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A CONTRIBUTION TO THE CHARACTERIZATION OF *RUPPIA DREPANENSIS* (RUPPIACEAE), A KEY SPECIES OF THREATENED MEDITERRANEAN WETLANDS[1]

Olga De Castro,[2] *Anna Geraci,*[3] *Anna Maria Mannino,*[3] *Nicolina Mormile,*[2] *Annalisa Santangelo,*[2] *and Angelo Troia*[3*]

Abstract

To elucidate the taxonomic status of *Ruppia drepanensis* Tineo ex Guss. (Alismatales, Ruppiaceae), we performed morphological analysis and DNA barcoding of historical materials (including the lectotype) and fresh samples (including those from a recently discovered population near the locus classicus in Sicily, Italy). We conclude that *R. drepanensis* is a separate species, closely related to *R. spiralis* L. ex Dumort., that occurs in temporary inland waters from the western to central sectors of the Mediterranean region. We also highlight the importance of vouchers and the need to link molecular investigations to field, ecological, and morphological investigations.

Key words: Aquatic meadows, DNA barcoding, herbarium, historical specimens, ITS, morphology, *Ruppia*, Ruppiaceae, seagrass, typification.

Mediterranean wetlands are under severe human pressure (Fraixedas et al., 2019; Geijzendorffer et al., 2019); in the framework of our studies on these threatened habitats and their flora (Mannino & Geraci, 2016; Troia & Lansdown, 2016; Guarino et al., 2019), we found a population of *Ruppia drepanensis* Tineo ex Guss. in Sicily (Italy), where it has not been recently reported.

[1] We thank the herbaria NAP and PAL for permission to collect samples from historical specimens; the manager of the "Riserva Naturale Saline di Trapani e Paceco" for permission to collect samples from Salina Culcasi; Nobuyuki Tanaka, senior curator of TNS, for high-resolution images of some herbarium specimens; and Vincenza Polizzano for help with arranging and drying the fresh material. The remarks and suggestions of three reviewers and the editor significantly improved the final version of this paper. None of the authors of this paper have any conflict of interest to declare. A. G., A. M. M., and A. T. conceived the idea; A. T. and A. G. collected fresh samples, A. G., A. M. M., A. T., A. S., and O. D. C. collected samples from herbarium specimens; O. D. C. designed the DNA barcoding analysis; O. D. C. and N. M. conducted molecular laboratory work; O. D. C. performed bioinformatic molecular analyses; all authors contributed to the experimental design, interpretation of the results, and commented on the manuscript. Additional supporting information may be found in the online version of this article. O. D. C., <https://orcid.org/0000-0002-4861-3960>; A. G., <https://orcid.org/0000-0002-4860-487X>; A. T., <https://orcid.org/0000-0001-5193-8865>; A. S., <http://orcid.org/0000-0001-5560-1725>; A. M. M., <http://orcid.org/0000-0001-6419-7066>

[2] University of Naples Federico II, Department of Biology, Via Foria 223 – Botanical Garden, 80139 Naples, Italy.

[3] University of Palermo, Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), via Archirafi 38, 90123 Palermo, Italy.

*Author for correspondence: angelo.troia@unipa.it

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The genus *Ruppia* L. (Alismatales, Ruppiaceae), considered to be closely related to other seagrass genera of Posidoniaceae and Cymodoceaceae (Les et al., 1997), has a cosmopolitan distribution and forms dense and generally monospecific aquatic meadows that play fundamental ecological roles (Verhoeven, 1979; Mannino et al., 2015; Mannino & Geraci, 2016). These meadows characterize "coastal lagoons," a priority habitat of community interest (Directive 92/43/ECC; Annex I, code 1150*) that is severely threatened in the Mediterranean region (European Environment Agency, 2012).

Ruppia taxonomy has been confusing because of several factors, such as its simplified morphology, high phenotypic plasticity, and the existence of polyploid and hybrid taxa (Mannino et al., 2015; Martínez-Garrido et al., 2016; Triest et al., 2018a). The difficulty in species delimitation has led to uncertainty in the exact number of species recognized worldwide. In the Mediterranean area, two taxa are usually recognized: *R. maritima* L. and *R. cirrhosa* (Petagna) Grande, the latter being the most widespread (Mannino et al., 2015; Den Hartog et al., 2016; Ito et al., 2017). According to Ito et al. (2017), the name *R. cirrhosa* is a homotypic synonym of *R. maritima*, and *R. spiralis* L. ex Dumort. should be applied to the long- and coiled-pedunculate species previously referred to as *R. cirrhosa*.

Another *Ruppia* species in the Mediterranean area is the enigmatic *R. drepanensis* Tineo ex Guss., whose locus classicus is the Trapani saltworks (Sicily, Italy). The treatment of *R. drepanensis* in recent floras is variable: it has been ignored (Dandy, 1980; Bartolucci et al., 2018), considered a synonym of *R. cirrhosa* (Zhao & Wu, 2008; Uotila, 2009), and accepted (Talavera & García-Murillo, 2010; Pignatti et al., 2017; in the latter case the species is treated as distinct from *R. cirrhosa* on the basis of leaf width and anther length characters).

The first molecular evidence that *Ruppia drepanensis* is a distinct genetic lineage appeared in Triest and Sierens (2009). A few years later Ito et al. (2013) also recognized "drepanensis" as a distinct genetic lineage (within the *R. maritima* s.l. complex sensu Ito et al. [2010]), but their phylogeny (Ito et al., 2013: 754) shows the single *R. drepanensis* sample in the same clade as a *R. cirrhosa* sample, leaving doubts about the correct rank. Similarly, Martínez-Garrido et al. (2016), while accepting *R. drepanensis* as a distinct species, show a nuclear ITS-based phylogeny where the *R. drepanensis* clade includes a *R. cirrhosa* sample. Finally, Mannino et al. (2015) and Triest et al. (2018a, 2018b) treat *R. drepanensis* as a separate species, but they include it within a "R. cirrhosa complex."

According to Marchioni Ortu (1982), *Ruppia drepanensis* is distributed in Spain, Morocco, Algeria, Tunisia, Sardinia, Sicily, and southeast Italy. Recent records mainly come from Spain (Ito et al., 2013; Triest & Sierens, 2013; Mannino & Geraci, 2016), but there are no recent records from Sicily where the species was originally described (Mannino & Sarà, 2006; Triest & Sierens, 2013; Mannino & Graziano, 2014).

In the present study, we conduct morphological analysis and DNA barcoding characterization of historical materials and recently collected samples from a population ascribed to *Ruppia drepanensis* near its locus classicus in Sicily (Table 1), with the aim of clarifying the taxonomic rank and phylogenetic position of this taxon.

Materials and Methods

plant materials

In addition to the analysis of relevant literature, we studied Italian Ruppiaceae specimens in FI, NAP, and PAL herbaria (abbreviations from Thiers, 2019). In all, 13 samples (from 11 collections) were analyzed, all of them from Sicily: nine were obtained from historical specimens kept in NAP and PAL and four were obtained from two populations sampled in spring 2017. Accessions, vouchers, and locality information of the specimens are reported in Table 1.

As mentioned above, phylogenies published in Ito et al. (2013) and Martínez-Garrido et al. (2016) show *Ruppia drepanensis* in a clade together with a sample of *R. cirrhosa* (now *R. spiralis*), leaving doubts about the correct taxonomic rank of "*R. drepanensis*." To clarify this situation, we examined an image of the voucher of that "*R. cirrhosa*" sample, preserved at TNS in Japan (Thiers, 2019). This voucher includes plants collected by Yu Ito and Pablo Garcia Murillo in Doñana National Park, Sevilla, Spain (coll. n. 1567-1). In addition, we examined images of other DNA vouchers to verify their taxonomic identity.

morphological analysis

The morphological traits we used to discriminate species, according to previous literature (Talavera & García Murillo, 2010; Mannino et al., 2015), were the form of the peduncle (straight in *Ruppia maritima*, lax spirally coiled in *R. spiralis*, and dense spirally coiled in *R. drepanensis*) and leaf width (0.2–0.3 mm in *R. maritima*, 0.5–1.2 mm in *R. spiralis*, 0.1–0.2 mm in *R. drepanensis*).

DNA extraction

Total genomic DNA was isolated from fresh and historical leaves by a modified cetyl trimethylammonium

Table 1. Sicilian *Ruppia* L. accessions used for molecular analyses with locality, collector, voucher information, ITS genotype, and corresponding GenBank number. In the column "Revised taxon" we confirm or revise (in bold) the identification of the specimen as reported in the herbarium label.

Code	Taxon	Revised taxon	Locality	Collector	Date	Voucher	Genotype	GenBank
Herbarium Neapolitanum (NAP)								
N1	*R. drepanensis*	*R. drepanensis*	Le Saline*, Trapani	Tineo	1828	NAPGS1 (lectotype)	ITS-1	MN958118
N2	*R. maritima*	***R. spiralis***	Mondello	Gussone	ca. 1850	NAPGS11	ITS-2	MN958119
N3	*R. spiralis*	*R. spiralis*	Mondello	Gussone	ca. 1850	NAPGS3	ITS-2	MN958120
N4	*R. spiralis*	*R. spiralis*	Siracusa	Gussone	ca. 1850	NAPGS5	ITS-2	MN958121
N5	*R. spiralis*	*R. spiralis*	Palermo	Pasquale	ca. 1850	NAPPasq2	ITS-2	MN958122
N6	*R. zosteroides*	***R. spiralis***	Messina	Gussone	ca. 1850	NAPGS9	ITS-2	MN958123
Herbarium Mediterraneum (PAL)								
P1	*R. drepanensis*	***R. maritima***	Favignana	Huet	May 1855	PAL70200-B	ITS-3	MN958124
P2	*R. maritima*	*R. maritima*	Mondello	unknown	July 1857	PAL70236-D	ITS-3	MN958125
P3	*R. maritima*	***R. spiralis***	Messina	Todaro	May ca. 1850	PAL70219-I	ITS-2	MN958126
Recent samples								
R1†	*R. drepanensis*	*R. drepanensis*	Pantano, Birgi Nuovo (Trapani)	Troia & Geraci	11 May 2017	PAL-J1/5	ITS-1	MN958127
R2†	*R. spiralis*	*R. spiralis*	Salina Culcasi, Nubia (Trapani)	Troia & Geraci	11 May 2017	PAL-K1/5	ITS-2	MN958128

*Locus classicus.

†Two individuals analyzed for population.

bromide (CTAB) 2× procedure (see Supplementary Appendix S1). Briefly, for historical specimens, a 6-day isopropanol precipitation was followed by an additional DNA purification using both additional ethanol precipitations and Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, Massachusetts, U.S.A.). DNA concentration was estimated using a Qubit 3 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

polymerase chain reaction (pcr) amplification, sequence analyses, and data analyses

After a preliminary screening of molecular markers from the literature, the internal transcribed spacer (ITS_T, ITS1+5.8S gene+ITS2) was selected for DNA barcoding because it was useful for discriminating among the taxa investigated here (Ito et al., 2013; Triest & Sierens, 2013).

ITS_T was amplified with JK14 (forward): 5′-GGA GAA GTC GTA ACA AGG TTT CCG-3′ (Aceto et al., 1999), SN3 (reverse): 5′ -TTC GCT CGC CGT TAC TAA GGG-3′ (De Castro et al., 2013), and new primers designed for this study for nested PCR (Ruppia18S_ITS1: 5′-TTT CCG TAG GTG AAC CTG C-3′ [at minus 32 bp at the end of 18S] and Ruppia26S_ITS2: 5′-TGG TCT TGC CTG ACC TGA-3′ [after 6 bp of the beginning of the 26S]).

For fresh tissue, each reaction used a volume of 25 µL, with 5 ng of DNA template, 0.25 µM of each primer, and a high-fidelity hot start DNA polymerase Kodaq 2× PCR MasterMix (Applied Biological Materials, Vancouver, Canada). The cycling parameters were programmed as follows: initial denaturation at 94°C for 3 min., followed by 35 cycles of denaturation at 94°C for 30 sec., annealing for 30 sec. at 55°C, and extension at 72°C for 45 sec. A final extension at 72°C for 5 min. was included.

Amplifications of historical DNA were performed in two amplification steps using the same DNA polymerase as reported above. The first PCR was carried out with 1–5 ng of DNA in a final volume of 50 µL; the nested PCR was performed with 1 µL from the first PCR (25 µL final volume) and using internal primers (Ta = 56°C) designed on the sequences of *Ruppia drepanensis/cirrhosa/maritima* present in GenBank.

The amplified fragments were purified using PEG8000 precipitation (PEG 15%, 2.5 M NaCl). Approximately 7–10 ng of purified template was sequenced in a final volume of 5 µL according to instructions from the BrightDye Terminator Cycle Sequencing Kit (MCLAB, San Francisco, California, U.S.A.). The reactions were

Table 2. List of GenBank ITS_T accessions (ITS1+5.8S+ITS2) used for the Bayesian phylogenetic analyses (taxon, origin, GenBank accession, and reference).

Taxon*	Origin	GenBank	Reference
Ruppia cirrhosa (Petagna) Grande†	Greece	JN113280 + JN113283	Triest & Sierens, 2013
R. cirrhosa	Italy: Marausa, Sicily	KX860107	Martínez-Garrido et al., 2017a
R. cirrhosa	Italy: Nubia, Sicily	KX860106	Martínez-Garrido et al., 2017a
R. cirrhosa	n.d.	AJ012292	GenBank database (submission by A. G. Ficca, 1999)
R. cirrhosa	Netherlands	KC505606	Yu et al., 2014
R. cirrhosa	Netherlands	JQ034335	Yu et al., 2014
R. cirrhosa	Portugal: Algarve	KX860112	Martínez-Garrido et al., 2017a
R. cirrhosa	Portugal: Óbidos	KX860114	Martínez-Garrido et al., 2017a
R. cirrhosa	Portugal: Quinta do Lago, Algarve	KR263119	Martínez-Garrido et al., 2016
R. cirrhosa	Spain: Cádiz	KX860113	Martínez-Garrido et al., 2017a
R. cirrhosa‡	Spain: Donana National Park, Sevilla	AB728740	Ito et al., 2013
R. cirrhosa	Spain: Murcia	KX860111	Martínez-Garrido et al., 2017a
R. cirrhosa	Spain: Murcia	KX860110	Martínez-Garrido et al., 2017a
R. cirrhosa	Spain: Murcia	KX860109	Martínez-Garrido et al., 2017a
R. cirrhosa	Spain: Palma de Mallorca	KX860108	Martínez-Garrido et al., 2017a
R. cirrhosa	Spain: Puerto Real, Cádiz	KR263118	Martínez-Garrido et al., 2016
R. cirrhosa	U.K.: Skye Island	AB728749	Ito et al., 2013
R. cirrhosa	U.K.: Skye Island	AB728748	Ito et al., 2013
R. drepanensis Tineo ex Guss.	Spain: Cádiz	KR263126	Martínez-Garrido et al., 2016
R. drepanensis†	Spain	JN113281 + JN113284	Triest & Sierens, 2013
R. maritima L.	Australia: Montecollina Bore, Strzelecki Track	AB728741	Ito et al., 2013
R. maritima	Canada: Cape Breton Island, Nova Scotia	AB728739	Ito et al., 2013
R. maritima	Cape Verde: Santiago Island	KY002069	Martínez-Garrido et al., 2017b
R. maritima	China: Yuhong, Sanya, Hainan	AB728735	Ito et al., 2013
R. maritima§	Croatia: Dubrovnik	AB728750	Ito et al., 2013
R. maritima†	France	JN113282 + JN113285	Triest & Sierens, 2013
R. maritima	India: Maharashtra	AB728736	Ito et al., 2013
R. maritima	Italy: Morgerra Salt Marsh, Marzanemi, Sicily	AB728747	Ito et al., 2013
R. maritima	Italy: Morgerra Salt Marsh, Marzanemi, Sicily	AB728746	Ito et al., 2013
R. maritima	Italy: Vendicari Natural Reserve, Noto, Sicily	AB728751	Ito et al., 2013
R. maritima	Japan: Shiokawa River, Okinawa	AB728743	Ito et al., 2013
R. maritima	Japan: Shiokawa River, Okinawa	AB728742	Ito et al., 2013
R. maritima	Malta: Gozo Island	AB728745	Ito et al., 2013
R. maritima	n.d.	JN034094	GenBank database (submission by L.Y Chen et al., 2012)
R. maritima	Netherlands	JQ034336	Yu et al., 2014
R. maritima	Spain: Bonba, Huelva	AB728752	Ito et al., 2013
R. maritima	Spain: Canatilla, Huelva	AB728744	Ito et al., 2013
R. maritima	U.S.A.: Chesapeake Bay, Maryland	AB728738	Ito et al., 2013
R. maritima	U.S.A.: Grand Bay, Gulf of Mexico, Jackson, Mississippi	AB728737	Ito et al., 2013

Taxon*	Origin	GenBank	Reference
R. maritima	Vanuatu: Anatom Island	AB728734	Ito et al., 2013
R. cf. *maritima*	Portugal: Olhão, Algarve	KR263125	Martínez-Garrido et al., 2016
R. cf. *maritima*	Spain: Jarana, Cadiz	KR263124	Martínez-Garrido et al., 2016
R. cirrhosa × *R.* cf. *maritima*	Portugal: Tavira, Algarve	KR263121	Martínez-Garrido et al., 2016
R. cirrhosa × *R.* cf. *maritima*	Portugal: Tavira, Algarve	KR263123	Martínez-Garrido et al., 2016
R. cirrhosa × *R.* cf. *maritima*	Spain: Calblanque, Murcia	KR263120	Martínez-Garrido et al., 2016
R. cirrhosa × *R.* cf. *maritima*	Spain: Calblanque, Murcia	KR263122	Martínez-Garrido et al., 2016
Outgroup			
R. megacarpa R. Mason	n.d.	JQ034337	Yu et al., 2014

n.d., No datum.

*Taxon's name as reported in the GenBank accession

†Two GenBank accessions are reported: ITS1 (+ 5′-5.8S) and ITS2 (+3′-5.8S), respectively.

‡Misidentification (= *R. drepanensis*).

§Misidentification (= *R. spiralis*).

purified using BigDye XTerminator Purification Kit (Applied Biosystems, Thermo Fisher Scientific) and read using an automated sequencer (3130 Genetic Analyzer, Life Technologies, Thermo Fisher Scientific). The sequences were analyzed using the AB DNA Sequencing Analysis Version 5.2 software (Applied Biosystems, Thermo Fisher Scientific), edited in the Chromas Lite Version 2.6.6 software (Technelysium Pty Ltd, South Brisbane, Australia), assembled in the Chromas Pro Version 2.1.8 software (Technelysium Pty Ltd), aligned and analyzed using BioEdit Version 7.2.5 software (Hall, 1999) and MEGA Version 10.1.8 (Kumar et al., 2018), respectively. Sequences obtained in this study have been deposited in GenBank under accession numbers MN958118–MN958128 (Table 1).

The phylogenetic relationships were assessed both with Bayesian and maximum likelihood (ML) inferences. MrBayes Version 3.2.6 software (Ronquist & Huelsenbeck, 2003) was run on a dataset comprising 47 previously published sequences (two *Ruppia drepanensis*; 22 *R. maritima*; 18 *R. spiralis*; four *R. spiralis* × *R.* cf. *maritima*; and one *R. megacarpa* R. Mason as outgroup; Table 2). Two runs of four Markov chains (three hot, one cold) were performed for 15,000,000 generations, sampling every 1500 generations and discarding the first 8.8% as burn-in (average of SD of split frequencies = $0.010873 \pm 1.01 \times 10^{-4}$ SD), under an HKY+G substitution model, as selected by jModeltest Version 2.1.10 (Darriba et al., 2012). Convergence diagnostics were also checked with Tracer Version 1.7.1 (Rambaut et al., 2018).

An ML tree search was performed under the same substitution model as above, in MEGA, using the default settings (i.e., five discrete gamma categories and the nearest-neighbor-interchange branch swapping). Support was assessed on 5000 bootstrap pseudoreplicated datasets.

Results

Type of *Ruppia drepanensis*

Marchioni Ortu (1982) typified *Ruppia drepanensis* Tineo ex Guss. (Fl. Sicul. Syn. 2[2]: 878. 1845), identifying as "holotypus" a specimen (Fig. 1) collected by Tineo ("In salsis stagnis maritimis; Trapani alle saline [Tin.]") and preserved in the herbarium of Naples (NAP). In fact, the type selected by Marchioni Ortu is not the holotype but a lectotype (as correctly cited by Talavera & García Murillo, 2010), since the protologue does not specify, and at least two other specimens, including PAL70199! and FI002953!, are "original material" in the sense of the Code (Art. 9.4, Turland et al., 2018).

Morphological analysis

Plants of *Ruppia drepanensis* collected near the locus classicus of the species were morphologically consistent and distinct from other Mediterranean species. They can be described as follows: submerged rooted annual herbaceous plants, 12–22(–40) cm tall; leaves 8–15(–20) cm × 0.1–0.2(–0.3) mm, narrowly linear, acute; flowering peduncle coiled, 3–6 cm long; inflorescence with two hermaphroditic flowers, anthers ca. 1 mm long, ovaries with four carpels; fruiting peduncle tightly coiled, 2–12(–20) cm long; fruitlet asymmetrical, (1.8–)2 × (1.2–)1.5 mm.

DNA barcoding

A total of three ITS genotypes were identified in our newly sequenced *Ruppia* accessions: genotype 1 is found in two samples of *R. drepanensis* (N1 and R1); genotype 2 is found in seven samples related to *R. spiralis* (N2–N6, P3, and R2); and genotype 3 is found in

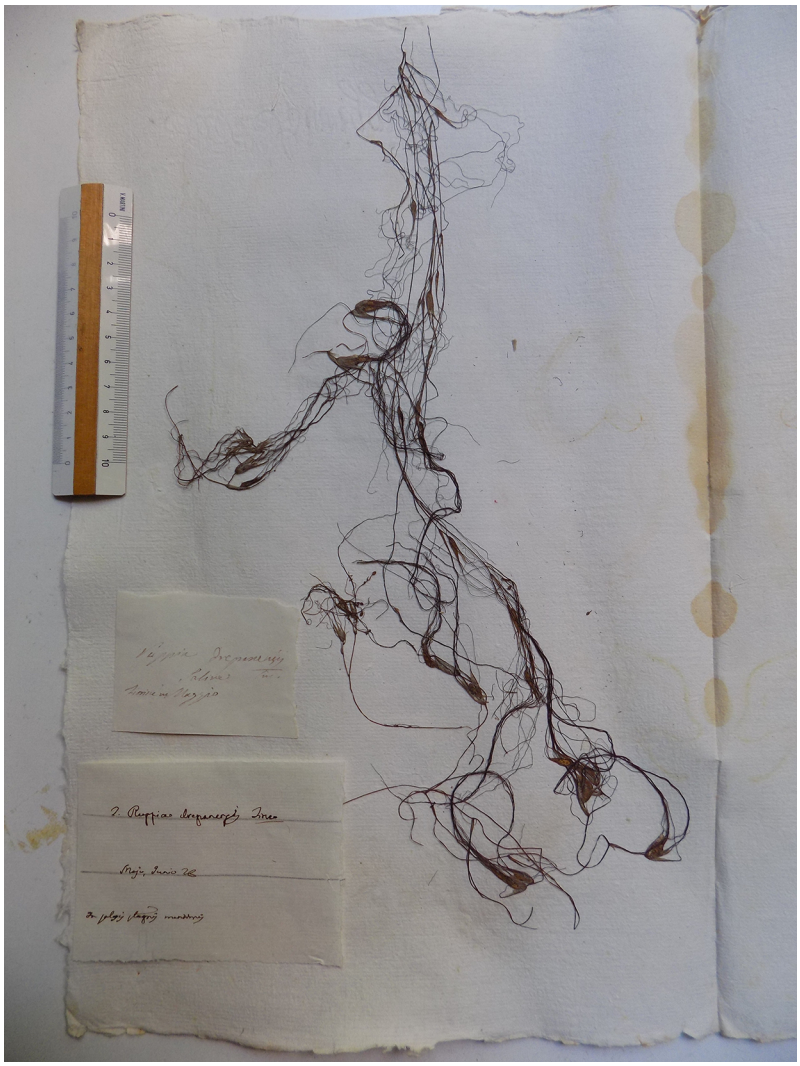

Figure 1. Lectotype of *Ruppia drepanensis* Tin. ex Guss. (stored in NAP).

two samples of *R. maritima* (P1 and P2; Table 1). The alignment of 58 ITS_T sequences resulted in a matrix of 680 characters (143 of which were variable and 49 of which were parsimony informative; Supplementary Appendix S2).

According to the phylogenetic analysis, our accessions fit into three well-defined clades: the *maritima* group (which is sister to the rest of samples), *drepanensis* group, and *spiralis* group (Fig. 2).

PLANT MATERIAL

The voucher TNS01178252, corresponding to the GenBank accession AB728740 and the sample analyzed as "*R. cirrhosa*" in Ito et al. (2013) and Martínez-Garrido et al. (2016), was originally determined as *Ruppia drepanensis* (probably by the collectors themselves, Ito and Garcia Murillo), and indeed exhibits the morphological characters (e.g., extremely narrow leaves) of *R. drepanensis*, consistent with its phylogenetic position. Correcting the determination of this "*R. cirrhosa*" sample (to *R. drepanensis*) means that *R. drepanensis* forms a clade in the analyses of both Ito et al. (2013) and Martínez-Garrido et al. (2016).

To determine if other samples were misidentified, we requested images of vouchers of "*R. maritima*" samples that fell in the *Ruppia spiralis* lineage, and "*R. spiralis*" samples in the *R. maritima* lineage. The GenBank accession AB728743, corresponding to a "*R. maritima*" sample in a small clade sister to the

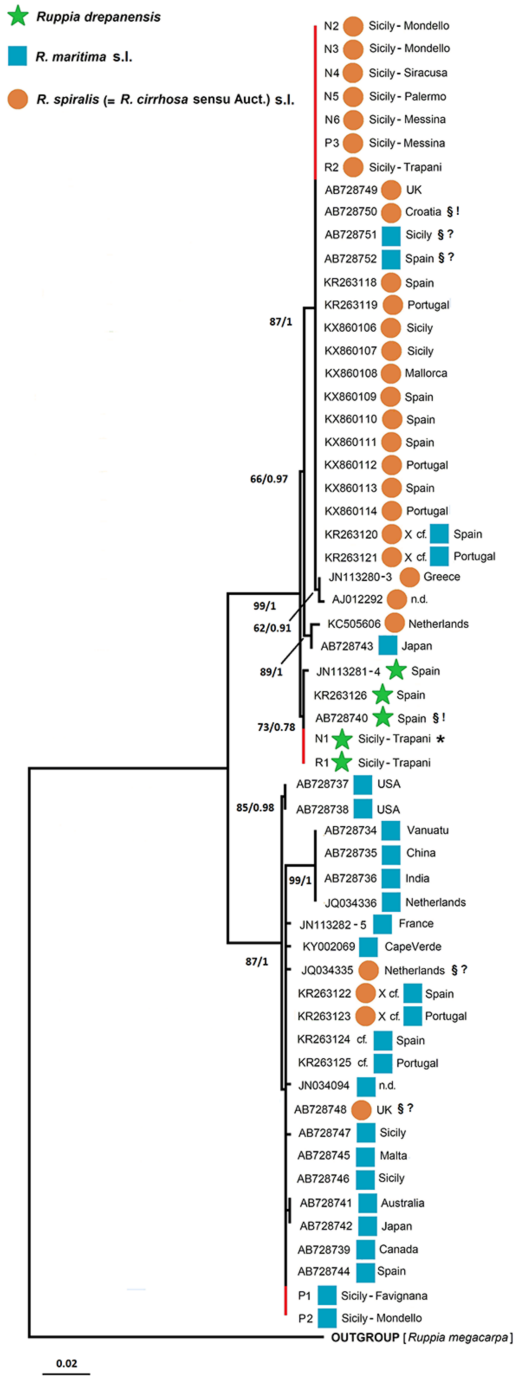

Figure 2. Maximum likelihood (ML) phylogram of newly generated and previously published ITS_1 sequences of *Ruppia drepanensis* (green stars), *R. maritima* s.l. (blue squares), and *R. spiralis* s.l. (orange circles). Newly generated sequences have red lines; ML bootstrap values followed by Bayesian posterior probabilities are shown below the branches (values > 50%). *, locus classicus; §!, misidentifications in GenBank (here corrected); §?, probable misidentifications in GenBank.

main *R. spiralis* group (Fig. 2), has straight flower peduncles and strongly asymmetric fruits (voucher herbarium specimen TNS01178605). This combination of characters is typical of *R. maritima*, so this specimen appears to be correctly identified on the basis of morphology, but needs further investigations. Of the three GenBank accessions (AB728752, AB728751, AB728750) labeled as *R. maritima* and falling in the main *R. spiralis* clade (Fig. 2), we could examine only TNS01178328, the voucher for GenBank accession AB728750. The leaf width of about 1 mm and the lax spirally coiled peduncle reveal that this sample is actually *R. spiralis* not *R. maritima*. If the other two "anomalous" *R. maritima* samples in the *R. spiralis* clade are also misidentifications, that would result in a clade containing only *R. spiralis*.

Discussion

Mediterranean *Ruppia* comprises two main lineages (Fig. 2), in agreement with recent literature (Triest & Sierens, 2014; Martínez-Garrido et al., 2016). One is the *R. maritima* clade, which may be limited to that species if the few "*R. spiralis*" accessions currently included within it prove to be misidentifications. This lineage is divided into two subgroups—one small, including only extra Mediterranean samples, and one large, including both Mediterranean and extra Mediterranean samples. However, the *R. maritima* clade is otherwise almost entirely unresolved, so the exact placement of the extra Mediterranean subclade cannot currently be determined. The second main lineage, the *R. spiralis* clade, includes three clear subgroups: a clade corresponding to *R. drepanensis* is sister to two clades consisting mostly of *R. spiralis* (s.l.). The main *R. spiralis* subgroup, including most of the samples of that species, presumably corresponds to *R. spiralis*, although it contains several possibly misidentified samples labeled as "*R. maritima*." Interestingly, the sample labeled *R. zosteroides* Lojac. (N6 in Fig. 2), a taxon described from Sicily and usually considered synonymous with *R. maritima* (cf. Uotila, 2009; Pignatti et al., 2017), falls in this *R. spiralis* subgroup; indeed, its morphology (ca. 1-mm-wide leaves, lax spirally coiled flowering peduncle) matches with this position. The second smaller *R. spiralis* subgroup includes two samples, one collected in the Netherlands and the other in Japan; as mentioned above, at least one of those samples does not fit the typical morphology of *R. spiralis*, so this clade deserves further investigation.

Both the type of *Ruppia drepanensis* and the recently collected Sicilian samples fall in the same clade, together with *R. drepanensis* specimens from Spain (including the sample from Doñana mentioned above).

So, we can confirm that *R. drepanensis* occurs in the central to western Mediterranean regions and that it is distinct from the rest of the *R. spiralis* lineage according to both molecular and morphological analyses.

Data from previous works (e.g., Marchioni Ortu, 1982; Santamaria Galdon, 1995; Talavera & García-Murillo, 2010) have been very useful to define the morphological and ecological traits of *Ruppia drepanensis*. We here confirm its ecology in Sicily: it is an annual species typically inhabiting temporary inland waters (Martínez-Garrido et al., 2016), where "inland waters" means waters not directly connected to the sea but including coastal wetlands. This assessment is in agreement with Santamaria Galdon (1995: 11), who wrote that "*R. drepanensis* inhabits temporary coastal and inland waters."

We think that the present contribution will be useful for future studies in clarifying the phylogenetic position of *Ruppia drepanensis*. In addition, the present work highlights the importance of basic taxonomic research for conservation planning, since *R. drepanensis* is a typical species of the priority habitat of community interest "coastal lagoons" (Biondi & Blasi, 2009), which is severely threatened in the Mediterranean region (European Environment Agency, 2012). The lack of knowledge about typical species has been highlighted as a critical point (Genovesi et al., 2014) in the assessment of conservation status of the habitat listed in annex 1 of The Council Directive 92/43/EEC.

Moreover, due to lack of taxonomic knowledge, current checklists are not correctly updated; for example, the Italian checklist (Bartolucci et al., 2018) does not list *Ruppia drepanensis*, the current Euro+Med Plantbase (Uotila, 2009) considers *R. drepanensis* a synonym of *R. cirrhosa*, and the recently updated Flora of Italy (Pignatti et al., 2017) combines a correct list of *Ruppia* species with wrong images (e.g., the image of *R. maritima* shows spirally coiled peduncles). Finally, the results of our investigation highlight the importance of vouchers in phylogenetic and conservation research, and the need to combine molecular analyses with field, ecological, and morphological investigations.

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