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Phylogeographic relationships between the mosses *Exsertotheca intermedia* from Macaronesian islands and *Neckera baetica* from southern glacial refugia of the Iberian Peninsula

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The phylogeographic relationships between the Macaronesian islands and southern glacial refugia of the Iberian Peninsula was studied using molecular markers and two mosses as model species: *Exsertotheca intermedia* from Macaronesia and the recently described *Neckera baetica* from the Iberian Peninsula. The new combination *Exsertotheca baetica* (Guerra) Draper, González-Mancebo, O. Werner, J. Patiño & Ros is proposed for the latter. The genus *Neckera* has recently undergone considerable systematic and taxonomic changes, but our results show that the European species *N. cephalonica* and *N. pumila* belong in *Neckera s. stricto*.

Introduction

The Macaronesian archipelagoes (Azores, Canary Islands and Madeira) are traditionally regarded as outstanding refugia for relict Tertiary lineages (Engler 1879, Axelrod 1975, Bramwell 1976, Sunding 1979, Rodríguez-Sánchez & Arroyo 2008, Rodríguez-Sánchez *et al*. 2009). However, mesic areas within the Mediterranean basin have also enabled long-term survival of some species in several glacial refugia (Vogel *et al*. 1999, Thompson 2005, Mejías *et al*. 2007, Rodríguez-Sánchez *et al*. 2008, 2009), as have also some Atlantic areas in the Iberian Peninsula (Sunding 1979, Désamoré *et al*. 2011).

The south-west of the Iberian Peninsula constitutes one of the main glacial refugia for the European fauna and flora (Hewitt 2000).

The Macaronesian region is characterized by high rates of endemicity in a vast array of organisms (Juan *et al*. 2000). This also applies to bryophytes, although this group generally exhibits much lower levels of endemism than vascular plants (Sotiaux *et al*. 2009, Aigoin *et al*. 2009, Vanderpoorten *et al*. 2011). The presence of endemics is precisely at the basis of the biogeographical concept of Macaronesia (Engler 1879, Sunding 1979). However, in addition to the strict Macaronesian endemic taxa, there is a group of species present both on the Macaronesian islands and in some glacial refugia of the Iberian Peninsula. Such taxa are called Ibero-Macaronesian endemics. In comparison with the strict Macaronesian endemics, there are few plant species with this type of distribution, such as *Convolvulus fernandesii* among the vascular plants (Carine *et al*. 2004), and the bryophytes *Frullania azorica*, *Porella canariensis*, *Tetrastichium fontanum*, *T. virens*, and *Isothecium algarvicum*. Until very recently, also *Exsertotheca intermedia* (Olsson *et al*. 2011) (= *Neckera intermedia*; *see* González-Mancebo *et al*. 2008) was considered an Ibero-Macaronesian endemic. However, the study of Guerra *et al*. (2010) rejected this concept, and they described a new species from the Iberian Peninsula, *Neckera baetica*, which had previously been overlooked.

Some previous studies on the Neckeraceae (Tsubota *et al*. 2002, Ignatov *et al*. 2007, Olsson *et al*. 2011) revealed that the genus *Neckera* as traditionally understood is paraphyletic. The results obtained by Olsson *et al*. (2011) suggest that it is phylogenetically divided into four clades that should be recognized as separate genera. The clade containing the generitype *N. pennata* is called *Neckera s. stricto*. Some species previously placed in *Neckera* became grouped in *Forsstroemia*. Finally, the rest of the '*Neckera*' species studied by Olsson *et al*. (2011) fall in two well-supported clades, and so those authors established two new genera to accommodate them: *Alleniella* (with ten species, including the European *A. besseri* and *A. complanata*) and *Exsertotheca* (including two species, *E. crispa* and *E. intermedia*).

In order to analyse the phylogeographic relationships between the Macaronesian islands and southern glacial refugia of the Iberian Peninsula we selected the Macaronesian endemic *Exsertotheca intermedia*, and the recently described *Neckera baetica* from the Iberian Peninsula. The first species was considered a suitable case study due to its relative abundance in Macaronesia (Hedenäs 1992). It has been reported from all of the Macaronesian archipelagoes (Hedenäs 1992, Patiño Llorente & González-Mancebo 2005, Frey *et al*. 2006) and was until very recently also thought to occur in the Iberian Peninsula (Guerra *et al*. 1980, 2003, Düll 1992, Frey *et al*. 2006). *Exsertotheca intermedia* was described as *Neckera intermedia* from material collected on Tenerife (Canary Islands), on the basis of the transversely undulate, oblong stem leaves with a rounded to obtuse apex (Bridel 1812). Due to its morphological variability several other taxa were described, but were later synonymised by Van der Wijk *et al*. (1964), De Sloover (1977) and Hedenäs (1992): *Neckera bolleana*, *N. elegans*, *N. elegans* var. *laevifolia* and *N. laeviuscula*. The recent segregation of the Iberian specimens as a separate species *N. baetica* (Guerra *et al*. 2010) renders *E. intermedia* a Macaronesian endemic, and arouses questions on the origin of the recently described species.

The description of *N. baetica* coincides temporally with our research on the phylogeographic relationships between the Macaronesian islands and the southern glacial refugia in the Iberian Peninsula using *Exsertotheca* species. Guerra *et al*. (2010) used nuclear ITS sequences and provided a morphological description of the species, but in our opinion some of the characters they propose are not completely useful for distinguishing the species. Therefore, the delimitation of *N. baetica* should be clarified under the framework of a most appropriate molecular approach (*see* Olsson *et al*. 2011).

Our main aim is to analyse the phylogenetic relationship between populations of *E. intermedia* from the Macaronesian islands and those of *N. baetica* from the Iberian Peninsula to infer the evolutionary origin of the latter. Secondly, we aim to clarify the present generic concept of *Neckera s. lato* species in Europe in the light of the proposal by Olsson *et al*. (2011).

Material and methods

Molecular study

Samples from seventeen populations of *E. inter-*

media and five populations of *N. baetica* were included in the molecular study. In addition, we included samples of other species traditionally included in *Neckera* and occurring in the Iberian Peninsula: *Alleniella besseri* (= *Neckera besseri*, two populations), *A. complanata* (= *N. complanata*, six populations), *Exsertotheca crispa* (= *N. crispa*, eight populations), *Neckera cephalonica* (two populations), and *N. pumila* (four populations). Other two *Neckera s. stricto* species that are present in the Iberian Peninsula (Sérgio *et al*. 2006), *N. menziesii* and *N. pennata*, are both phylogenetically clearly separated from *E. intermedia* (Olsson *et al*. 2011). Molecular sequences of these two species that are available from GenBank were also included in the present study in order to picture the phylogenetic relationship of all the Iberian species. Finally, a sample from one population of *Leptodon smithii* was used as outgroup. Details for all the sequenced specimens are provided in the Appendix.

We used two molecular markers, the nuclear ITS and the chloroplast intron *trn*L-F. Total DNA was extracted using the NaOH method of Werner *et al*. (2002). PCR was performed in an Eppendorf Mastercycler using PuReTaqTM Ready-To-Go™ PCR Beads (GE Healthcare) in a 25 μl reaction volume according to the manufacturers' instructions. For the two molecular markers, the PCR programs given below were initiated by a melting step of 5 min at 95 °C and were followed by a final extension period of 8 min at 72 °C. For the internal transcribed spacer (ITS) the PCR program employed was 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min 45 s at 72 °C, with the primers 'AB101F' and 'AB102R' (Douzery *et al*. 1999) or '25R' (Stech & Frahm 1999) and '18F-ISO'. The latter is slightly modified from the '18F' designed by Stech and Frahm (1999) and is constituted of the following base sequence: 5´GGA TGG AGA AGT CGT AAC AAG G 3´. For the chloroplast intron *trn*L-F, 35 cycles of 30 s at 95 °C, 45 s at 51 °C and 1 min 30 s at 72 °C were employed, with the primers 'TabC' and 'TabF' (Taberlet *et al*. 1991). Aliquots (5 μl) of the amplification products were visualized on 1% agarose gel. Successful amplifications were cleaned with a GenElute™ PCR Clean-Up kit (Sigma-Aldrich Biotechnology). The amplification primers were used in the sequencing reactions with the Big Dye sequencing kit and separated on an ABI-Prism 3700 using standard protocols.

Nucleotide sequence fragments were edited and assembled for each DNA region using PhyDE v0.995 (Müller *et al*. 2006). The assembled sequences were manually aligned, on the basis of the criteria provided by Kelchner (2000), and regions of incomplete data at the beginning and end of the sequences were excluded from subsequent analyses. The sequence alignments used in the analyses are available on request.

Phylogenetic analyses

Phylogenetic reconstructions were made on the basis of Maximum Parsimony (MP) and Bayesian Inference (BI) optimality criteria using the programs TNT 1.0 (Goloboff *et al*. 2003) for MP, and MrBayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) for BI. MP phylogeny was analysed by a traditional heuristic search, selecting tree bisection reconnection (TBR) as swapping algorithm, with one tree held in memory. All characters were equally weighted. Clade support was assessed via non-parametric bootstrapping using the default settings in TNT, except for the number of replicates, which was set to 1000. The output results requested were the absolute frequencies. For all generated maximum parsimony trees the consistency index (Ci) and retention index (Ri), as well as tree length were calculated.

The nucleotide substitution model used for the BI analysis was selected with jModeltest 0.1.1 (Posada 2008), based on Bayesian information criteria. BI analysis used one cold and three incrementally heated Monte Carlo Markov chains (MCMC) on two simultaneous runs. MCMC run until the standard deviation of split frequencies was below 0.01 (1 000 000 generations), with one tree sampled every 100th generation, each using a random tree as a starting point and a temperature parameter value of 0.2 (the default in MrBayes). The first 25% of the total sampled trees of each run were discarded as burn-in, in order to achieve the MCMC log-likelihoods that had become stationary and converged. The remaining sampled trees were used to construct a Bayesian consensus tree and to infer posterior probabilities (PP).

While gaps in non-coding regions are sometimes difficult to assess (Kelchner 2000), all the analyses were run both with the insertions and deletions coded as informative characters and without coding the insertions and deletions. The indels were coded using Simple Indel Coding (SIC) strategy, by Simmons and Ochoterena (2000), as implemented in SeqState (Müller 2004).

Results

Molecular analyses

The nucleotide substitution model that best suits our data set was K80+G. Length variation for the ITS dataset (ITS1, 5.8S rDNA, ITS2) ranges from a low of 725 nucleotides to a high of 777 nucleotides (missing data, length polymorphic regions, 3´ end of SSU rDNA and 5´ end of LSU rDNA excluded), of which 64 sites are variable, and of these 45 are parsimony-informative. Out of the 462 to 463 nucleotides included in the *trn*L-F, 19 are variable and 16 of these are parsimony informative. The simple indel coding doubled the number of parsimony-informative characters in the case of the nuclear data sets, increasing them with 34 characters. In the case of the chloroplast data sets, the simple indel coding yielded only one additional parsimony-informative character.

The two optimality criteria used (MP and BI) yielded similar results in all the analyses run, although BI resolves some of the relationships that remain unclear under the MP analysis. Separate analyses of the nuclear and the chloroplast datasets result in clearly congruent tree topologies that differ only in unsupported clades, and so the ITS and *trn*L-F datasets are combined. The topology of the trees resulting from the analyses coding and without coding the insertions and deletions as informative characters are also congruent, although MP trees obtained without the indels coded are markedly shorter than those obtained with indels coded. The tree shown (Fig. 1) is therefore the result of the BI analysis of the dataset that combines the two molecular markers, not including the insertions and deletions coded as informative characters. The MP analysis of the same combined dataset yields 693 equally parsimonious trees of 160 steps (Ci = 0.869, Ri = 0.954) and has an overall similar topology, albeit lacking significant support for some of the clades (bootstrap values for well supported clades are indicated in Fig. 1).

In all the analyses, the studied samples of *N. baetica* were positioned together in a clade that is clearly separated (PP = 1.00) from the clade in which all the *E. intermedia* samples from Macaronesia gather (Fig. 1). Moreover, when the ITS marker is separately analysed, *E. crispa* is resolved in a well supported clade (PP = 1.00, bootstrap value = 100), and the *N. baetica* populations are placed as a sister clade (with PP = 0.95) to this *E. crispa* group, instead of together with the Macaronesian *E. intermedia*, as was also observed by Guerra *et al*. (2010). Within the studied taxa, *N. baetica* is phylogenetically closely related to the Macaronesian *E. intermedia* and the European-Asiatic *E. crispa*. These three species constitute a well-supported clade in all the analyses (PP = 1.00), which according to the chloroplast marker *trn*L-F is sister to *A. besseri* and *A. complanata*. Finally, *Neckera pumila* and *N. cephalonica* are closely related to *N. pennata* (PP = 0.99 according to the *trn*L-F marker) and *N. menziesii* (PP = 1.00, bootstrap value = 91), constituting a monophyletic basal clade, which is apparently not more closely related to *Alleniella* or *Exsertotheca* than the outgroup taxon *Leptodon smithii*.

Discussion

Our results (Fig. 1) fully agree with the generic circumscription proposed by Olsson *et al*. (2011). In addition, the study of two molecular markers, including one nuclear and one chloroplast, and the increased number of sequences analysed, give support to the description of a separate species for the Iberian Peninsula, as Guerra *et al*. (2010) suggested on the basis of the study of ITS markers of two specimens for each species included in their study. All the studied samples of *Exsertotheca crispa* and *E. intermedia* are grouped in a well-resolved clade, together with the samples of *Neckera baetica*.

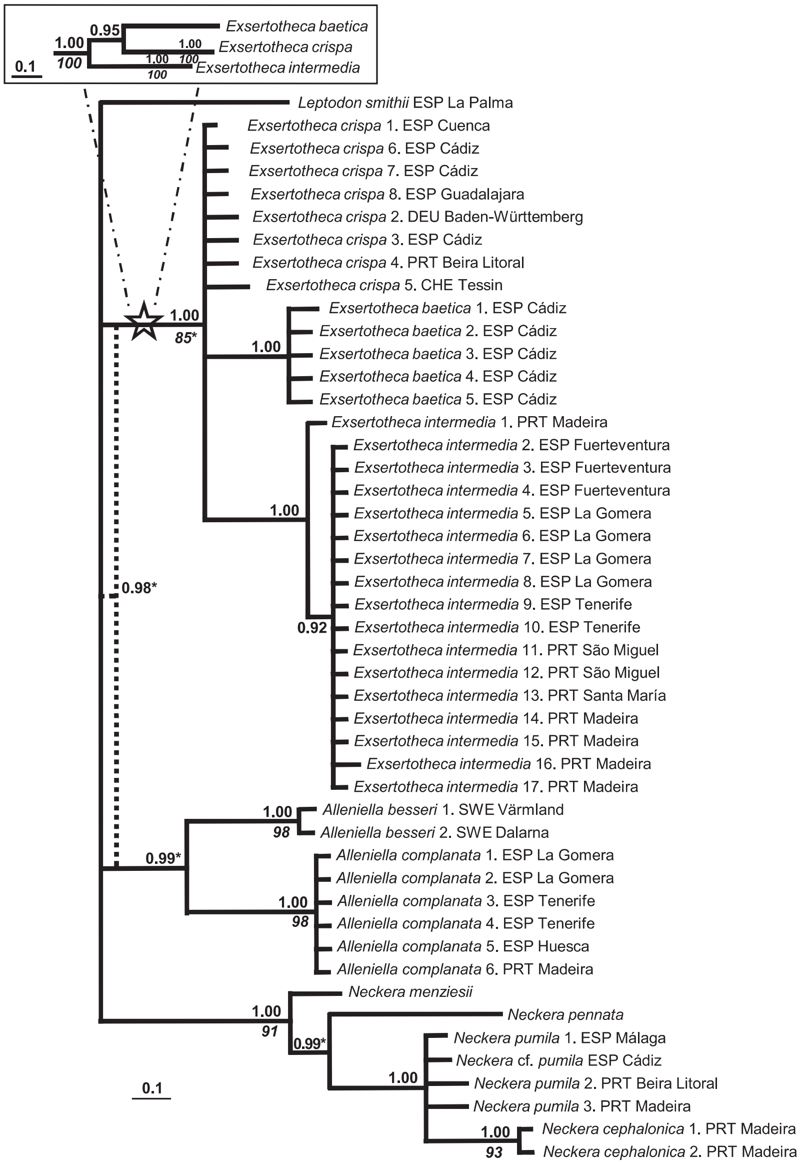

Fig. 1. Tree obtained from the BI analysis of the dataset combining ITS and *trn*L-F molecular markers. CHE = Switzerland; DEU = Germany; ESP = Spain; PRT = Portugal; SWE = Sweden. Sample numbers correspond to those given in the Appendix. PP values (≥ 0.95) are given above the branches and bootstrap values (≥ 85) obtained for the equivalent clades in MP analysis are given below the branches; * = clade support and relationship recovered only on the analysis based on the *trn*L-F dataset. ★ = topology of the ingroup clade recovered only on the analysis of the ITS dataset (note that species names represent several specimens with nearly identical sequences).

The included specimens of *Alleniella besseri* and *A. complanata* constitute a separate clade, which is sister to the one including *Exsertotheca*. Finally, our samples of *Neckera pumila* and *N. cephalonica* fall into a clade containing *Neckera s. stricto* species (*N. menziesii* and *N. pennata*), and which is clearly separated both from *Exsertotheca* and from *Alleniella*.

The close relationship of the studied samples of *N. baetica* with *E. intermedia* and *E. crispa* justifies placing the recently described *N. baetica* into the genus *Exsertotheca*. In order to better understand the phylogenetic position of *N. baetica*, we have also run MP and BI analyses of the ITS dataset used in the present study including the wider Neckeraceae ITS sampling of Olsson *et al*. (2011), available in GenBank. As expected, *N. baetica* falls into the *Exsertotheca* clade of Olsson *et al*. (2011), as sister clade of *E. crispa* and closely related to *E. intermedia*. The resulting trees show identical topologies to those shown in the present study and by Olsson *et al*. (2011) and are therefore not shown. Regarding the other samples sequenced for this study, our

Alleniella besseri and *A. complanata* specimens are grouped together with Olsson's *et al.* (2011) samples of the corresponding species. As for *Neckera pumila* and *N. cephalonica*, our samples fall into Olsson's *et al.* (2011) *Neckera s. stricto* clade, which precludes any taxonomic change for these two species not previously studied by Olsson *et al.* (2011). The interest of the confirmation of these two species as belonging to *Neckera* has to be highlighted in the taxonomic frame of the Neckeraceae shown by Olsson *et al.* (2011).

After our contribution, from the nine species of *Neckera* accepted by Hill *et al.* (2006) for Europe and Macaronesia, *N. oligocarpa* (known from Scandinavia, Austria and Italy according to Frey *et al.* 2006; also from North America according to Anderson *et al.* 1990) is the only one for which the generic placement remains to be tested by molecular data. Taxonomically, this species appears to be close to *N. pennata*, with which it has been synonymized (Ignatov & Afonina 1992) or treated at varietal rank (*N. pennata* var. *tenera*). We therefore think that it probably belongs to *Neckera s. stricto*, although the verification is still necessary.

Exsertotheca baetica (Guerra) Draper, González-Mancebo, O. Werner, J. Patiño & Ros, *comb. nova*

Neckera baetica Guerra, Nova Hedwigia 91: 259, *f. 4–6, 7, 9, 12, 14*. 2010.

We agree with Guerra *et al.* (2010) that *Exsertotheca baetica* is well separated from *E. intermedia*, not only by the studied molecular markers, but also morphologically. These authors consider that the species is mainly characterized by its large shoots (up to 35 cm), yellowish green, ovate-oblong leaves, which are transversely undulate or plane when dry, obtuse to rounded leaf apex, plane leaf margins, eporose upper and middle laminal cells with 1.5–2 μm thick walls, and a slightly papillose and spiculose endostome. They characterize *E. intermedia* by its dark green-brown to coppery colour, mostly oblong or elongate oblong leaves, which are occasionally lightly constricted in the upper third and strongly transversely undulate, porose laminal cells with the mid-leaf cells walls (2)3.5–3.7(4.5) μm thick, and strongly papillose, not spiculose endostome processes. Guerra *et al.* (2010) also state that *E. baetica* is similar to *E. intermedia* in the leaf apex, the plane leaf margins, and the size and shape of the laminal cells. Our morphological study allows us to add some morphological differences between these species. In *E. baetica* the plants are small to large (no more than 30–35 cm long), but never as large as *E. intermedia*, which can reach a length of 1 m in the Canary Islands. The stems of *E. baetica* are up to 3 mm wide including the leaves, whereas they usually reach 4–5 mm in *E. intermedia*. It has to be mentioned that both species are highly variable in size depending on the environmental conditions in *Quercus canariensis* forests (*E. baetica*) or in laurel forests (*E. intermedia*). The stem leaves are generally asymmetric, spirally arranged on the branches and only loosely complanate on the old stems in *E. baetica*, and slightly asymmetric and complanate in *E. intermedia*; the stem leaf apex is slightly denticulate in *E. baetica* and generally entire in *E. intermedia*. It is also important to mention that, according to our observations, *E. baetica* has a slightly to strongly concave leaf lamina, not undulate when moist, not undulate or rugose and with 1–2 undulations when dry, while the leaves of *E. intermedia* are slightly transversely undulate when moist and strongly so when dry. Therefore, the placement of *E. baetica* in the group of undulate-leaved *Neckera* species by Guerra *et al.* (2010) cannot be sustained.

The recent description of *E. baetica*, an Iberian endemic, renders *E. intermedia* a Macaronesian endemic. Morphologically *E. baetica* is closer to *E. intermedia* than to *E. crispa*, in spite of the fact that the sampled *E. baetica* populations form a sister clade to the sampled *E. crispa* populations (Fig. 1). Even if further studies are necessary in order to reliably assess the origin for *E. baetica*, this morphological similarity suggests that it shared a common ancestor with *E. intermedia*. It is possible that *E. baetica* represents a recently diverging species in a Mediterranean glacial refuge, as was recently proposed for the Corsican endemic *Leptodon corsicus* (Sotiaux *et al.* 2009). If so, the origin of *E. baetica* might be more associated with ecological differentiation than with geographical disjunction, since the area where it thrives in the south of the Iberian Peninsula has a very different climate than the surrounding areas. The climate is Mediterranean, but with a strong oceanic influence, and it has already been pinpointed as one of the main hotspots for plant biodiversity, both because of the presence of a relict flora and for its high rate of endemism (Rodríguez-Sánchez *et al*. 2008). Nevertheless, another possible explanation for the presence of the species in this area is that it is a relict of an originally Tertiary species. Studies on vascular plants have shown that endemics in the southwest of the Iberian Peninsula have followed different evolutionary patterns, including both long-term isolation and multiple active processes of speciation (Rodríguez-Sanchez *et al*. 2008, 2009). Further studies are therefore necessary in order to be able to date the time of divergence of *E. baetica*.

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Appendix. Herbarium vouchers, studied specimens' geographic origin and Genbank accession numbers (ITS; *trn*L-F).

Alleniella besseri. **1**. S-B106203, Sweden, Värmland (JF690782; JF690819); **2**. S-B125094, Sweden, Dalarna (JF690783; JF690820).

Alleniella complanata. **1**. MUB-30336/TFCBry-15241, Spain, Canary Islands, La Gomera (JF690784; JF690821); **2**. MUB-30337/TFCBry-15233, Spain, Canary Islands, La Gomera (JF690785; JF690822); **3**. MUB-30335/TFCBry-15256, Spain, Canary Islands, Tenerife (JF690786; JF690823); **4**. MUB-30334/TFCBry-15253, Spain, Canary Islands, Tenerife (JF690787; JF690824); **5**. MUB-34552, Spain, Huesca (JF690788; JF690825); **6**. S-B9726, Portugal, Madeira (JF690789; JF690826).

Exsertotheca baetica. **1**. MUB-34553, Spain, Cádiz (JF690800; JF690841); **2**. MUB-34554, Spain, Cádiz (–; JF690842); **3**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Cádiz (–; JF690843); **4**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Cádiz (–; JF690844); **5**. S-B100277, Spain, Cádiz (–; JF690845).

Exsertotheca crispa. **1**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Cuenca (JF690796; JF690834); **2**. S-B125096, Germany, Baden-Württemberg (JF690795; JF690833); **3**. MUB-10055, Spain, Cádiz (JF690797; JF690835); **4**. S-B44528, Portugal, Beira Litoral, Coimbra (JF690798; JF690836); **5**. S-B111464, Switzerland, Tessin (JF690799; JF690837); **6**. MUB-34555, Spain, Cádiz (–; JF690838); **7**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Cádiz (–; JF690839); **8**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Guadalajara (–; JF690840).

Exsertotheca intermedia. **1**. S-B9730, Portugal, Madeira (JF690816; JF690861); **2**. MUB-310007/TFCBry-17120, Spain, Canary Islands, Fuerteventura (JF690801; JF690846); **3**. MUB-310008/TFCBry-17123, Spain, Canary Islands, Fuerteventura (JF690802; JF690847); **4**. MUB-310009/TFCBry-17126, Spain, Canary Islands, Fuerteventura (JF690803; JF690848); **5**. MUB-31001/TFCBry-15242, Spain, Canary Islands, La Gomera (JF690804; JF690849); **6**. MUB-31002/TFCBry-15231, Spain, Canary Islands, La Gomera (JF690805; JF690850); **7**. MUB-31006/TFCBry-15234, Spain, Canary Islands, La Gomera (JF690806; JF690851F); **8**. MUB-31005/TFCBry-15801, Spain, Canary Islands, La Gomera (JF690807; JF690852); **9**. MUB-31004/TFCBry-15250, Spain, Canary Islands, Tenerife (JF690808; JF690853); **10**. MUB-31003/TFCBry-15252, Spain, Canary Islands, Tenerife (JF690809; JF690854); **11**. S-B42781, Portugal, Azores, São Miguel (JF690810; JF690855); **12.** S-B42782, Portugal, Azores, São Miguel (JF690811; JF690856); **13**. AZU, Portugal, Azores, Santa María (JF690812; JF690857); **14**. MUB-30332/TFCBry-15264, Portugal, Madeira (JF690813; JF690858); **15**. E-00266405, Portugal, Madeira (JF690814; JF690859); **16**. MUB-30333/TFCBry-15263, Portugal, Madeira (JF690815; JF690860); **17**. MUB-34556, Portugal, Madeira (JF690817; JF690862).

Leptodon smithii. MUB-30331/TFCBry-15246, Spain, Canary Islands, La Palma (JF690781; JF690818).

Neckera cephalonica. **1**. S-B9806, Portugal, Madeira (JF690793; JF690831); **2**. S-B9824, Portugal, Madeira (JF690794; JF690832).

Neckera menziesii. GenBank FM161167; FM210305.

Neckera pennata. GenBank FM161169; AM990414.

Neckera pumila. **1**. Autonomous University of Madrid Bryophyte Herbarium, Spain, Málaga (JF690790; JF690827); **2**. S-B44531, Portugal, Beira Litoral, Coimbra (JF690791; JF690828); **3**. S-B9798, Portugal, Madeira (JF690792; JF690829). ***Neckera cf. pumila***. Autonomous University of Madrid Bryophyte Herbarium, Spain, Cádiz (–; JF690830).