions of the substances, little variation; sekikaic acid is the major product, followed by homosekikaic acid. Usnic acid is usually present in trace amounts; it is rarely accompanied by a trace of atranorin.

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TABLE 2. *Main differences between* Ramalina panizzei *and similar species.**

Characters	*R. panizzei*	*R. fastigiata*	*R. subgeniculata*	*R. elegans*	*R. calicaris*	*R. pusilla*
Thallus	More or less hollow	Solid; partly hollow	Hollow	More or less hollow	Solid	Hollow
Branches	Robust and/or irregularly branched forms frequent; partly inflated, compressed	Often palmately branched; compressed and partly inflated, longitudinally furrowed	Usually narrow, often compressed, more or less canaliculate	Shrubby and rigid; moderately branched; usually compressed, partly inflated	Usually narrow; moderately to densely branched, canaliculate	Irregularly branched; inflated
Apothecia	Apical-subapical, lateral	Apical-subapical, more rarely lateral	Apical, subapical, lateral	Apical-subapical	Subapical, lateral	Apical
Apical spur	Often present	Often present	Often present	Present	Present	Absent
Perforations	Present	Often present	Present	Present	Absent	+/− Orbicular; longitudinal cracks
Fenestrations	Present	Sometimes present	Present	Absent	Absent	Absent
Pseudocyphellae	Absent (?)	Absent	Absent	Present (linear) or absent	Punctiform-linear or absent	Absent
Other characteristics	Often disintegrating or cracked cortex	—	Often with blackened areas	—	Spores mostly straight	Cortex with black patches
Chemistry (diagnostic acids)	Sekikaic aggr.	Evernic	Divaricatic	Sekikaic aggr.	Sekikaic aggr./no medullary substances	Sekikaic, terpenoids/ salazinic
Known distribution	? Central European—Mediterranean? montane (?)	Southern boreal—Mediterranean	Mediterranean, Macaronesian	Southern boreal—Mediterranean; montane	Southern boreal—Mediterranean; western	Mediterranean, Macaronesian

*Details from Poelt (1969), Krog & James (1977), Krog & Østhagen (1980), Clauzade & Roux (1985), Skytén (1993), Arroyo *et al.* (1995).

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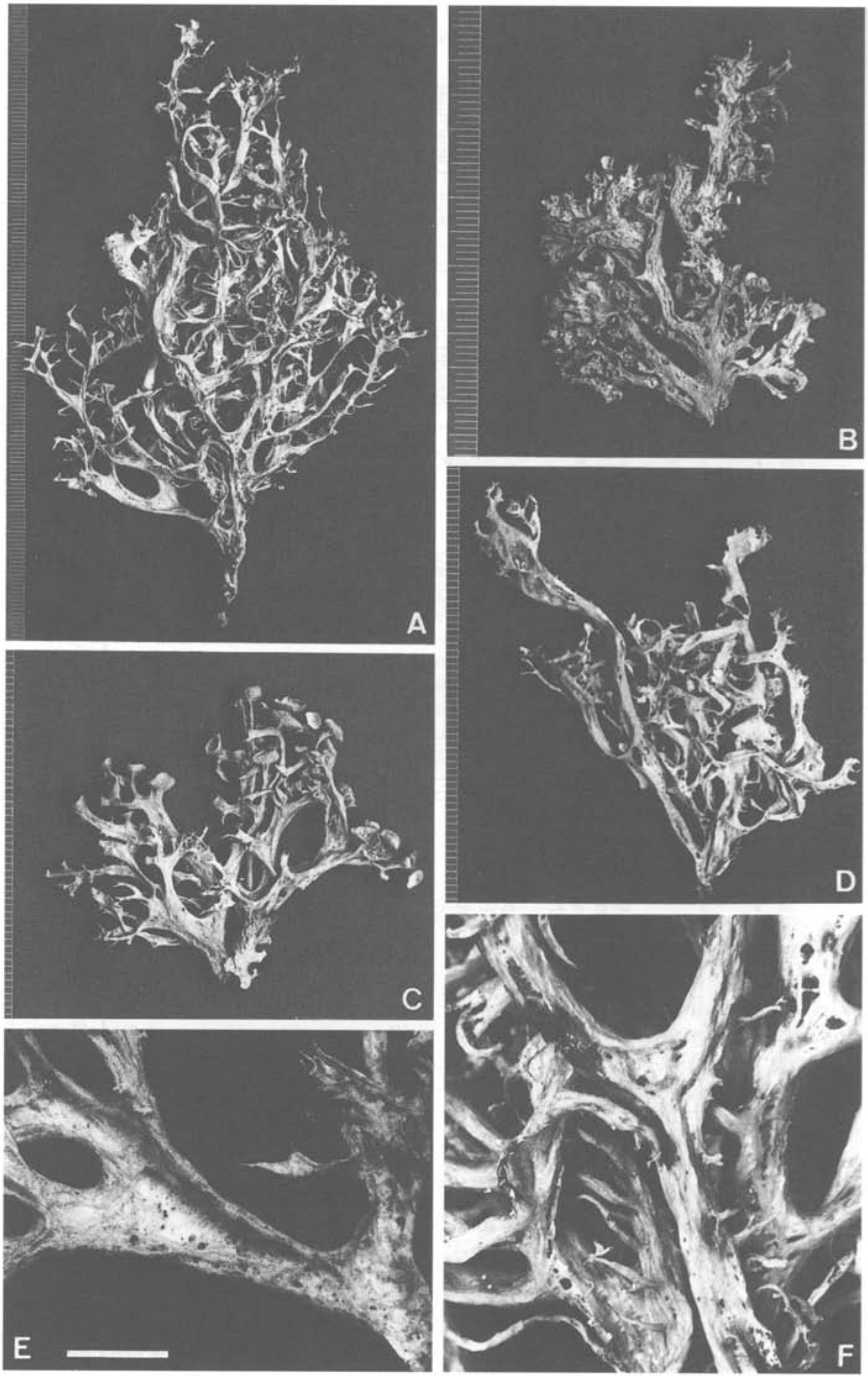

FIG. 1. *Ramalina panizzei* (*Groner* 614). A–D, Different morphotypes from a single tree. E, Disintegrating cortex on underside of thallus. F, Irregular branching, perforations and cracking cortex. Scales: A–D rule in mm; E & F = 5 mm.

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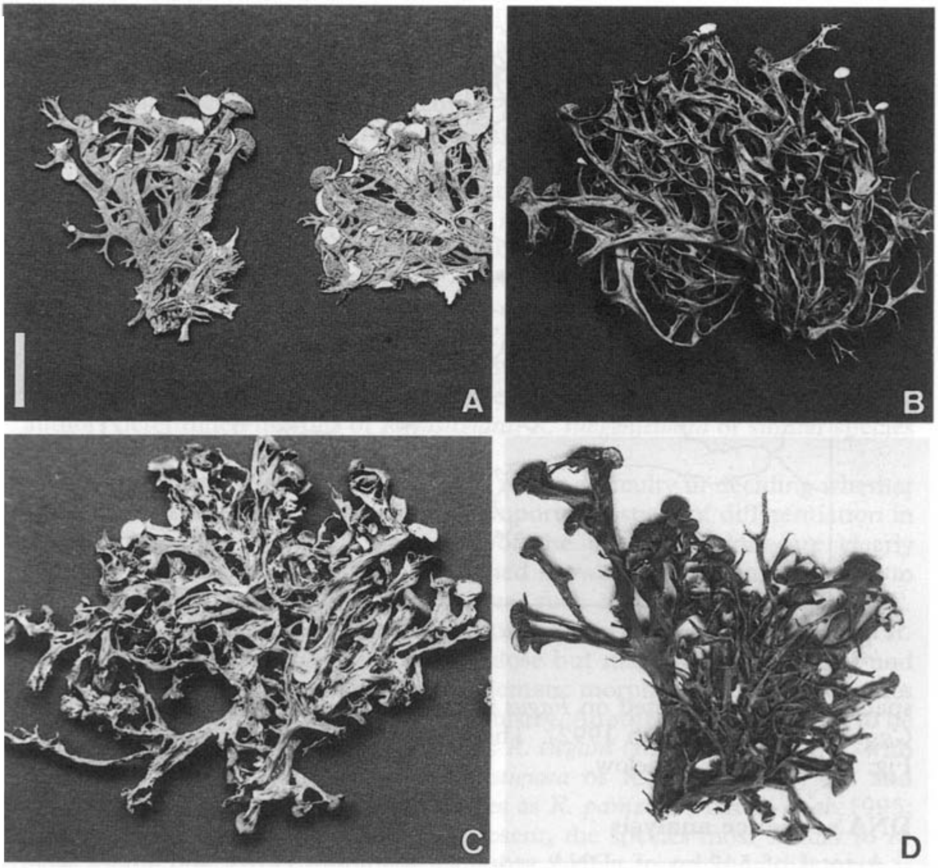

FIG. 2. Morphs of *Ramalina panizzei* from old collections. A, *Egli* (Z); B, *Frey* 23.418 (BERN); C, hb. Frey 15.905 (BERN); D, *Rome* (G). Scale=10 mm.

Localities and habitat ecology

Recent collections of *R. panizzei* have been made in three different parts of Switzerland. Muotatal, east of Lake Lucerne in central Switzerland, is part of the Northern Calcareous Alps (details in Groner 1990). The second locality, Naye, is close to Lake Geneva in the Préalpes region (western Switzerland), and Bellelay, the third, is situated in the Jura Mountains in the northwestern part of the country. Common to all three localities are more or less calcareous rocks of Jurassic or Cretaceous age; these sediments are covered by a Quaternary peat bog at Bellelay. The collection sites (900 to 1350 m a.s.l.) are in the montane-upper montane zone of fir-beech forest, where suboceanic and oceanic lichens occur. *Ramalina panizzei* is found on trunks and lower branches of *Acer pseudoplatanus*; the pertaining epiphytic communities are attributed to a *Ramalinetum fastigiatae* Duvign. Collection data are scarce or incomplete on three of the four examined herbarium specimens; *Acer pseudoplatanus* and *Fagus sylvatica*, respectively, are mentioned on two labels. Italian

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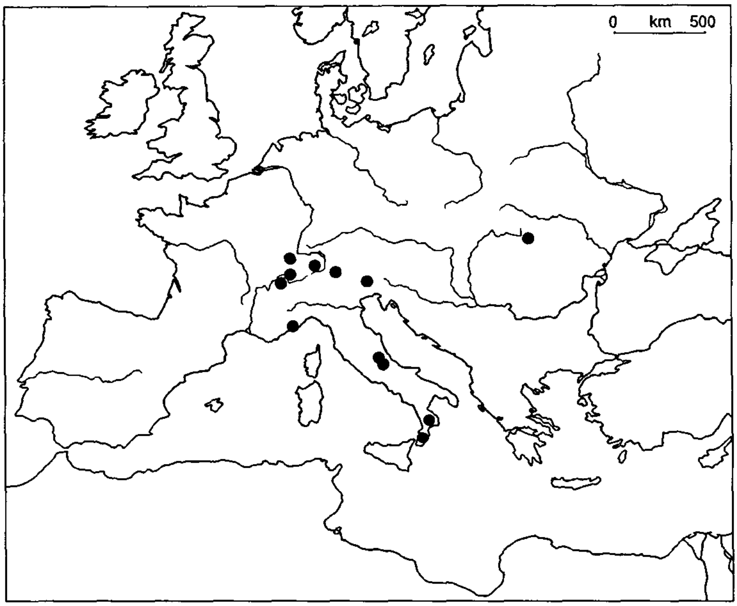

FIG. 3. Known distribution of *Ramalina panizzei*. Italian locations from Bartsch (1992).

specimens were collected on *Fagus* in montane forests, a single specimen on *Castanea sativa* (Bartsch 1992). The distribution of *R. panizzei* is shown in Fig. 3 and discussed below.

DNA sequence analysis

A total of 542 bp of rDNA sequence including ITS-1 and ITS-2 were determined for all six samples. Complete ITS-1 and ITS-2 sequences, 159 bp and 166 bp long, respectively, are given in Table 3. These sequences are readily alignable over their entire lengths; excluding the 22 positions with gaps, they are 85·2% identical (277 nucleotides out of 325). The average nucleotide composition calculated over all six sequences is: 19·2%A, 31·8%C, 24·7%G, and 24·3%T. Average pairwise genetic-distance estimates within species is 0·0023 and between species is 0·0600 (Table 4).

Discussion

There are considerable differences between the original diagnosis of *R. panizzei* (De Notaris 1846) and the description summarized in Table 2. Based on De Notaris' description, it seems likely that the specimens he examined were poorly developed or that they originated from a population with diminutive thalli (3 cm). While the herbarium specimens roughly match the diagnosis (Fig. 2A–B, D), the new collections represent unusually well-developed, robust individuals up to 8(− 13) cm long (Fig. 1). In addition, De Notaris did not mention the perforations and fenestrations present in the type.

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This could be because the material examined was possibly heterogeneous since Panizzi collected both *R. panizzei* and *R. fastigiata* at the type locality (see De Notaris 1846; Nylander 1870, and specimen mentioned below). Also, the original description did not mention the variability of the species nor its delimitation from similar, fertile shrubby species such as *R. fastigiata* and *R. subgeniculata*. The confusion of *R. panizzei*, *R. subgeniculata* (Krog & Østhagen 1980; Nimis & Poelt 1987) and *R. fastigiata*, seems to derive from the monograph of Nylander (1870). His rather short diagnosis of *R. panizzei* was obviously based on De Notaris' description and on specimens ex herb. Lenormand collected by Panizzi (the corresponding tiny specimen in H-NYL is *R. fastigiata*). Probably, like Nylander, none of the earlier authors citing *R. panizzei* had seen the original material of De Notaris. Later authors referring to Nylander's work (e.g. Stizenberger 1891; Harmand 1907; Steiner 1920), did so without seeing Nylander's reference specimens. In consequence, several authors determined morphs of *R. fastigiata*, *R. subgeniculata* or similar species as *R. panizzei.*

This confusion of species is partly due to the difficulty in deciding whether a thallus is fistulose or not, which is an important aspect of differentiation in this species group. Several specimens of the new collections are clearly fistulose, whereas the other thalli examined showed a wide range of solid to partly hollow to entirely hollow branches, such as Italian specimens of *R. panizzei* (Bartsch 1992). The variation of this feature is well known for *R. fastigiata*; *R. subgeniculata* is usually fistulose but may have compressed and canaliculate thallus parts (Table 2). Problematic morphs of these three species may be separated by their distinctive chemistry. Another species that has to be considered in this regard (Krog, *in litt.*) is *R. elegans* (Bagl. & Car.) Jatta, with thalli similar to some morphs of *R. fastigiata* or *R. calicaris* (L.) Fr. and apparently the same medullary substances as *R. panizzei* (Arroyo *et al.* 1995; Skytén 1993). *Ramalina elegans* is, at present, the species most similar to *R. panizzei* and may prove to be a synonym, but further research on this taxon is necessary to clarify its identity. Concerning subfistulose specimens in general, the question ' solid or hollow? ' cannot definitely be answered (see also discussion in Krog & Østhagen 1980). *Ramalina panizzei* must be retained within *Fistularia* until more results are available.

At first sight, specimens of *R. panizzei*, especially of the *fastigiata* morph (Fig. 2A-B), might be regarded as a chemotype of *R. fastigiata*, as they lack the striking combination of morphological characteristics of recent collections (Fig. 1). Other *Ramalina* species also show considerable morphological and chemical variation; see for example Culberson *et al.* (1990) and LaGreca (1997). Moreover, the two species have been collected together at the type locality and the same is true for herbarium specimens, because *R. panizzei* was found among *R. fastigiata* thalli. For instance, E. Frey's collection in the Swiss Jura Mountains (*Frey* 23.418) contains 18 thalli of *R. panizzei* and two of *R. fastigiata*. We have recently collected two *R. panizzei* and 19 *R. fastigiata* thalli on the same tree; the species were indistinguishable in the field.

DNA sequence data, however, are inconsistent with these arguments. The low levels of intraspecific ITS sequence divergence found in this study (Tables

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TABLE 3. *Complete, aligned DNA sequence data of the ITS regions for the six samples. Dots (.) in the sequences indicate bases which are identical to the* R.fas.a *sequence. Dashes (−) indicate gaps*

ITS-1:

```
                    1  1111111112  2222222223  3333333334  4444444445  5555555556
           1234567890  1234567890  1234567890  1234567890  1234567890  1234567890
R.fas.a    AAGAGAGGGG  CTTCGCGCTC  CAGGGGATTC  CGGTCCCCGC  CTCTACACCC  TGTGATTACG
R.fas.b    ..........  ..........  ..........  ..........  ..........  ..........
R.pan.a    C.........  .?........  .......C.T  .....T....  ..........  .........T
R.pan.b    ??????????  .?........  .......C.T  .....T....  ..........  .........T
R.sil.st   ..........  ..........  .......C.T  ...?......  ....G.....  C........C
R.sil.nst  ..........  ..........  .......C.T  ..........  ....G.....  C........C

                                                        1  1111111111  1111111111
           6666666667  7777777778  8888888889  9999999990  0000000011  1111111112
           1234567890  1234567890  1234567890  1234567890  1234567890  1234567890
R.fas.a    TTACCCTTT-  GTTGCTTTGG  CGGGGGCACT  CCC-CGCCAG  CAGCCCACCG  AAACTCGTTT
R.fas.b    ..........  ..........  ..........  ..........  ..........  ..........
R.pan.a    .....T....  ..........  ......G.G.  ...C......  ..........  ..........
R.pan.b    .....T....  ..........  ......G.G.  ...C......  ..........  ..........
R.sil.st   .....T...T  ..........  ..A..--.G.  ...T......  ..........  ..........
R.sil.nst  .....T...T  ..........  ..A..--.G.  ...T......  ..........  ..........

           1111111111  1111111111  1111111111  111111111
           2222222223  3333333334  4444444445  555555555
           1234567890  1234567890  1234567890  123456789
R.fas.a    TATCCATGTT  CGTCCGAGT-  C--TAATTCA  TAATGAATC
R.fas.b    ..........  ..........  ..........  .........
R.pan.a    ........CC  ....T..--A  .AA-......  .........
R.pan.b    ........CC  ....T..--A  .AA-......  .........
R.sil.st   ........CC  ....T....A  -..G......  .........
R.sil.nst  ........CC  ....T....A  -..G......  .........
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TABLE 3. *Continued.*

ITS-2:

	1	1111111112	2222222223	3333333334	4444444445	5555555556
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
R.fas.a	GCACCACTCA	AGCGTAGCTT	GGTATTGGGT	CCATCGCCCG	GGATCTCCCC	GCGGCGTGCC
R.fas.b	....	....	....	....	....	....
R.pan.a	....	....	....	..T.....G.	..-..A....	C-........
R.pan.b	....	....	....	..T.....G.	..-..A....	C-........
R.sil.st	A.....T...	....	....	.-T.....G.	..-..C....	C-..T.....
R.sil.nst	......T...	....	....	.-T.....G.	..-..C....	C-........
				1	1111111111	1111111111
	6666666667	7777777778	8888888889	9999999990	0000000011	1111111112
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
R.fas.a	CGAAAAGCAT	TGGCGGTCCG	GTGTGACTTT	AAGCGTAGTA	AATTTTCTCC	CGCTTTGAAA
R.fas.b	....	....	....	....	....	....
R.pan.a	....	....	....	.....C....	....	....
R.pan.b	....	....	....	.....C....	....	....
R.sil.st	....	....	....	....	.......G..	....
R.sil.nst	....	....	....	....	.......G..	....
	1111111111	1111111111	1111111111	1111111111	111111	
	2222222223	3333333334	4444444445	5555555556	666666	
	1234567890	1234567890	1234567890	1234567890	123456	
R.fas.a	GCCTCACGCC	GTGGCCGGCC	AGACAACCCC	CACA-TATTA	--TCAC	
R.fas.b	....	....	....	....	......	
R.pan.a	.TT.T.....	....	....	..T..-....	......	
R.pan.b	.TT.T.....	....	....	..T..-....	......	
R.sil.st	....	....	....	-...C---.-	TC....	
R.sil.nst	....	....	....	-...C---.-	TC....	

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TABLE 4. *Total number of nucleotide differences (upper right) and Kimura 2-parameter genetic distance estimates (lower left) for pairs of samples from Table 3*

Samples	1	2	3	4	5	6
1 R.fas.a	–	0·0000	18·0000	18·0000	19·0000	17·0000
2 R.fas.b	0·0000	–	18·0000	18·0000	19·0000	17·0000
3 R.pan.a	0·0649	0·0649	–	0·0000	15·0000	13·0000
4 R.pan.b	0·0649	0·0649	0·0000	–	15·0000	13·0000
5 R.sil.st	0·0688	0·0688	0·0540	0·0540	–	2·0000
6 R.sil.nst	0·0611	0·0611	0·0465	0·0465	0·0069	–

3 & 4) agree with those for many other *Ramalina* species (LaGreca 1997) and are comparable with those reported for various angiosperms (review: Baldwin *et al.* 1995) and the parasitic ascomycetes *Botrytis* (Carbone & Kohn 1993) and *Cladosporium* (Curtis *et al.* 1994). The interspecific differences, however, between the three *Ramalina* species here are almost ten times as great (Table 4). Another, similar study of the lichen species *Lasallia papulosa* and *L. rossica* (Niu & Wei 1993) showed a much higher (24%) interspecific sequence difference (ITS-2 only), but this may reflect the fact that those two species were collected on two different, distant continents (North America and Asia, respectively). The identity of the within-species sequences from *R. panizzei* and *R. fastigiata* and the substantial average between-species genetic distance (0·0649) of these two species (Table 4) indicate that they are distinct and separate from each other. This evidence is especially compelling because, as mentioned above, one sample of each species was collected from a tree where both species grow intermixed (Table 1), a situation where a high incidence of gene-flow between them might be expected. In addition, both species are approximately as different from each other (genetic-distance-wise) as either is from *R. siliquosa s.l.*, a morphologically and ecologically dissimilar European species. An emendation of *R. panizzei* is presently not possible because Italian specimens have not been sequenced, nor have specimens of *R. elegans*. However, the results of this study demonstrate the potential of utilizing DNA sequence data for resolving species complexes in lichens.

The occurrence of *Ramalina panizzei* north of the Alps and in the Carpathians (Fig. 3) considerably extends its known distribution, no longer being restricted to the Mediterranean or southern Europe. Its range and ecological preferences may be similar to that of *R. fastigiata*. More material should be looked for under conditions like those of the recent discoveries: well-lit places in rather undisturbed forests, with high air humidity and precipitation levels and trees with more or less base-rich bark. The appropriate climatic conditions in central Europe are found in montane and upper montane zones or in humid montane forests in southern Europe (Nimis, *in litt.*). While a broad-based taxonomic revision is impossible given the limited scope of this study, *R. panizzei* is retained here as distinct on the (although provisional) basis of morphological, chemical and DNA sequence data. The re-examination of old and recent *Ramalina* collections, as well as additional

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field and molecular work including related taxa, are encouraged to provide a better understanding of this species.

Excluded specimens (labelled *Ramalina panizzei*):

Ramalina calicaris (L.) Fr.: **Portugal:** without location; Basel Rheinhafen, 1961, *W. Baumgartner* (G).

Ramalina canariensis Steiner: **France:** *Corsica*: *Requien* a reliq. b. Schaereri (H-NYL 36874).

Ramalina fastigiata (Pers.) Ach.: **Italy:** *Liguria occid.*: In sylvis supra S. Remo, *Panizzi* (H-NYL 36876); *Liguria*: 1962, *M. Steiner* [*Lichenes Alpium/München* no. 335] (BERN, MARSSJ); *Carnica*: Lago di Sauris, 1990, *A. Vězda* (MARSSJ).—**France:** *Var*: Estérel, 1965, *J. Lambinon* (MARSSJ).—**Spain:** Route de Murcie à Grenade, 1962, *H. Derval*, hb. Bouly de Lesdain (MARSSJ).—**Greece:** *Rhodos*, 1886, *Dr. Forsyth* (G).—**Algeria:** Sommet de l'Atlas, 1844, *Durieu* (H-NYL 36875).

Ramalina pusilla Duby: **Portugal:** without location, hb. Bouly de Lesdain? (MARSSJ).

Ramalina subgeniculata Nyl.: **France:** *Corsica*: *Requien* (H-NYL 36873); *Corsica*: Bonifacio, 1878, *J. P. Norrlin* (H-NYL 36872); *Var*: Ile de Porquerolles, 1955, *C. Sbarbaro*, hb. Bouly de Lesdain (MARSSJ); *Var*: Ile de Porquerolles, 1955, *G. Clauzade*, hb. Bouly de Lesdain (MARSSJ); *Var*: Port-Cros, 1971, *Y. Rondon* [Vězda, *Lichenes selecti* no. 1018] (G).—**Algeria:** Oran, 1852, 1853, *Balansa* (H-NYL 36870).

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