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Banks of Oued Zen river, habitat of *Heterocladium flaccidum*, within the Kroumirian zeen oak forest (N.W. Tunisia) / Rives de l'Oued Zen, habitat de *Heterocladium flaccidum*, au sein de la zéenaie de Kroumirie (N.O. Tunisie)

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A range extension of *Heterocladium flaccidum* (Schimp.) A.J.E.Sm. to Africa and Asia and confirmation of its specific status

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ABSTRACT

Recent collections of *Heterocladium flaccidum* (Schimp.) A.J.E.Sm. in Tunisia and in Georgia (Caucasus) represent the first records of this species both in Africa and Asia. Molecular phylogenetic analysis of samples from France, Great Britain, Tunisia and Georgia placed the species in a maximally supported clade sister to or nested in other species of *Heterocladium* s.str., i.e., excluding recently segregated *Heterocladiella* species. This genetic distinction of *H. flaccidum* from *H. heteropterum* (Brid.) Schimp., found in separate analyses of ITS and *trnS-F* markers, strongly supports the specific recognition of *H. flaccidum*. Representative African and Asian specimens are illustrated and described. Ecological and chorological details are provided.

KEY WORDS

Heterocladium heteropterum,
geographical distribution,
molecular phylogenetics,
ITS,
trnS-F,
Lembophyllaceae,
Neckeraceae.

RÉSUMÉ

Élargissement de l'aire de répartition de Heterocladium flaccidum (Schimp.) A.J.E.Sm. en Afrique et en Asie, et confirmation de son statut spécifique.

La présence de *Heterocladium flaccidum* (Schimp.) A.J.E.Sm. est signalée en Tunisie et en Géorgie (Caucase) ce qui représente les premières mentions de cette espèce à la fois en Afrique et en Asie. Une analyse phylogénétique moléculaire de spécimens originaires de France, Grande Bretagne, Tunisie et Géorgie place l'espèce dans un clade à support maximal, en position sœur ou parmi d'autres espèces du genre *Heterocladium* s.str., c'est-à-dire excluant les espèces considérées comme appartenant au genre récemment exclus *Heterocradiella*. La différence génétique entre *H. flaccidum* et *H. heteropterum* (Brid.) Schimp., obtenue par les analyses des marqueurs ITS et *trnS-F*, est largement en faveur d'un statut spécifique de *H. flaccidum*. Des spécimens représentatifs en provenance d'Afrique et d'Asie sont illustrés et décrits. Des détails écologiques et chorologiques sont fournis.

MOTS CLÉS

Heterocladium heteropterum,
distribution géographique,
phylogénétique moléculaire,
ITS,
trnS-F,
Lembophyllaceae,
Neckeraceae.

INTRODUCTION

The genus *Heterocladium* Schimp. was introduced in “Bryologia Europaea” (Bruch *et al.* 1851-1855) for three species, *Heterocladium dimorphum* (Brid.) Schimp., *H. heteropterum* (Brid.) Schimp. and *H. kurzii* Schimp, the latter subsequently synonymized with *H. dimorphum* (Wijk *et al.* 1962). The Tropicos database (<http://legacy.tropicos.org/namesearch.aspx>, accessed 15 April 2020) accepts 10 species of the genus in current use. Recent molecular phylogenetic studies revealed the heterogeneity of the genus, placing *H. dimorphum* and *H. procurrans* (Mitt.) A. Jaeger in a clade with *Anomodontella longifolia* (Schleich. ex Brid.) Ignatov & Fedosov (synonym of *Anomodon longifolius* (Schleich. ex Brid.) Hartm.) (Ignatov *et al.* 2007). The same results were found in many subsequent analyses (e.g. Enroth *et al.* 2019), and latterly Ignatov *et al.* (2019) segregated these two species in a new genus *Heterocradiella* Ignatov & Fedosov, rendering *Heterocladium* morphologically more homogeneous. Only one of the remaining eight species, *H. heteropterum*, was thought to have wide distribution throughout the Holarctic (Wijk *et al.* 1962). However in recent floras, it is excluded from East Asia and North America, so all the species of the genus are confined in their distribution to a single longitudinal sector: *H. macounii* Best occurs in North America, *H. angustifolium* (Dixon) R.Watan., *H. capillaceum* Broth. ex Ihsiba, *H. pilicuspis* Broth. ex Ihsiba, and *H. tenellum* Deguchi & H.Suzuki in East Asia, and *H. flaccidum* (Schimp.) A.J.E.Sm. and *H. wulfsbergii* I.Hagen are European endemics, while *H. heteropterum* extends from Europe to West Asia. These small saxicolous mosses are rather rare in most regions.

The genus *Heterocladium*, either *s.l.* or *s. str.*, has never been the subject of a revision at a worldwide scale. The status of the two endemic European species (*H. flaccidum* and *H. wulfsbergii*), their recognition at species level, and their separation from the more widespread *H. heteropterum* are still debated (Crundwell & Smith 2000; Hedenäs & Isoviita 2008). The paucity of available morphological characters and the rarity of sporophytes impede the identification of these taxa. It is not rare to encounter difficulties in identification between *H. flaccidum* and filiform morphs of *H. heteropterum* (Crundwell & Smith 2000; Blockeel *et al.* 2014).

Material attributable to *Heterocladium flaccidum* was recently collected in Georgia in the Caucasus and in Tunisia in North Africa. In addition to our morphologically-based identifications, we undertook a DNA study to verify these records as the first for *H. flaccidum* in Africa and Asia, to clarify the taxonomic status of this taxon, and to elucidate its relationships to other *Heterocladium* species.

MATERIAL AND METHODS

One nuclear and one plastid marker were used: nuclear ribosomal region, including internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, 921 bp in alignment (hereafter ITS) and plastid region, including tRNA-Ser gene (*trnS*), partial sequence; *rps4* gene, complete sequence; tRNA-Thr gene (*trnT*), complete sequence; tRNA-Leu gene (*trnL*), complete sequence; and tRNA-Phe gene (*trnF*), partial sequence, 1792 bp in alignment (hereafter *trnS-F*). These markers were chosen because the phylogenetic signals from them are among the strongest for bryophytes (Olsson *et al.* 2009; Huttunen *et al.* 2013), and therefore they have maintained a leading position in the number of sequences for mosses in GenBank over twenty years. DNA extraction, PCR and sequencing follow the standard protocols, as described e.g. in Gardiner *et al.* (2005). Sample information and GenBank Accession numbers are provided in Table 1. The sequences obtained were analyzed in sets that were largely composed of GenBank sequences that showed the highest similarity in a BLAST search. Therefore, the respective sets for nuclear and plastid markers consisted of somewhat different species.

The analyses of nuclear and plastid datasets were conducted separately, as the topologies of phylogenetic trees from the different markers were not fully consistent. Sequences were aligned by Clustal and modified manually using BioEdit 7.0 (Hall 1999). Bayesian analysis for both the ITS dataset and *trnS-F* dataset was conducted in MrBayes 2 (Huelsenbeck & Ronquist 2001) using the GTR+G model following *MrModeltest2.3* (Nylander 2004). It was run for 20 000 000 generations with sampling every 1000 generations, the chain

TABLE 1. — Voucher information of sequenced specimens of *Heterocladium flaccidum* (Schimp.) A.J.E.Sm. and GenBank accession numbers.

| Voucher specimen | Isolate | ITS | trnS-F |
|---|---------|----------|----------|
| France, Pyrenees, coll. V. Hugonnot, VH15818, MHA | OK2480 | MT456272 | MT559314 |
| France, Saverne, coll. V. Hugonnot, VH18619, MHA | OK2477 | MT456273 | MT559312 |
| Georgia, Adjara, coll. V.A. Bakalin G-12-23-13 VBGI, MHA | OK598 | MT456270 | MT559310 |
| Tunisia, Oued Zen, coll. V. Hugonnot and I. Ben Osman, T2019-150, MHA | OK2478 | MT456274 | MT559313 |
| United Kingdom, Wales, coll. M.S. Ignatov s.n., 8.IX.2004, MHA | OK597 | MT456271 | MT559311 |

temperature was set at 0.02. The first 25% of sampled trees were discarded for the burn-in. In addition, maximum parsimony analysis was performed in Nona (Goloboff 1994) under the Winclada shell (Nixon 1999), and bootstrap was calculated for 2000 iterations.

RESULTS

Molecular phylogenetic analyses based on nuclear and on plastid markers result in trees with supported nodes for Anomodontaceae and Heterocladiales, while the complex group of Neckeraceae plus Lembophyllaceae is poorly resolved, with most clades having weak support and different overall topology in the two trees. However, *Heterocladium* is resolved as the maximally supported clade within Neckeraceae in Bayesian analyses of *trnS-F* and within Lembophyllaceae in ITS tree (Figs 1; 2).

The *Heterocladium* clade in the ITS tree includes two highly supported subclades: one of *H. flaccidum* (PP = 1, BS = 100) and the second with *H. heteropterum*+*H. wulfsbergii* (PP = 1, BS = 99). In the *trnS-F* tree, the *Heterocladium* clade includes *H. macounii* in a position sister to all other species. The latter clade is weakly supported and includes the trichotomy of 1) one specimen of *H. wulfsbergii*; 2) three specimens of *H. heteropterum*, with very lower support (PP = 0.71; BS = 62); and 3) the clade of *H. flaccidum* (PP = 1, BS = 100).

DISCUSSION

The taxonomic status of *Heterocladium flaccidum* has varied much through time and it is still debated. It was most commonly considered a variety of *H. heteropterum* (Limpricht 1895; Crundwell & Smith 2000; Blockeel *et al.* 2014). Our molecular results support the specific distinctiveness of *H. flaccidum* with a high degree of confidence; yet, strict morphological differentiation of this species from *H. heteropterum* can be difficult.

Well-developed morphs of *H. heteropterum* are immediately distinct, even in the field. The plants are rather regularly pinnately branched, with well-developed leaves slightly cordate-amplexicaul and sheathing the stem at the base. The stem and branch leaves are often secund and the shoot apices are clearly hooked. Well-developed leaves have an ovate-oblong base and a wide triangular acumen with a slight constriction between the two parts; they are rounded to the insertion and have long decurrencies.

By contrast, *H. flaccidum* is characterized by a much denser habit, with many pectinate filiform branches. Well-developed leaves are much more triangular in outline, without apical differentiation; they are truncate at the base and not sheathing the stem, and not or feebly decurrent. The apices of the shoots are occasionally hooked but the leaves are not falciform.

Difficulties may arise when *H. heteropterum* is more profusely branched with filiform last-order ramifications, which appear to be similar to normal axes of *H. flaccidum*. Those filiform branches apparently always emerge from more typical axes. However, the most reduced morphs of *H. heteropterum* (and *H. wulfsbergii*) may not be confidently separable from *H. flaccidum* by morphology alone.

Although *H. wulfsbergii* was not the focus of the present study, this endemic of W. Europe and Macaronesia was also found outside the *H. heteropterum* clade, though its genetic distance from the latter species is markedly lower than that of *H. flaccidum*. However, insufficient sampling precludes further discussion of *H. wulfsbergii*.

TAXONOMY

The plants from Tunisia look rather different from the specimens from France, United Kingdom and the Caucasus, having a somewhat more robust stature, mostly erect-spreading leaves vs mostly erect-appressed, and lacking long filiform branches with minute long-acuminate leaves. The difference however may be explained by the habitat which experiences intermittent flooding, being half-buried in sand. Other studied specimens had the appearance of thin mats which grew on wet, shaded cliffs and never experienced mechanical damage.

Because the Tunisian specimens could well have been erroneously identified as depauperate *H. heteropterum*, we consequently describe below the Tunisian material.

Heterocladium flaccidum (Schimp.) A.J.E.Sm. (Fig. 3B, G-H, M-N)

SPECIMENS EXAMINED. — **North Africa.** Tunisia, Kroumiria, Jendouba Governorate, delegation of Ain Draham, Oued Zen National Park, Oued Zen, 36°48.43'.08"N, 08°50'.41.34"E, 368 m alt., in deep valley along the watercourse, leg. Vincent Hugonnot and Imen Ben Osman, 4.IV.2019, T2019-150.

Caucasus. Georgia, Adjara, Mtirala National Park, upper course of Chakvistavi River, c. 4 km upstream of Chakvistavi Village, the buffer zone of the park, 41°40'30.1"N, 41°52'58.1"E, 400 m alt., Broadleaf Colchis forest in the stream valley (right tributary of Chakvistavi River), stones and cliffs along stream, leg. Vadim Bakalin, 12.V.2013, coll. # G-12-23-13. MHA ex VBGI.

DESCRIPTION

Plants

Very slender, forming dull green wiry wefts.

Stems

Irregularly and repeatedly branched (often as a result of apical damage), with fasciculate secondary branching, with weak differentiation of branches and stems; foliage terete to indistinctly complanate.

Branches

Fragile, rupturing at base, at junction with the stem.

Rhizoids

Clustered below leaf insertion, brown, smooth, simple or with a few ramifications, in small fascicles at base of old stems.

Stolons

With distant leaves occasionally present, strongly rhizoidous at apex.

Stem leaves

Erecto-patent, ovate- to lanceolate-triangular, acute, feebly decurrent, 225-290 × 65-110 µm; margins serrate throughout, sometimes more strongly in distal half, or on one side; costa 20-25 µm wide, double or very short and forked distally, reaching 0.2-0.3 the leaf length; alar cells quadrate to oblate in small rectangular ascendant group, 2-3 cells wide; median cells quadrate-hexagonal to ovate, 1-2 times as long as wide, 8-11(-15) × 6-7(-8) µm, with groups of feebly prorate cells dispersed on dorsal side; apical cell triangular, 17-19 × 8-10 µm.

Branch leaves

Similar to stem leaves, but smaller, narrower, and erect to somewhat appressed.

Gametangia or sporophytes

Not observed.

REMARKS

Plants from the Caucasus (Fig. 3A, C-F, I-L) are similar to the Tunisian plants in the narrowly triangular shape of leaves, which are truncate at base and not decurrent, and in sharply serrate leaf margins. However, Caucasian plants are smaller, more irregularly branched; leaves are slightly smaller, 225-260 × 65-100 µm; and laminal cells are more strongly prorate at upper angles on dorsal surface of the leaves. They are very similar in these characters to plants from France and United Kingdom.

HABITAT

In Tunisia, *Heterocladium flaccidum* was discovered on the banks of a permanent wadi, Oued Zen, in Kroumiria (N.W. Tunisia). Kroumiria is a mountainous massif with alternating sandstone and clay from the Numidian flysch. It is located in the humid Meso-Mediterranean belt but reaches the Supra-Mediterranean belt at its highest elevations (INRF 1975). It is

the wettest region of Tunisia: rainfall ranges between 1000 and 1500 mm/year, and it regularly snows in the mountains from December to February. Annual average temperatures range from 16 to 20°C, with minimum values between 2-7°C (January), and relatively strong daily, seasonal fluctuations. The regional vegetation is dominated by *Quercus suber* L. woodlands at low altitudes, and by *Q. canariensis* Willd. forests at higher elevations. Understoreys are constituted of numerous shrubs, including *Arbutus unedo* L., *Calicotome villosa* (Poir.) Link, *Erica arborea* L., *Myrtus communis* L., *Phillyrea media* L. and *Pistacia lentiscus* L. Despite the summer drought in Kroumiria, the high annual rainfall permits the existence of some permanent watercourses in the central part of the region. In Oued Zen region, the bottom of the valleys and small flat areas are occupied by riparian forests of *Alnus glutinosa* (L.) Gaertn. Individual Alders extend narrowly and discontinuously along the main axis of wadi beds and do not occur beyond their confines. These riparian forests represent the North-African *Campanulo alatae-Alnenion glutinosae* classified within the *Osmundo-Alnion* and the *Populetalia albae* (Géhu *et al.* 1994).

The most abundant populations of *Heterocladium flaccidum* were found at the interface between *Alnus glutinosa* and *Quercus canariensis* woodlands, on the higher banks that are characterized by a certain instability and the occurrence of small rock outcrops. These are entangled with aerial roots and trunks. There *H. flaccidum* grows on soil between rocks and roots. Accompanying bryophyte species are: *Calypogeia fissa* (L.) Raddi, *Dicranella heteromalla* (Hedw.) Schimp., *Fissidens serrulatus* Brid., *F. taxifolius* Hedw., *Homalia lusitanica* Schimp., *Isotheceum algarvicum* W.E.Nicholson & Dixon, *Lejeunea cavifolia* (Ehrh.) Lindb., *Lophocolea fragrans* (Moris & De Not.) Gottsche, Lindenb. & Nees, *Lunularia cruciata* (L.) Dumort. ex Lindb., *Microeurhynchium pumilum* (Wilson) Ignatov & Vanderp., *Plagiomnium undulatum* (Hedw.) T.J.Kop., *Plagiothecium nemorale* (Mitt.) A.Jaeger, *Ptychostomum donianum* (Grev.) Holyoak & N.Pedersen, *Rhynchostegium confertum* (Dicks.) Schimp., *Saccogyna viticulosa* (L.) Dumort., and *Thamnobryum alopecurum* (Hedw.) Gangulee.

CONCLUSION

The genus *Heterocladium* and *H. flaccidum* were previously unknown in Africa (O'Shea 1995; Ros *et al.* 1999, 2013) and in Asia (Ignatov *et al.* 2006; Kürschner & Frey 2011; Erdağ & Kürschner 2017), respectively. *Heterocladium flaccidum* is predominantly a west European species, reaching Sweden and Norway to the north, Germany and Austria to the east, some Mediterranean islands (e.g. Corsica) and the Iberian Peninsula to the south, and also occurring in the Azores (Blockeel *et al.* 2014; Hodgetts & Lockhart 2020). The occurrence of *H. flaccidum* in the ravines of Kroumiria (N.W. Tunisia, Africa) is consistent with the recent discovery, in the same region, of other oceanic species, such as *Saccogyna viticulosa* and *Lophocolea fragrans* (Hugonnot & Ben Osman unpublished data).

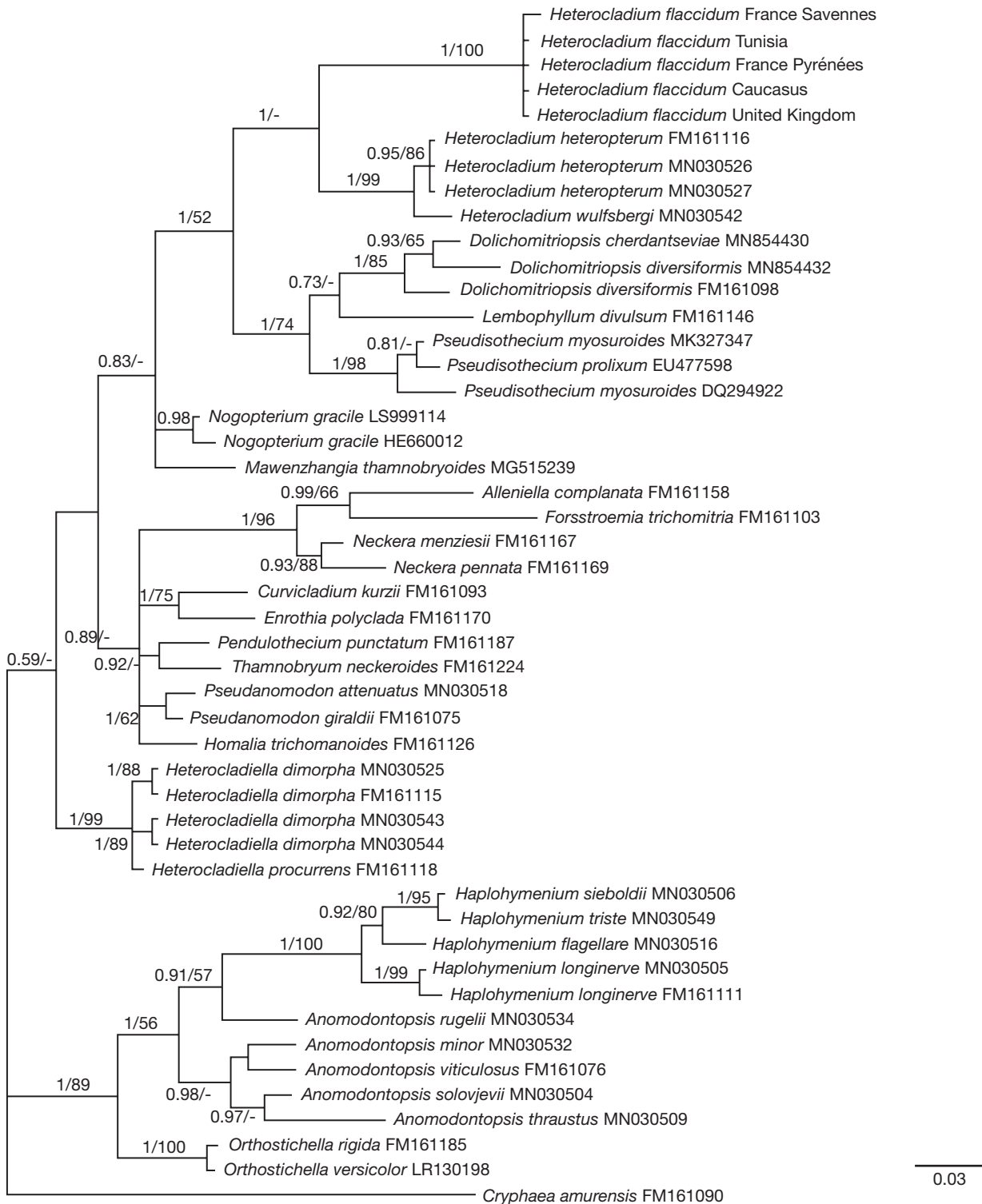


Fig. 1. — Bayesian tree from nrITS analysis. Bayesian posterior probabilities and MP bootstrap support are shown at branches.

Sporophytes are rarely produced in the genus *Heterocladium*: they are rare in *H. wulfsbergii* (Hedenäs & Isoviita 2008; V. Hugonnot, pers. obs. in France) and *H. heteropterum* (Nebel & Philippi 2001; Blockeel *et al.* 2014), and are currently unknown in *H. flaccidum*. This species may

have recently (i.e., in historical times) stopped producing sporophytes like it is the case for *Rhytidium rugosum* (Hedw.) Kindb. in the Alps (Hedenäs 2017). Therefore, relatively recent long-range colonization events by means of spores cannot be excluded. An alternative explanation would be

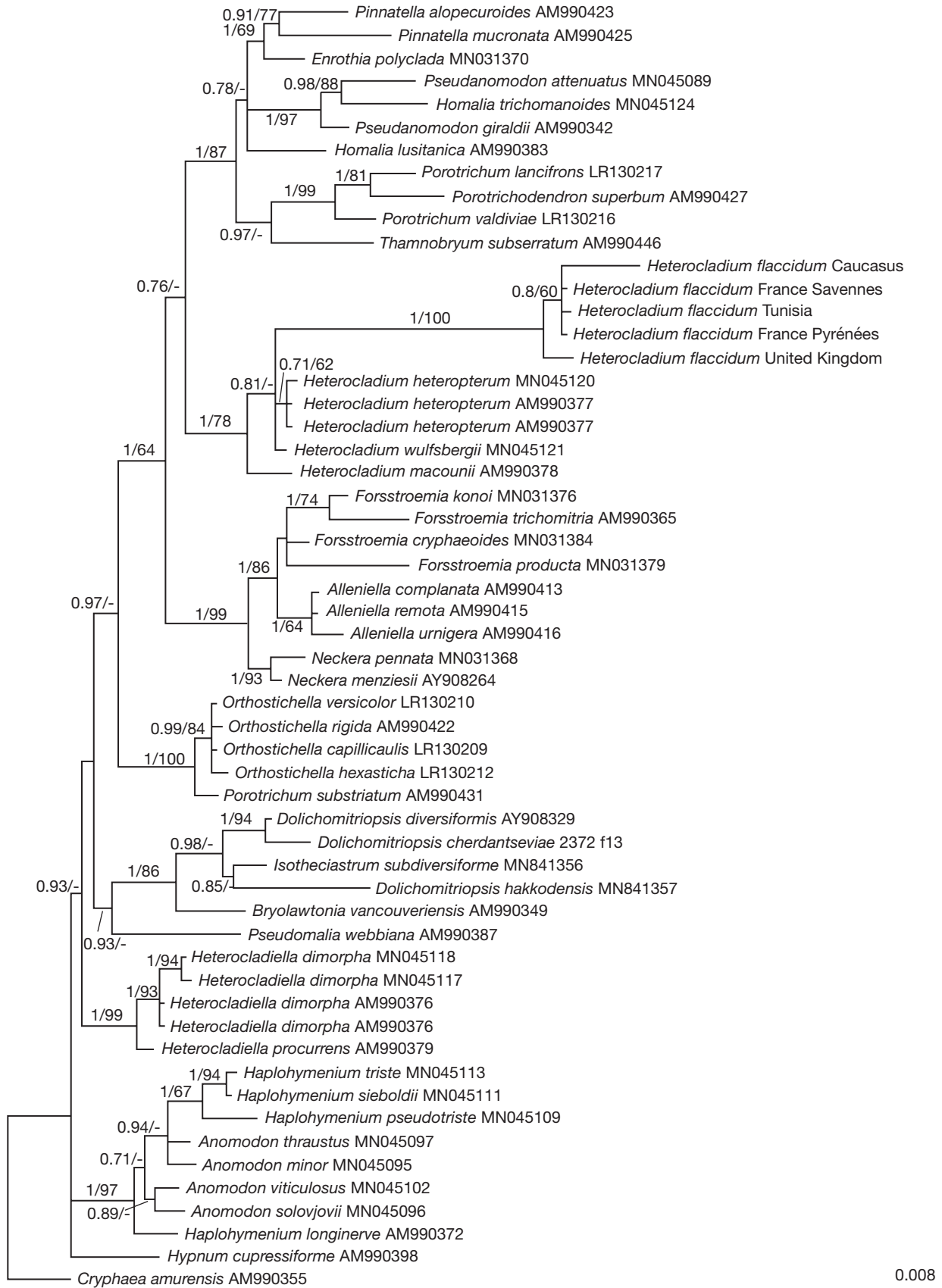


FIG. 2. — Bayesian tree from trnS-F analysis. Bayesian posterior probabilities and MP bootstrap support are shown at branches.



FIG. 3. — *Heterocladium flaccidum* (Schimp.) A.J.E.Sm.: **A-C, E-G, J-N:** leaves; **D, H, I:** habits, dry. (**A, C-F, I-L,** Georgia, Bakalin G-12-23-13; **B, G, H, M-N,** Tunisia, Hugonnot T2019-150) Scale bars: A-C, E-G, J, K, M, 0.2 mm; D, 2 mm; H, I, L, N, 100 μm.

that *H. flaccidum* is a long-established relict at the edge of its distribution area.

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