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## **From European Priority Species to Invasive Weed: *Marsilea azorica* (Marsileaceae) is a Misidentified Alien**

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## From European Priority Species to Invasive Weed: *Marsilea azorica* (Marsileaceae) is a Misidentified Alien

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**Abstract**—The clover fern *Marsilea azorica* was described in 1983 from the isolated Azores archipelago in the northern Atlantic, where it is restricted to a single roadside pond. Thought to be an extremely local endemic, it was subsequently listed as a conservation priority species for the Azores, Macaronesia, and Europe, included as ‘critically endangered’ on the IUCN red list, and as ‘strictly protected’ species by the Bern convention and the European Union’s habitats directive. However, we present morphological and molecular data (*rbcL* gene, *rps4* gene, *rps4-trnS* spacer and *trnL-trnF* spacer sequences), which demonstrate that *M. azorica* is conspecific with *M. hirsuta*, a species native to Australia, but widely cultivated and locally invasive in the southern U. S. A. Based on our DNA data, we conclude that these plants are most likely a recent introduction to the Azores from Florida. We recommend removal of Azorean *Marsilea* from conservation priority lists. While there is no evidence that the small existing population threatens native species, further spread in the Azores should be prevented.

**Keywords**—Azores, Azorean endemic, DNA barcoding, invasive ferns, molecular phylogenetics.

The clover ferns or waterclovers, *Marsilea* L., are a genus of 45–50 aquatic, heterosporous fern species (Nagalingum et al. 2007). Together with the pillworts, *Pilularia* L. (five species), and the monotypic *Regnellidium* Lindm., they are placed in the family Marsileaceae, which is the sister group to the Salviniaceae (Johnson 1986; Kubitzki 1990; Nagalingum et al. 2007; Nagalingum et al. 2008). Waterclovers are most diverse in tropical and subtropical Africa and America, where they grow in seasonal and permanent ponds, and in shore regions of larger fresh water bodies (Launert 1968; Johnson 1986). *Marsilea* species are easily recognized by their clover-like leaves, which are composed of four leaflets on a long petiole. Most species show extreme morphological plasticity in their vegetative organs and sterile material is often impossible to identify based on morphology alone (Launert 1968). The current classification is instead based on morphology of the sporocarps, long-lived, drought-resistant reproductive structures produced near or at the petiole base (Launert 1968; Johnson 1986; Kubitzki 1990). The sporocarps are the primary dispersal and dormancy units in Marsileaceae and release their spores only in water, where they germinate rapidly (Schneider and Pryer 2002). Even though these sporocarps suggest Marsileaceae is well adapted to long-distance dispersal by waterfowl, a population-level study in the Mediterranean region found extremely reduced gene flow between distant or close populations of the clover fern *M. strigosa* Willd., pointing to a low dispersal ability, at least in that species (Vitalis et al. 2002).

In the middle-Atlantic islands, *Marsilea* has been recorded from the Cape Verde archipelago, the Canary Islands, and the Azores. In the Cape Verdes, the African species *Marsilea coromandelina* Willd. is listed as native on Branco island (Arechavaleta et al. 2005). In the Canarian archipelago, the genus was known from a single locality at Arucas on Gran Canaria. Initially identified as *M. quadrifolia* (Webb and Berthelot 1840), the Arucas species was identified by most later authors to be *M. diffusa* A. Br. (= *M. minuta* L.; Milde 1867; Christ 1888; Burchard 1929; Kunkel 1971), with Braun (1870) regarding it as a distinct form (f. *canariensis*). However, Hansen (1972) considered it to be *M. quadrifolia* and some

doubt therefore exists over the identity of the Canarian plant. The plant was last collected from a wetland area at Arucas in 1897 (*O. Gelert s. n.*, Arucas, 22 April 1897, (C) [sterile specimen]). More recently, the genus has been considered an introduction to the archipelago (Izquierdo et al. 2004), although evidence for this is unclear. Complete loss of the wetland area on Gran Canaria, as already noted by Burchard (1929), means it is likely that the genus is now extinct in the Canaries.

In contrast to the situation in the Canaries, *Marsilea* was not mentioned in any of the 19th century plant inventories for the Azores (Seubert and Hochstetter 1843; Seubert 1844; Drouet 1866; Watson 1870; Trelease 1897; Gandoger 1899). Clover ferns are also missing in all Azores plant lists based on fieldwork from the first 70 yr of the 20th century (Christ 1906; Druce 1911; Gonçalves da Cunha and Sobrinho 1938; Palhinha 1943; Palhinha et al. 1946; Tardieu-Blot 1946; Dansereau 1961; Palhinha 1966; Vasconcellos de Carvalho 1968; Sjögren 1973). The first collection from the Azores was made on Terceira Island in the central group of the archipelago by I. Botelho Gonçalves, an inspector of the local forest department (*Gonçalves 3259*, 12.7.1971 (BM)). It was identified as *M. quadrifolia*, and reported as such in *Atlas Flora Europaea* (Jalas and Suominen 1972). At the same time, the plant was independently discovered and the find published by Hansen (1972, 1973), who found the plants “abundant in mud-bottom of a small temporary lake or pond near Pico de Bagacina (about 600 m),” which remains the only known *Marsilea* population in the Azores. Hansen identified the plants as *M. strigosa* Willd. and, commenting on the record in Jalas and Suominen (1972), said “[it] may very well be the same plant and the same locality, yet its identity as *M. strigosa* is quite clear!” In 1983, based on examination of an herbarium specimen in Copenhagen (*Hansen 308*, 4 July 1972 (C)), E. Launert concluded that the Azorean material belonged to an undescribed species closely related to the western European *M. strigosa*, but differing in sporocarp morphology. Consequently, it was described as *Marsilea azorica* Launert & Paiva (Launert and Paiva 1983; our Fig. 1).

Even though it is restricted to a single, small, roadside pond in a heavily grazed pasture, the Azorean *Marsilea* population



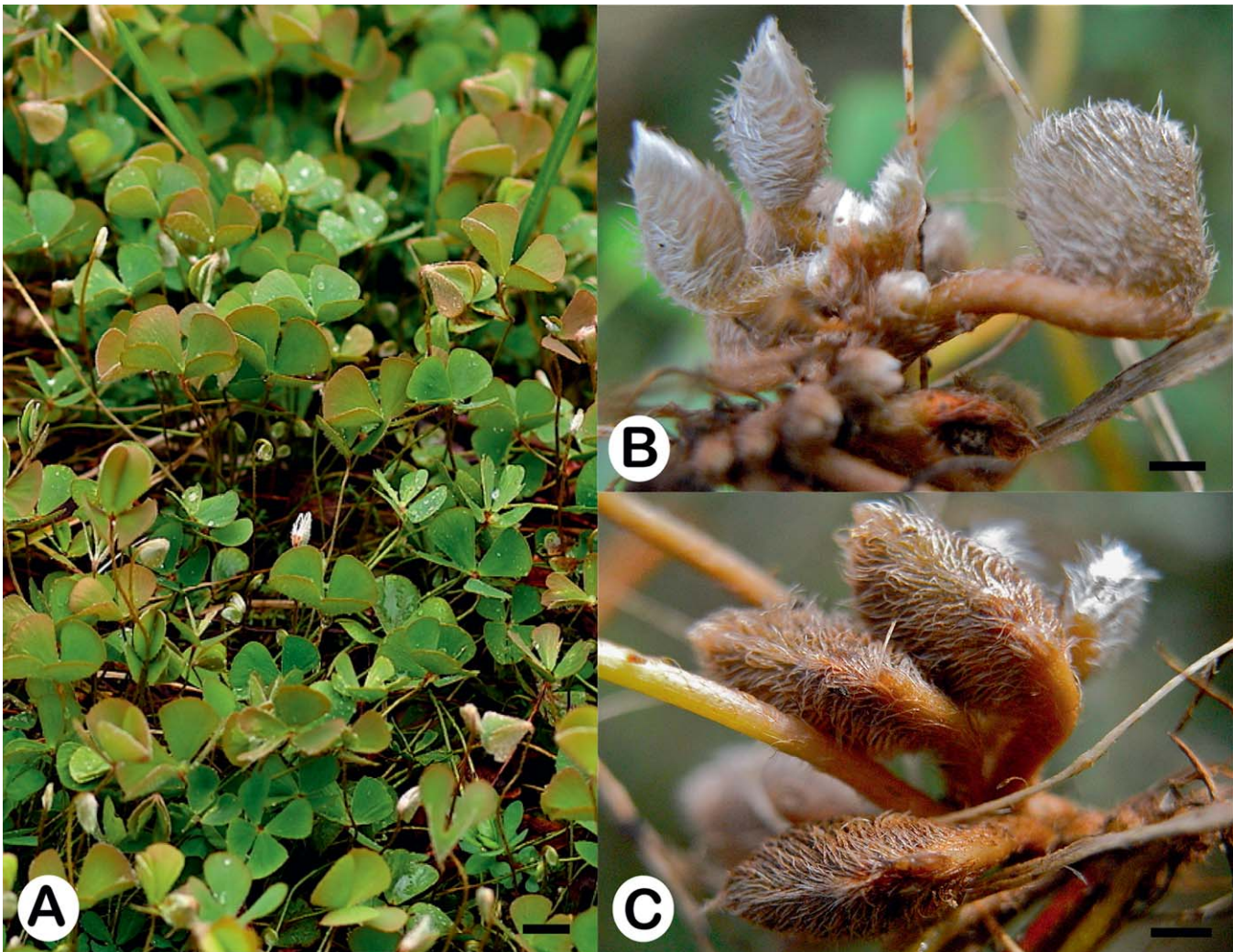


FIG 1. *Marsilea hirsuta* (= *M. azorica*), Terceira, Azores (Portugal). A. habit (scale bar 10 mm). B, C. sporocarps (scale bars 1 mm); photos 1A: H. Schaefer; 1B, C: F. Rumsey).

has remained stable since the 1970s. *Marsilea azorica* was classified as a conservation priority species in the Azores, Macaronesia, and Europe (Martín et al. 2008) and the population is managed by the local conservation agency. In a recently published 'taxonomically unbiased' approach to prioritize conservation resources in Macaronesia, it reached the highest scoring of all organisms included in the ranking (Martín et al. 2010). It was listed as 'critically endangered' in the IUCN red list (IUCN 2010) and was included in Appendix 1 of the 'Bern convention on the conservation of European wildlife and natural habitats' and Annex 2 of the 'Habitats Directive' (Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora) as a strictly protected species (Council of Europe 2010).

In contrast to the considerable effort focused on the conservation of *M. azorica*, its taxonomy and relationships have been largely neglected and all recent treatments of the Azorean flora and vegetation (e.g. Dias 1989, 1996; Hansen and Sunding 1993; Schaefer 2002, 2003, 2005a, b; Silva et al. 2010) have adopted the view of Launert and Paiva (1983) without a critical re-evaluation of the plant. Nevertheless, its late discovery, disturbed habitat, and highly localized distribution have seemed suspicious to many. In this paper, we use DNA data, in combination with a morphological reassessment of

*M. azorica*, to determine its status and specifically to establish whether or not it is a genetically and morphologically distinct entity endemic to the Azores that deserves special protection and management.

#### MATERIALS AND METHODS

**Sampling for Molecular Analysis and DNA Extraction**—We included 57 accessions of 39 species of Marsileaceae. Fourteen sequences for accessions of *M. aegyptiaca* Willd., *M. azorica*, *M. hirsuta*, and *M. strigosa* were generated for this study and deposited in Genbank (accession numbers HQ 728338–48; Appendix 2). The remaining sequences, produced by Nagalingum et al. (2007, 2008) and Whitten and Jacono (2009), were downloaded from Genbank. Trees were rooted with *Pilularia* and *Regnellidium* based on Pryer (1999). DNA isolation from silica-dried leaves and herbarium material, amplification, and sequencing of the *rbcL*, *rps4*, *rps4-trnS* and *trnL-trnF* regions (all from the chloroplast) followed the protocol in Nagalingum et al. (2007). Primer sequences and PCR conditions are as in Whitten and Jacono (2009).

**Sequence Alignment and Phylogenetic Analyses**—Sequences were edited using Geneious pro 5.0.4 (Drummond et al. 2010), and aligned with MAFFT (Kato et al. 2005). The final alignments were checked in MacClade 4.08 (Maddison and Maddison 2005). There were no ambiguously aligned regions and therefore nothing had to be excluded from subsequent analyses.

Equally weighted maximum parsimony (MP) analyses for matrices of nucleotides were conducted using PAUP 4.0b10 (Swofford 2002). The search strategy involved 100 random addition replicates with TBR



branch swapping, saving all optimal trees. Gaps were treated as missing data. To assess node support, parsimony bootstrap analyses (Felsenstein 1985) were performed using 1,000 replicate heuristic searches, each with 10 random addition replicates and TBR branch swapping, saving all optimal trees. We detected no statistically supported topological conflicts between the data sets for the individual markers and therefore combined all sequences in one alignment used in all subsequent analyses.

Maximum likelihood (ML; Felsenstein 1973) tree searches and ML bootstrap searches (Felsenstein 1985) for the individual and combined data sets were performed using RAxML-HPC2 vs. 7.2.6 (Stamatakis et al. 2008) on the CIPRES cluster (Miller et al. 2009). Based on the Akaike information criterion (Akaike 1974) and the Bayesian information criterion (Schwarz 1978) as implemented in jModeltest (Posada 2008), we selected the GTR +  $\Gamma$  model (six general time-reversible substitution rates, assuming gamma rate heterogeneity), with model parameters estimated over the duration of specified runs. Bayesian Markov chain Monte Carlo (MCMC) inference (Yang and Rannala 1997) used the GTR +  $\Gamma$  model (with the default four rate categories) plus a proportion of invariable sites, and relied on MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) on the CIPRES cluster (Miller et al. 2009). The MCMC runs started from independent random trees, were repeated twice, and extended for ten million generations, with trees sampled every 100th generation. We used the default priors in MrBayes, namely a flat Dirichlet prior for the relative nucleotide frequencies and rate parameters, a discrete uniform prior for topologies, and an exponential distribution (mean 1.0) for the gamma-shape parameter and branch lengths. Convergence of the Bayesian analyses was assessed by checking that final likelihoods and majority rule topologies in different runs were similar, that the standard deviations of split frequencies were  $< 0.01$ , and by examining the plot of generation number versus log probability of the data.

The data matrix and trees have been deposited in TreeBASE (study number 11196).

**Morphology**—*Marsilea azorica* was studied in the field during several visits to Terceira in 1994, 1998, 2000, 2005, 2008, and 2010. Herbarium specimens of *Marsilea* from the Azores and other origins were studied at BM and GH (Appendix 1). While we screened through all available *Marsilea* material at the two herbaria, we focused especially on comparisons of the Azorean taxon with the following: (i) specimens of *M. quadrifolia* and *M. strigosa*/*M. batardae* because of previous determinations; (ii) *M. minuta* specimens from the Canaries and elsewhere to exclude the possibility that the same taxon occurs in both archipelagos, (iii) genetically similar species from Australia as revealed here by our molecular phylogenetic analyses: *M. hirsuta* R. Br., *M. drummondii* A. Braun, *M. exarata* A. Braun (incl. *M. paradoxa* Diels), and *M. angustifolia* R. Br. We have not seen or sequenced material of the Australian endemics *M. costulifera* D. L. Jones, *M. latzii* D. L. Jones, and *M. cryptocarpa* Albr. & Chinnock, which are all uncommon. However, based on descriptions in Entwisle (1993), Jones (1998), and Albrecht and Chinnock (2008), they are close to *M. hirsuta* and comparisons with the Azorean material were therefore made using the published descriptions of these taxa.

Our examination of morphology (Table 1) encompassed both leaflet and sporocarp traits. In general, vegetative features, especially leaflet size and shape, vary among *Marsilea* species but also within a species (Johnson 1986). The presence, structure, abundance, and persistence of an indumentum may also vary. The sporocarps may be borne singly or in clusters (although poor production by taxa that normally produce multiple sporocarps may cause confusion), and may be sessile or more usually pedicellate. The characters of the pedicel, notably its branching or lack thereof, seem to be informative. The sporocarp itself provides many characters: shape, size, surface detail (ribbing), and the number and relative size of the so-called teeth on its dorsal surface would appear to be among the most reliable and regularly used taxonomic discriminants.

## RESULTS

**Phylogenetic Analyses**—The aligned matrix comprised 2,532 nucleotides with a total of 5.3% gaps or missing data in the combined alignment and 525 variable characters, 404 of them parsimony informative. Maximum parsimony, ML, and Bayesian analyses resulted in highly congruent topologies, so we only show the ML tree (Fig. 2). We recover six highly supported clades, one comprising *M. mutica* and a number of other Neotropical species with branched pedicels corresponding to 'group I' of Nagalingum et al. (2007), and five

other clades with usually unbranched pedicels corresponding to 'group II' of these authors. The latter group consists of five well-supported clades and a few species (*M. quadrifolia*, *M. strigosa*, and *M. nubica*) placed among them with relatively poor support. One of those five clades, the Neotropical *M. mollis* clade, is sister to the remaining African, Asian, and Australian clades (even though it appears to be a polytomy in Fig. 2 because of the short branches).

The *rbcL*, *rps4*, *rps4-trnS*, and *trnL-trnF* sequences for the Azorean *Marsilea* are completely identical to the sequences of two *M. hirsuta* accessions from invasive populations in Florida, U. S. A., and a native population in New South Wales, Australia (Fig. 2). Together with other accessions of invasive *M. hirsuta* from southern U. S. A., they are nested in a well-supported clade of Australian species ('*M. hirsuta* clade' in Fig. 2; 92% likelihood bootstrap (BS), 0.99 Bayesian posterior probability (PP)).

**Morphology**—The Azorean *Marsilea* forms dense mats in shallow water, creeping with relatively robust rhizomes of ca. 1 mm diameter, with slightly hairy nodes and internode distances between 5–62 mm. The leaves are on petioles 12–125 mm long, and the four leaflets are fresh green, obovate-cuneate, entire, 8–24  $\times$  6–22 mm, densely hairy when young, later almost glabrous. The sporocarps are solitary or in groups of 2–3 at the base of the petioles, but not adnate to them, on relatively robust, unbranched pedicels that are as long as or up to twice as long as the sporocarp ( $< 5$  mm), erect, recurved, and densely hairy with multicellular, uniseriate hairs. The sporocarp is 3.8–4.8  $\times$  2.9–3.8 mm, oblong-ellipsoid, with six to eight faint lateral ridges when young. Each sporocarp contains six to eight sori on each side. There are two teeth on the sporocarp dorsal wall; the lower tooth is prominent, conical, and slightly truncate; the upper tooth is also well-developed, broadly conical, obtuse, and elliptic in outline.

The Azorean plant is clearly different from both *M. quadrifolia* and *M. strigosa*/*M. batardae* in the degree of pubescence of the leaflets on both surfaces and the possession of two, equal-sized sporocarp teeth (Table 1). Leaflet pubescence, together with the larger sporocarps, also distinguish the Azorean plants from *M. minuta*.

All members of the '*M. hirsuta*' clade in our phylogenetic tree (Fig. 2), plus the three unsequenced Australian endemics (*M. costulifera*, *M. cryptocarpa*, and *M. latzii*), share the feature of pubescent leaves (Table 1). However, only *M. hirsuta* possesses the two, equal-sized sporocarp teeth evident in Azorean plants, and using our morphological traits (Table 1) the Azorean plants and *M. hirsuta* are indistinguishable, suggesting that the two are conspecific.

**Habitat**—The only *Marsilea* population in the Azores is restricted to a small roadside pond in a pasture at ca. 460 m, where it grows together with *Agrostis stolonifera* L., *Cyperus eragrostis* Willd., *Cyperus longus* L., *Eleocharis multicaulis* (Sm.) Desv., *Galium palustre* L., *Hydrocotyle vulgaris* L., *Isolepis fluitans* (L.) R. Br., *Juncus articulatus* L., *J. bulbosus* L., *J. effusus* L., *Mentha suaveolens* Ehrh., *Myosotis ramosissima* Rochel ex Schult., *Polygonum hydropiperoides* Michx., *Potamogeton polygonifolius* Pourr., *Ranunculus flammula* L., *R. repens* L., *Rumex conglomeratus* Murray, and *Trifolium repens* L.

## DISCUSSION

**The Taxonomic Status of '*M. azorica*'**—Morphological and molecular data both indicate that *M. azorica* is conspecific

TABLE 1. Morphological comparison of selected *Marsilea* species based on herbarium material (Appendix 1) and literature (Johnson 1986; Paiva 1986; Akeroyd 1993; Entwistle 1993; Jones 1998; Jacono and Johnson 2006; Albrecht and Chinnock 2008).

	<i>Marsilea azorica</i> Launert & Paiva	<i>Marsilea strigosa</i> Willd.	<i>Marsilea batardae</i> Launert	<i>Marsilea quadrifolia</i> L.	<i>Marsilea minuta</i> L. (incl. M. crenata C.Presl)	<i>Marsilea hirsuta</i> R. Br.	<i>Marsilea drummondii</i> A. Braun	<i>Marsilea exarata</i> A. Braun (incl. M. paradoxa Diels)	<i>Marsilea angustifolia</i> R. Br.	<i>Marsilea latzii</i> D. L. Jones	<i>Marsilea costulifera</i> D. L. Jones	<i>Marsilea cryptocarpa</i> Albr. & Chinnock
<b>Range</b>	Portugal, Azores	Western Europe, Southwest Russia	Iberian peninsula	Eurasia, Africa, introduced in the Americas	Africa, Asia, Australia, introduced in the Americas	Australia, wide spread, introduced in North America	Australia, wide-spread	Australia, wide-spread	Australia, Northern Territory & Western Australia	Australia, Northern Territory, Tanami Desert	Australia, wide-spread	Australia, Northern Territory & South Australia
<b>Leaflet shape</b>	obovate-cuneate	obdeltate-cuneate	obdeltate-cuneate	obovate-cuneate	broadly obovate-cuneate	obovate-cuneate	broadly obovate-cuneate	oblanceolate-cuneate	narrowly cuneate	(broadly) cuneate	oblanceolate-cuneate	obdeltate
<b>Leaflet pubescence</b>	hairy becoming glabrescent	glabrous to sparsely hairy	glabrescent	glabrous or with few hairs only	glabrous to sparsely hairy	hairy to glabrous	hairy	glabrescent	glabrescent	glabrous to hairy	hairy	(densely) hairy
<b>Leaflet size</b>	8–24 × 6–22 mm	2–15(-30) × 2–15(-25) mm	2–8(-11) × 2–7(-9) mm	7–30 × 6–31 mm	12–20 × 8–21 mm	5–21 × 5–21 mm	5–39 × 3–32 mm	5–13 × 2–8 mm	7–20 × 3–5 mm	1–12 × 1–5 mm	4–12 × 1–5(-8) mm	8–35 × 8–35 mm
<b>Sporo-carp shape</b>	oblong-ellipsoid	oblong-ellipsoid	oblong-ellipsoid	oblong-ellipsoid	oblong-ellipsoid	oblong-ellipsoid	oblong	oblong-ellipsoid	oblong-ellipsoid	ellipsoid	oblong-ellipsoid	oblong-ellipsoid
<b>Sporo-carp size</b>	3.8–4.8 × 2.9–3.8 mm	2.5–4.7 × 3–4 mm	3–4.5 × 2.5–4.5 mm	4.0–5.6 × 3.1–4.0 mm	2.6–4.1 × <2.0 mm	3–7 × 4–6 mm	(4)–8–9 × 6–9 mm	4–5 × 3–5 mm	5–5.5 × 3–5 mm	3–4 × 2–4 mm	3–5 × 2–4 mm	2–3 × 2–3 mm
<b>Sporo-carp surface</b>	not or faintly ribbed	not or faintly ribbed	not or faintly ribbed	not ribbed	not ribbed	not or faintly ribbed	faintly to distinctly ribbed	ribbed	indistinctly ribbed	distinctly ribbed	distinctly ribbed	faintly ribbed
<b>Sporo-carp teeth</b>	2, equal in size	1, rarely a second smaller tooth	1, rarely a second smaller tooth	0 or upper tooth present, very small	2, equal in size or lower tooth absent	2, equal in size	2, upper tooth < lower tooth	2, the lower ± recurved	1, rarely a second smaller tooth	1, base of stalk forming second tooth like structure	1, rarely a second smaller tooth	0 or 1–2 slight protuberances
<b>Sporo-carp stalk (pedicel)</b>	< 5 mm, unbranched	< 1–2.5 mm, unbranched	2–6(-8) mm, unbranched	3–16 mm, often branched	2.6–6 mm, sometimes branched	1.5–6 mm, unbranched	10–60(-100) mm, unbranched	1–4 mm, unbranched	1–3 mm, unbranched	4–6 mm, unbranched	< 5 mm, unbranched	1–3 mm, unbranched
<b>Grouping of sporo-carps</b>	solitary or clustered (1–3)	mostly solitary	mostly solitary	clustered 2–3(-4), rarely solitary	Usually paired, solitary or clustered (1–4)	solitary or clustered	solitary or clustered	solitary	clustered	solitary or in small clusters	clustered	6–12 in a dense row at base of stipe

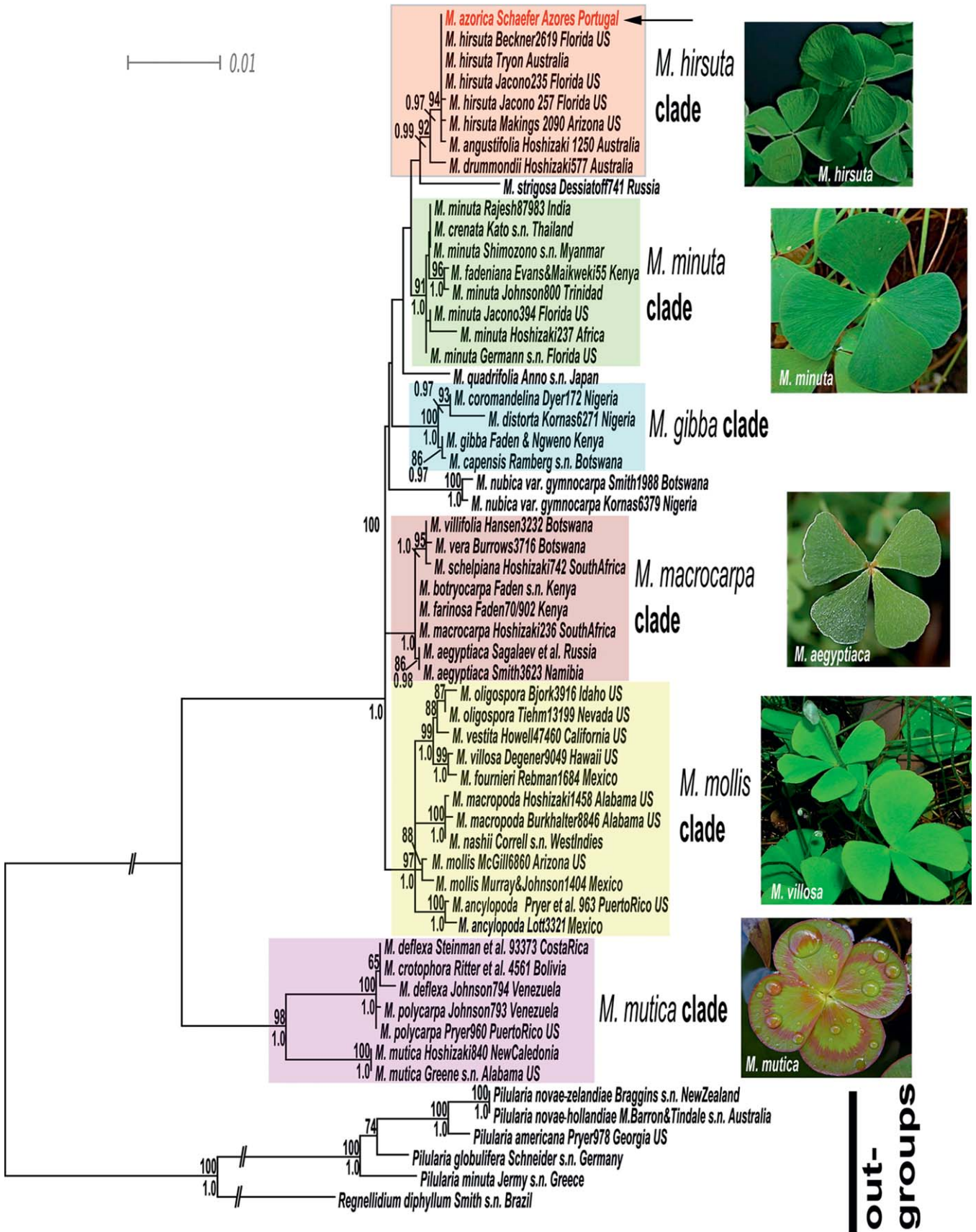


FIG. 2. Maximum likelihood phylogram of combined *rbcl*, *rps4*, *rps4-trnS*, and *trnL-F* sequences (2,532 aligned nucleotides) analyzed under the GTR +  $\Gamma$  model. Likelihood bootstrap support values  $\geq 60\%$  are indicated above branches at each node, Bayesian posterior probability values  $> 0.95$  below the branches. Arrow points to position of *Marsilea azorica* in *M. hirsuta* clade (photographs by Andreea Filip, Jenny Hsu, Armin Jagel, Forest and Kim Starr, and Barbara Quinn (top to bottom)).



with the variable Australian species *M. hirsuta*. By virtue of a suite of pedicel and sporocarp characters (Table 1), the Azorean population of *Marsilea* is clearly distinct from *M. strigosa* with which it was compared by Launert and Paiva (1983), and the few European (Akeroyd 1993) and significantly greater number of African *Marsilea* species (Launert 1984, not in our overview table). Of the geographically closest taxa, arguably the greatest resemblance is with the Canarian taxon (most likely *M. minuta*), which shares its stature, widely creeping growth form, and the presence of two definite teeth on the sporocarp. However, *M. minuta* is less densely sericeous in both foliage and particularly the sporocarp, and the pedicels are not entirely free.

Specimens of *M. hirsuta* examined at BM and GH show considerable variability in growth form and stature. Smaller leaved, sub-caespitose plants look different, but other larger, laxer expressions are indistinguishable in habit from typical Azorean material.

It is not unreasonable to suppose that Launert and Paiva (1983) did not make detailed comparisons with the geographically remote Australasian taxa, which were not known to be invasive elsewhere at that time, and this may be why the obvious similarities to *M. hirsuta* were overlooked when describing *M. azorica* as new. However, in light of the results reported here, it is clear that *M. azorica* should be treated as a synonym of *M. hirsuta*.

*Marsilea hirsuta* was described by Brown (1810) based on material he collected in 1802 on the Flanders expedition to Australia. Jones (1998) indicated that the syntypes of this species were at BM. All three (*R. Brown s. n., Iter Austral. 138–140*) possess sporocarps. The specimen *R. Brown s. n., Iter Austral. 139* was collected from Broad Sound and his diary entry for 15 September 1802 indicates that this was the first time he encountered this plant (given as *M. quadrifolia* both in the diary entry and initially on the label; Vallance et al. 2001). This specimen not only agrees well with the protologue and has all of the important features necessary for identification, but also is the best localized and with the most supporting additional literature information. It is therefore selected as lectotype for *M. hirsuta* and we propose the following synonymy:

*M. HIRSUTA* R. Br., Prodr. Fl. Nov. Holland. 167. 1810.—TYPE: AUSTRALIA. Broad Sound in pratis depressis [sestris] temporibus inundatis, *R. Brown, Iter Austral. 139*, (lectotype, here designated: BM!).

*M. azorica* Launert & Paiva, Iconogr. Selecta Fl. Azor. 2: 159. 1983.—TYPE: PORTUGAL. Terceira, Azores, *A. Hansen 308* (holotype: C [photograph!]; isotype: LISI [photograph!]).

***Marsilea hirsuta* in the Azores**—Given that *M. azorica* is conspecific with *M. hirsuta*, it should clearly be removed from all conservation priority lists and the IUCN red list. Within the Azores, the relatively recent discovery of *M. hirsuta* suggests that it may be the result of recent human introduction and it is therefore most appropriate to consider it as an introduced species in the archipelago.

*Marsilea hirsuta* is invasive in Florida, southeastern U. S. A. (Jacono and Johnson 2006; Whitten and Jacono 2009). Indeed, it is one of several species of the genus that are invasive in tropical Asia and North and South America: *M. minuta* and *M. quadrifolia* are rice field weeds in Asia (Ampong-Nyarko and De Datta 1991), and *M. minuta* is also invasive in some places in Brazil and Trinidad (Johnson 1986). In the U. S. A.,

*M. minuta*, *M. quadrifolia*, and *M. mutica* are invasive in natural and disturbed wetland habitats from New England to Florida (Jacono and Johnson 2006). Even though there is no evidence that *M. hirsuta* is spreading in the Azores, the potential to become invasive and a threat to endemic shallow water species such as the endangered quillwort, *Isoetes azorica* Durieu, should be recognized.

How *M. hirsuta* was transported to the Azores remains unclear. Given that DNA sequences are identical to those obtained from two populations in Florida, it is plausible that the Azorean plants were not introduced from within its native range in Australia, but came from Florida (Fig. 3). *Marsilea hirsuta* is a popular ornamental plant for fish tanks and ponds, and is commonly traded via the internet. In Florida, it was introduced with the horticultural trade before becoming locally invasive in natural habitats (Jacono and Johnson 2006; Whitten and Jacono 2009). The horticultural trade, however, seems an unlikely source of introduction into the Azores since *M. hirsuta* is not on sale in the few Azorean nurseries and was not observed in any park or private garden during any of our visits (but it might be present in private fish tanks). Furthermore, introduction to the Azores must have happened before the 1970s (there was already a population covering several square meters of ground, when it was first recorded in 1970), well before *M. hirsuta* was 'discovered' for the horticultural industry. However, one of us (FR) recently found the related water fern *Salvinia molesta* D. Mitch as an introduced ornamental in an artificial pond on Flores Island, Azores, so even if it seems unlikely, intentional introduction for ornament followed by spread to the actual habitat remains a possibility. Introduction by vagrant waterfowl blown to the Azores from the southeastern U. S. A. is another possible, but not very likely, explanation for its dispersal to the Azores. Several species of American ducks, waders, and herons or egrets are reported every year from the Azores and they might carry diaspores of water plants attached to their feet or plumage as was recently shown in a study of migrating waterfowl on the European mainland (Figuerola and Green 2002). However, those vagrants are usually observed on the bigger and undisturbed lakes or wetland areas in other parts of the archipelago and not on small ponds next to busy roads. Finally, an obvious connection between Florida and the Azores is the U. S. A. airbase on Terceira Island: introduction of sporocarps attached to the tyres of machines or vehicles brought from the U. S. A. to Terceira is also possible.

The case of *M. azorica* is similar to that of *Diplazium allorgei* Tardieu, which was described as an Azorean endemic in 1938. Some 40 yr later it was shown to be conspecific with *Deparia petersenii* (Kunze) M. Kato, an introduced species of Asian origin (Sledge 1975, 1977). In other groups, however, recent molecular data suggest that current species circumscriptions may be under-estimating the levels of endemic diversity (Schaefer et al. 2011). The status of many other taxa in the Azorean flora remains unclear. *Selaginella kraussiana* (Kunze) A. Braun, for example, was thought to be an invasive introduced from the African continent (Schaefer 2003), until its native status was confirmed through sediment core analyses (Van Leeuwen et al. 2005).

Even the most sophisticated conservation priority ranking schemes, like the one developed by Martín et al. (2010), will fail if there is no solid taxonomic basis. If we want to avoid directing precious conservation resources to misidentified alien species, we urgently need a comprehensive and detailed





FIG. 3. Overview map showing most likely scenario for origin of the Azorean *Marsilea* population: native to Australia (1), primary introduction to southeastern U. S. A. (2), and from there a further introduction to the Azores (3).

reassessment of the taxonomy and status of the Azorean flora based on approaches that combine molecular and morphological data.

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APPENDIX 1. Representative Specimens Examined for Morphological Analysis (determination of all specimens confirmed by the authors).

*Marsilea aegyptiaca* Willd. EGYPT. Zagazig, L. Boulos s. n. (GH). RUSSIA. Astrachan, Volga Delta, *Sagalaev et al. s. n.* (GH). *Marsilea angustifolia* R. Br. AUSTRALIA. New South Wales, Bethungra, Cootamundra district, K. Mair P2022 (GH); Royal Botanic Gardens Sydney (cult. material from Bethungra), A. Rodd P9410 (GH). *Marsilea azorica* Launert & Paiva. PORTUGAL. Azores, Terceira, [I.] B[otelho] Gonçalves 3259 (BM); Terceira, M. G. Belo Maciel et al. (BM; AZU); Terceira, H. Schaefer 2008/923 (M). *Marsilea burchellii* (Kunze) A. Braun. SOUTH AFRICA. Cape Province, E. A. Schelpe 4552 (GH). *Marsilea drummondii* A. Braun. AUSTRALIA. New South Wales, Narrabri, J. L. Boorman s. n. (GH); Queensland, Rifle Creek, southwest of Port Douglas, C. B. Hellquist 15117

- (GH). *Marsilea ephippiocarpa* Alston. NAMIBIA. West of Otavi, L. Smook 10000 (GH). *Marsilea exarata* A. Braun. AUSTRALIA. Queensland, A. Fraser 333 (GH); New South Wales, Mundi Mundi station near Broken Hill, E. F. Constable P237 (GH). *Marsilea hirsuta* R. Br. AUSTRALIA. Iter Australiense 1802-5, R. Brown 138-140 (BM); New South Wales, E. F. Constable 4069A (BM); New South Wales, A. & R. Tryon 7317 (GH); Northern Territory, Prof. Sir Baldwin Spencer s. n. (BM); Pickanjinie Creek, E. H. Belson (BM); Queensland (cultivated at Botanical Garden of Berlin, Germany), A. Braun 15, 16, 17, 18, 19 (GH); Queensland, N. Michael 1430 (GH); Queensland, M. S. Clemens s. n. (GH); Queensland, L. J. Brass 19752 (GH); Victoria, W. Watts 1458 (GH); Queensland, Proserpine, river bed, N. Michael 1430 (BM, GH); Western Australia far eastern section, R. H. Kuchel 259 (BM); Western Australia, C. B. Hellquist 15103 (GH). *Marsilea macrocarpa* Presl. KENYA. Kajiado district, road to Masai Lodge, R. B. & A. J. Faden 77/295 (GH). SOUTH AFRICA. Cape Colony, pr. Grahamstown, R. Schlechter 2612 (GH); Cape Province, Komgha, E. A. Schelpe 5025 (GH). *Marsilea minuta* L. (incl. *M. crenata* Presl). AUSTRALIA. Queensland, Townsville, K. Kennedy & M. Tindale P7967 (GH); Queensland, Inkerman, S. E. Stephens P2924 (GH). MALAYSIA: Perak, B. E. G. Molesworth-Allen 4714 & 4516 (GH); fields near Kodianq, B. E. G. Molesworth-Allen 2819 (GH). PAPUA NEW GUINEA. Western district, near Bula village, R. Pullen 7031 (GH). PHILIPPINES. Luzon, Bayninan, D. R. Mendoza & Buwaya 76795 (GH). SENEGAL. R. Berhaut 4926 (GH) [as *M. diffusa*]. SPAIN. Canary Islands, Gran Canaria, E. Bourgeau Plantae Canarienses 1168 (BM, GH) [as *M. quadrifolia* L., the BM specimen redet. *M. minuta* by E. Launert, 1968]; Gran Canaria, Arucas, O. Gelert s. n. (C, photograph seen) [as *M. quadrifolia* L.]. *Marsilea mutica* Mett. AUSTRALIA. New South Wales, St. Albans Lagoon, E. F. Constable P8312 (GH); New South Wales, Emu Plains, E. F. Constable NSW 9770 (GH); Sullivan's creek, A.C.T., J. Pulley 1218 (GH); New South Wales, Coorabong, E. F. Constable P8142 (GH). *Marsilea quadrifolia* L. FRANCE. Montbison (Loire), Le Grand s. n. (GH). HUNGARY. Holt Körös prope pagum Vesztö, Borbás s. n. (GH). INDONESIA. Java, Horsfield 174 (GH). ROMANIA: Muntenia, Vlasca district, I. Prodan s. n. (GH). RUSSIA. Astrachanj, Volga Delta, G. Malutin s. n. (GH). *Marsilea polycarpa* Hook. & Grev. FRENCH POLYNESIA. Society Islands, Borabora, Turapuo H. St. John 17414 (GH). *Marsilea schelpeana* Launert. SOUTH AFRICA. Cape Province, Zwartskops River valley, R. Anderson 28 (GH). *Marsilea strigosa* Willd. ALGERIA. Oran, Mare du Djebel-Santo, Clauson s. n. (GH) [as *M. pubescens* Ten.]. FRANCE. Dept. Hérault, J. de Vichet 81 (GH) [as *M. pubescens* Ten.]; B. Lugardon s. n. (GH); Agde, pr. Montpellier, A. Braun s. n. (BM). MOROCCO. Oued Issen, E. Jahandiez 298 (BM). RUSSIA. Astrachanj, Volga Delta, N. Dessiatoff 741 (GH); Saratow, Sarepta, A. Becker s. n. (GH). SPAIN. Islas Baleares, Menorca, N. of Mahon, C. Jermy 18172 (BM). *Marsilea villosa* Kaulf. U. S. A. Hawai'i, Oahu, F. R. Fosberg 9704 (GH); Oahu, D. F. Topping 3409 & 3518 (GH); West of Mokia, Molokai, O. Degener & H. Wiebke 3215 (GH); Oahu, H. L. Lyon s. n. (GH).

APPENDIX 2. Material sequenced with GenBank accession number (*rbcL*, *trnL-trnF*, *rps4* + *rps4-trnS*).

*Marsilea aegyptiaca*. Russia, Sagalaev et al. s. n. (GH) (HQ728338, HQ728342, HQ728346); *Marsilea azorica*. Portugal, Azores, H. Schaefer 2008/923 (M) (HQ728339, HQ728343, HQ728347); *Marsilea hirsuta*. Australia, A. & R. Tryon 7317 (GH) (HQ728340, HQ728344, HQ728348); *Marsilea strigosa*. Russia, N. Dessiatoff 741 (GH) (HQ728341, HQ728345, —).