



Short communication

Assessing the genuine identity of alien *Vallisneria* (Hydrocharitaceae) species in EuropeA. Mesterházy^{a,*}, G. Somogyi^b, A. Efremov^c, F. Verloove^d^a Centre for Ecological Research, Wetland Ecology Research Group, H-4026, Bem tér18/C, Debrecen, Hungary^b Szent István University, Faculty of Horticultural Science, Department of Botany and Soroksár Botanical Garden, H-1118, Villányi út 29-43, Budapest, Hungary^c Research Center of Fundamental and Applied Problems of Bioecology and Biotechnology of Ulyanovsk State Pedagogical University, 4/5, Lenin Square, 432071 Ulyanovsk, Russia^d Meise Botanic Garden, B-1860, Nieuwelaan 38, Meise, Belgium

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ABSTRACT

The genus *Vallisneria* consists of linear-leaved aquatic plants within the Hydrocharitaceae family. Only one indigenous member (*V. spiralis*) occurs in Europe. Populations of alien species appeared in some European countries in recent decades and these were named in various ways. To clarify the identity of alien *Vallisneria* we collected samples for a molecular phylogenetic analysis from six populations in five European countries. We investigated the nuclear ribosomal Internal Transcribed Spacer (ITS) region. Based on our results we found that samples from Belgium, Germany, Hungary and Italy belong to the recently described *V. australis*, a species native to Australia, while a sample from Russia is identical with *V. neotropicalis*. We provide a key to distinguish these from other similar species. All populations probably derived from dumped plant parts in the drainage water from aquariums. Most populations of *V. australis* are established and this species might therefore become invasive in the future. The investigated *V. australis* populations proved to be not uniform genetically.

1. Introduction

Vallisneria L. (Hydrocharitaceae) is a genus of annual or perennial, submersed, dioecious, freshwater herbs. It has an essentially cosmopolitan distribution, but the highest number of species is reported from Australia. The extensive morphological variability has caused considerable taxonomic disarray in the genus, and the systematic relationships are poorly understood. Species delimitation is also controversial within the genus (Lowden, 1982).

According to the most recent revision of the genus there are 15 extant species (Les et al., 2008). Based on this work the phylogenetic relationship between the species was resolved, but a key for the identification of species was missing.

Vallisneria species are highly valued commercially as ornamental aquarium plants. They sometimes escape from cultivation or (more often) are released/dumped in the drainage water from aquariums. In some cases they can establish self-sustaining populations, mainly in warm waters. *V. spiralis* L. is the only native species in Europe. It is widely dispersed throughout southern Europe with more scattered, non-native occurrences further north (Hussner, 2012). While the identity and

taxonomic status of *V. spiralis* are straightforward, the identity of alien species recorded in some European countries has remained questionable so far.

The first record of alien *Vallisneria* in Europe was reported from the thermal springs of Budapest (Hungary) in 1891 by L. Simonkai (Lukács et al., 2016). The species was identified by him as *V. gigantea* Graebn. Since that time this species was detected in most of the thermally heated canals in Hungary (Hévíz, Tapolca, Tata, Eger) (Király and Vidéki, 2009) and became invasive in at least some of these. For unknown reasons the Hungarian species appeared under the name *V. nana* R.Br. in Hussner (2012). The author mentioned *V. nana* only from Hungary, based on Mesterházy et al. (2009), although the authors of the latter publication used the name *V. gigantea* for the Hungarian plants.

The second record of *V. gigantea* in Europe was from Italy. It was first found in a rice field in Pavia in 1954 (Pirola, 1964) and later also in a canal near Brescia (Frattini, 2008). Thereafter these populations disappeared, but recently (2010) it was found in a pond in Milano. In a work about the alien flora of Lombardia (Banfi and Galasso, 2010), this species was referred to *V. americana* Michx.

Another alien *Vallisneria* was discovered in Russia in the summer of

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2010 in the Pechorka River, at the water discharge point of the Lyubertsy aeration station near Moscow, where it formed a thicket covering an area of 4 m². By 2011 this thicket increased to 30 m² and in 2012 this species already formed a large (about 0.5 ha) 'spot' in the backwater below the spillway, as well as a two-meter wide vegetation border along both banks of the channel (Mayorov et al., 2012; Scherbakov and Mayorov, 2013). Flowering plants were observed only with female inflorescences (author's observation in 2016) suggesting that reproduction was only vegetative. These plants with wider leaves, which are popular in aquariums, were deliberately brought into the Pechorka river (Mayorov et al., 2012). They were attributed to the Cuban-Florida species *V. neotropicalis* Victorin by Scherbakov and Mayorov (2013).

A small (ca. 60 m²) population assigned to *V. americana* was discovered in a canal in Geel in Belgium in 2016. Its presence there was confirmed in 2018 and the size of the population increased to ca. 180 m² (Verloove, 2019). The species apparently survived the rather cold winter 2020–2021, with temperatures down to -15 °C at night, without any problem (author's observation April 2021).

Finally, *V. gigantea* was found in Germany in 2016, where a small population was detected in an artificial lake near Frankfurt (Van de Weyer and Schmidt, 2018).

Based on these records it seems that alien *Vallisneria* species recently have become more widespread in Europe, and all of the populations are considered to have escaped from cultivation. These occur in thermally heated waterbodies in the continental part of Europe, while in western Europe populations are also found in non-heated water bodies. All populations were detected in artificial or strongly modified water bodies; no plants were found so far in unregulated water systems. As these plants have been observed across multiple growing seasons, these can be considered as self-sustaining populations. However, the survival of these plants in all known populations, pointing at. While, at least at present, continental cold winters are posing a barrier for the "giant" alien *Vallisneria* species, these may become invasive species in western Europe in the future.

From the above, it is clear that the taxonomy and nomenclature of the alien species are quite controversial in European countries. At least four different names have been applied (*V. neotropicalis*, *V. nana*, *V. americana* and *V. gigantea*). As most of these populations do not produce generative parts, the determination is very difficult and, as a result, their identification requires critical reassessment, using molecular techniques.

The purpose of our study is twofold: to determine how many alien taxa of broad leaved *Vallisneria* are occurring in Europe and to which species do they belong.

As Les et al. (2008) demonstrated, the analysis of the nrITS region is an appropriate tool to differentiate *Vallisneria* species from each other. The topology of the MP cladogram based on their combined molecular data set (ITS and cpDNA) was similar to that solely generated by ITS data. In the ITS tree almost all species are forming separate clades. However, the exact affiliation of some species remained unresolved, both in the ITS and combined dataset (e.g. the Australian *V. annua* – *V. erecta* – *V. gracilis* species trio, and the Asian *V. asiatica* – *V. natans* species). These unresolved clades on the ITS tree do not affect our investigations about the molecular genetic relationship of the collected samples. On the other hand, by using ITS marker, our results are comparable with the large dataset generated by Les et al. (2008). Therefore, we have chosen ITS for identifying *Vallisneria* specimens collected from anthropogenic localities across Europe.

2. Material and methods

The plant material was collected from known extant populations in Belgium (1 locality), Germany (1 locality), Italy (1 locality), Russia (1 locality) and we also sampled two out of four extant populations in Hungary. Because of clonal propagation leaf material was collected from a single specimen per population. It was dried and stored in silica gel

until usage. The sampling localities and voucher details of the investigated *Vallisneria* specimens are shown in Table 1. From the samples we extracted total genomic DNA by using the modified CTAB method of Xu et al. (2004). The nuclear ribosomal Internal Transcribed Spacer (ITS) region was amplified using the primers ITS-A (Blattner, 1999) and ITS-4 (White et al., 1990). This marker is appropriate to distinguish also newly described *Vallisneria* species from each other (Les et al., 2008; Wasekura et al., 2016). PCRs were performed in 25 µl total volume using 17.8 µl Milli-Q ultrapure water (Merckmillipore, Billerica, MA, USA), 0.2 µl (1 unit) Taq polymerase (Fermentas, Biocenter Ltd., Szeged, Hungary), 2.5 µl of the 10 × Fermentas PCR buffer, 1.25 µl (25 mM) MgCl₂, 1 µl (10 mM) of each primer (Sigma-Aldrich, Budapest, Hungary), 0.25 µl (10 mM) dNTP (Fermentas) and 1 µl of the template DNA of unknown concentration. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 0.5 min at 95 °C, 0.5 min at 55 °C, 1 min at 72 °C, ending with a final elongation of 8 min at 72 °C. Amplifications were carried out in AERIS-BG096 Thermal Cycler (ESCO, UK, Hoyland). PCR products were purified by PCR Viogene PCR Advanced Clean Up System (ZENON Bio, Szeged, Hungary) according to the manufacturer's protocol. Purified DNAs were directly sequenced. Cycle sequencing reactions were performed in 10 µl volume, using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Norwalk, Connecticut, USA). The reactions contained 1 µl (50–60 ng) of PCR products, 4 µl Milli-Q ultrapure water, 2 µl 5 × BigDye Sequencing Buffer, 2 µl BigDye 3.1, and 1 µl (10 mM) of the same primers as used for PCR. Cycle sequencing products were purified following the manufacturer's protocols (BigDye Terminator v 3.1 Purification Kit) and complementary strands were sequenced on an ABI 3130 automated sequencer. Our 6 newly sequenced samples, whose taxonomic affiliation was questionable, were compared with ITS sequences of *Vallisneria* species previously published by Les et al. (2008).

Staden Package 2.0 (Staden et al., 2000) was used to assemble and edit the contigs and sequence alignment was carried out by MEGA X software (Kumar et al., 2018). GenBank accession numbers of the used sequences are given in the Appendix.

A Bayesian analysis was performed employing MrBayes 3.2.1 (Ronquist et al., 2012) applying the GTR + T substitution model proposed by the Akaike information criterion implemented in MrAIC.pl 1.4

Table 1
Details of sampled localities.

Nr.	Locality data	Date of collection	GenBank accession number	Collector
V1	Hévíz lake (Hévíz-tó), Zala county, Hungary (46.465224 °N, 17.114939 °E)	15. 02.2018	MT273070	A. Mesterházy
V2	Geel, Ten Aard, Kempisch kanaal (Kanaal St.-Jozef-Olen/Dessel), Province of Antwerp, Belgium (51.12250 °N, 4.584039 °E)	14.06.2018	MT273071	F. Verloove
V3	Pechorka River, 1 km NE of Lubertsy, Lubertsy district, Moscow region, Russia (55.61371 °N, 37.98,980 °E)	31.07.2010	MT273072	A.Sherbakov, S. Lednev
V6	Eger, Heves county, Hungary (47.535564 °N, 20.225787 °E)	19.03.2019	MT273073	A. Mesterházy
V7	Milan, Lombardy region, Italy (45.484139 °N, 9.193578 °E)	24.03.2019	MT273074	G. Galasso
V9	Mönchwaldsee, Kelsterbach, Hessen, Germany (54.64586 °N, 5.545454 °E)	02.09.2019	MT273075	J. Bruinsma

(Nylander, 2004). Values for all parameters, such as the shape of the gamma distribution, were estimated during the analyses. The Markov Chain Monte Carlo process included four runs with four chains each (three heated ones using the default heating scheme), run simultaneously for 2,000,000 generations each, trees were sampled every 1,000th generation using default priors. Posterior probabilities (PPs) were determined from the combined set of trees, discarding the first 25 % of each run as burn-in. We generated a 50 % majority rule consensus tree from the remaining trees, in which the percentage of nodes recovered represented their posterior probability (PP).

The Maximum Parsimony (MP) analyses were performed using MEGA X (Kumar et al., 2018). The most parsimonious trees were searched by using the TBR method with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). *Nechamandra alternifolia* (EF142957) was used as an out-group species to reroute phylogenetic trees.

We present here only the Bayesian tree of the dataset (Fig. 1.) because of the good congruence of the trees generated by the different methods.

In the Bayesian tree both posterior probability (above 0.95) and bootstrap (above 50 %) support values are shown.

Herbarium material, including from online resources, was studied from several different herbaria like BP, FI, IBIW, HK, K, LE, MW, P, QBG, RWBG, SART, VLA and W (herbarium acronyms following Thiers, 2020). Morphological descriptions given in publications by Lowden (1982), Les et al. (2008) and observations of the authors were used for building an identification key. The studied specimens we used for morphological descriptions are presented in Appendix 2.

3. Results and discussion

A total of 655 aligned base pairs were obtained from the ITS dataset of the 6 newly investigated *Vallisneria* specimens and the additional 52 accessions originating from GenBank (*Maidenia rubra* Rendle was re-evaluated as *Vallisneria rubra* by Les et al., 2008). The phylogenetic reconstruction of this dataset based on Bayesian analysis is presented in Fig. 1. The general topology of the Bayesian tree obtained is

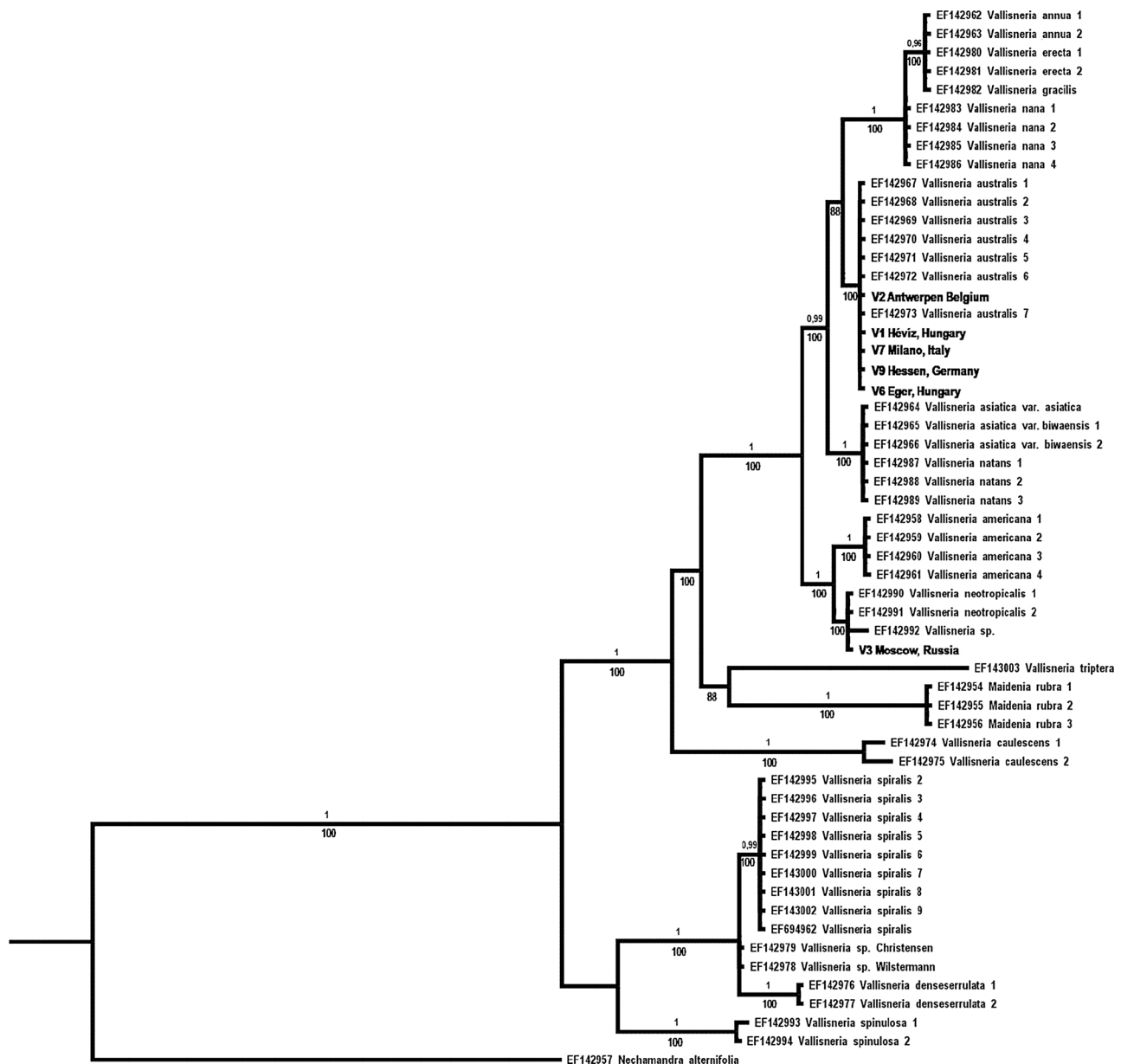


Fig. 1. 50 % majority rule consensus tree based on the Bayesian analysis of the ITS dataset. Posterior probabilities (if >0.95) are shown above, bootstrap support values (>55 %) below branches.

substantially identical with the ITS tree published by Les et al. (2008). Some clades with low support in the original tree are positioned elsewhere here. For instance, the *V. australis* clade is in our Bayesian tree sister to the clade which includes all the rosulate taxa originating from Australia. Despite the changes in topology, these do not influence the position of the newly investigated samples and the conclusions about their taxonomic identity.

Based on our molecular research the investigated *Vallisneria* populations from Belgium, Germany, Hungary and Italy are all identified as *V. australis* S.W.L. Jacobs & Les, which is a recently described species originating from Australia (see Les et al., 2008) and introduced in China (Wasekura et al., 2016). This is not a well-supported clade in the Bayesian tree (PP = 0.83; BS = 100 %), because the investigated *V. australis* sequences differ from each other in two positions. Les et al. (2008) indicated previously that, based on ITS sequences, the natural and cultivated populations of *V. australis* (accessions 1, 2, 3 and 4, 5, 6, 7 respectively) can be distinguished. Among the newly sequenced specimens one sample from Hungary (V1) is identical with *V. australis* 7 (EF142973), the other four specimens are heterozygous for both of the above mentioned positions (Table 2). Because of the direct sequencing, it cannot be completely excluded that this is caused by sequencing errors; however, it is more likely that these sites are polymorphic. Interestingly, a population from Russia (near Moscow) turned out to belong indeed to *V. neotropicalis* as initially reported by Scherbakov and Mayorov (2013).

Both *V. australis* and *V. neotropicalis* are often used by aquarists and *V. australis* has already become an invasive species in Japan (Wasekura et al., 2016). *V. australis* has been known in Europe since the late 1800's as the herbarium specimen of *V. gigantea* collected at the end of the 19th century at „Lukács-fürdő” (BP) in fact corresponds with this species (Lukács et al., 2016), while *V. neotropicalis* was discovered in Russia in 2010. Our study confirms that *V. australis* has become established in some parts of Europe. Firstly, this species naturalised in Hungary although in this country it only occurs in thermally heated canals. Later, this species was also recorded from Italy, Belgium and Germany where it occurs in strongly modified water bodies (rice fields, canals, gravel pits), but these are not thermally heated. This means that this species can adapt to the Atlantic climate and potentially may become an invasive species in the near future. According to our experience in Hungarian populations, this species is a very strong competitor and can replace other submerged invasive species (*Hydrilla verticillata* (L.f.) Royle, *Cabomba caroliniana* A. Gray) in thermal water canals. It always forms monodominant stands. According to our observations both female and male populations are known in Hungary but these are found in different sites, while only female specimens were collected formerly in Italy and currently in Belgium. In a German population no inflorescences were observed so far (J. Bruinsma, pers. comm.). *V. neotropicalis* has been known in Europe for 10 years and during this time the population hardly extended. The occurrence is very local and doubtlessly this species can survive the continental winter only in heated water.

All known populations probably resulted from plant remains released in drainage water from aquariums. Aquarists cultivate these

and other tropical species from natural waters. Some of these have already become serious invasive species like *Hydrilla verticillata*, *Elodea* spp., *Ludwigia* spp. (see: Regulation (EU) No1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species). Therefore, specific regulations to prevent the introduction of new populations via the aquarium trade, and to guide the management of population throughout Europe, seem appropriate.

According to our morphological study some vegetative characters, such as the leaf width and length, pigmentation, apical teeth and apex shape (see: Fig. 2) are useful characters for the determination of some species. Jacobs and Frank (1997) already indicated that in some species the shape of leaf apex and leaf margin can be used as a distinguishing character, while leaf venation is not a useful character. The generative parts would be more suitable for an accurate determination, but flowers are sometimes not produced in Europe. We attempted to build a key, based on both vegetative and generative traits, to distinguish the species found in Europe (*V. spiralis*, *V. neotropicalis* and *V. australis*) from some similar species that may also appear as an introduction in the future. Due to extensive phenotypic plasticity (Lowden, 1982; Les et al., 2008) multiple defining characteristics (leaf tip, leaf margin, pigmentation, etc.) should be used for an accurate identification.

1.1 Max. leaf width 10–15 mm; max. leaf length usually 50–80 cm, rarely more

Vallisneria spiralis L.

Leaf apex obtuse (or broadly acute) in fully developed leaves; veins 5–9; red/brown pigmented leaf striae absent or present; apical teeth sparse < 2/mm; max. peduncle length 13–80 cm; max. fruit length 1–15 cm; stigma divided $> \frac{2}{3}$ to base; hairs at base of androecium (incl. stamens and staminodia) absent, filaments free or united only at base, V-shaped; free portion of staminode arising below upper edge of adjacent stigma margins.

Vallisneria natans (Lour.) H. Hara is similar to this species but differs in leaf apex acute in fully developed leaves, max. fruit length 16–27 cm; fruit cross-section flat/linear (not ovoid!); hairs at base of androecium present; filaments partially/fully fused Y-shaped; free portion of staminode arising at upper edge of adjacent stigma margins.

1.2 Max. leaf width 16–35 mm; max. leaf length 100–300 cm 2

2.1. Red/brown pigmented leaf striae present; apical teeth dense $> 2/\text{mm}$ (margins entire to serrate)

Vallisneria neotropicalis Vict.

Leaf apex obtuse (or broadly acute); veins 5–9; max. peduncle length 100–300 cm; stigma divided $< \frac{1}{2}$ to base; max. fruit length 1–15 cm; hairs at base of androecium present, filaments partially or fully fused, Y-shaped; free portion of staminode arising at upper edge of adjacent stigma margins.

Vallisneria americana Michx. is similar to *V. neotropicalis* but differs in leaves to 15 mm wide; apical teeth < 2/mm; red/brown pigmented leaf striae absent, veins (3)5–9, max. peduncle length 13–80 cm.

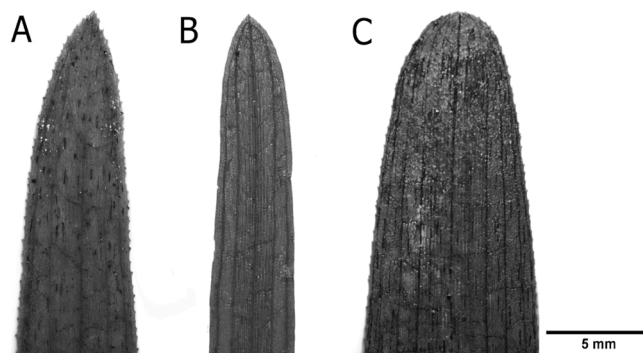


Fig. 2. Leaf apex of three *Vallisneria* species in Europe (A.: *V. neotropicalis*, B.: *V. spiralis*, C.: *V. australis*).

Table 2

ITS nucleotide differences between the newly sequenced specimens in naturalised populations from different parts of Europe, and the *Vallisneria australis* sequences in GenBank.

Sample	Nucleotide positions	
	87	545
V1	C	A
V2, V6, V7, V9	Y	R
<i>V. australis</i> 1, 2, 3 (EF142967-EF142969)	C	G
<i>V. australis</i> 4 (EF142970)	T	A
<i>V. australis</i> 5, 6 (EF142971-EF142972)	T	R
<i>V. australis</i> 7 (EF142973)	C	A

***Vallisneria nana* R.Br.** (syn: *V. gigantea* Graebn.) is similar to *V. neotropicalis* but differs in leaf apex acute in fully developed leaves; veins 3–5 (7), hairs at base of androecium absent.

2.2. Red/brown pigmented leaf striae absent; apical teeth dense >2/mm or < 2/mm

***Vallisneria australis* S.W.L. Jacobs & Les**

Leaf apex obtuse or rounded; veins 5–7; max. peduncle length 100–300 cm; stigma bifid for 50–70 % of their length; max. fruit length 2–16 cm; hairs at base of androecium absent, filaments partially or fully fused, Y-shaped; free portion of staminode arising at upper edge of adjacent stigma margins

Authors' contributions

The study was planned by AM and FV; fieldwork was organized and performed by AM and FV; DNA analyses were performed by GS; the manuscript was written by AM, SG, AE and FV, all other authors made essential contributions to revise the text.

Declaration of Competing Interest

We have no conflict of Interest.

Acknowledgements

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Appendix 1 List of taxa, vouchers and GenBank accession numbers for specimens examined

Maidenia rubra (1) voucher Wilson 8871 (NSW) (EF142954); *Maidenia rubra* (2) voucher Wilson 8872 (NSW) (EF142955); *Maidenia rubra* (3) voucher Hellquist s.n. (CONN) (EF142956); *Nechamandra alternifolia* voucher Cook s.n. (Z) (EF142957); *Vallisneria americana* (1) voucher Les 510 (CONN) (EF142958); *Vallisneria americana* (2) voucher Les Weiss s. n. (CONN) (EF142959); *Vallisneria americana* (3) voucher Les Capers s.n. (CONN) (EF142960); *Vallisneria americana* (4) voucher Moody 148 (CONN) (EF142961); *Vallisneria annua* (1) voucher Jacobs 8578 Les 602 (CONN NSW) (EF142962); *Vallisneria annua* (2) voucher Jacobs 8586 Les 608 (CONN NSW) (EF142963); *Vallisneria asiatica* var. *asiatica* voucher Jacobs 8874 (CONN NSW) (EF142964); *Vallisneria asiatica* var. *biwaensis* (1) voucher Jacobs 8873 (CONN NSW) (EF142965); *Vallisneria asiatica* var. *biwaensis* (2) voucher Gabel s.n. (CONN) (EF142966); *Vallisneria australis* (1) Jacobs 8548 & Les 574 (CONN, NSW) (EF142967); *Vallisneria australis* (2) Jacobs 8554 & Les 580 (CONN, NSW) (EF142968); *Vallisneria australis* Italy, voucher V7 (MT273074); *Vallisneria australis* (4) Wilstermann s.n., 14 Oct 2005 (CONN) (EF142970); *Vallisneria australis* (5) Wilstermann s.n., 30 Jan 2006 (CONN) (EF142971); *Vallisneria australis* (6) Wilstermanns.n., 30 Jan 2006 (CONN) (EF142972); *Vallisneria australis* (7) Wilstermanns.n., 10 Oct 2005 (CONN) (EF142973); *Vallisneria australis* Hévíz, Hungary voucher V1 (MT273070); *Vallisneria australis* Belgium voucher V2 (MT273071); *Vallisneria australis* Eger, Hungary voucher V6 (MT273073); *Vallisneria australis* Italy, voucher V7 (MT273074); *Vallisneria australis* Germany voucher V9 (MT273075); *Vallisneria caulescens* (1) voucher Jacobs 8764 (NSW) (EF142974); *Vallisneria caulescens* (2) voucher Jacobs 8765 (NSW) (EF142975); *Vallisneria denseserrulata* (1) voucher Chen 20 (WBG) (EF142976); *Vallisneria denseserrulata* (2) voucher Chen 21 (WBG) (EF142977); *Vallisneria* sp. Wilstermann s.n. (EF1429798); *Vallisneria* sp. Christensen s.n. (EF1429799); *Vallisneria erecta* (1) Jacobs 8531 & Les 557 (CONN, NSW) (EF142980); *Vallisneria erecta* (2) Jacobs 8584 & Les 607 (CONN, NSW) (EF142981); *Vallisneria gracilis* voucher Jacobs 8549 Les 575 (CONN NSW) (EF142982); *Vallisneria nana* (1) voucher Jacobs 8511 Les 538 (CONN NSW) (EF142983);

Vallisneria nana (2) voucher Martine 863 W.R. Barker s.n. (CONN) (EF142984); *Vallisneria nana* (3) voucher Wilstermann s.n. (CONN) (EF142985); *Vallisneria nana* (4) voucher Gabel s.n. (CONN) (EF142986); *Vallisneria natans* (1) voucher Chen 1 (WBG) (EF142987); *Vallisneria natans* (2) voucher Chen 2 (WBG) (EF142988); *Vallisneria natans* (3) voucher Chen 4 (WBG) (EF142989); *Vallisneria neotropicalis* (1) voucher Harms s.n. (CONN) (EF142990); *Vallisneria neotropicalis* (2) voucher Padgett s.n. (CONN) (EF142991); *Vallisneria neotropicalis* Russia voucher V3 (MT273072); *Vallisneria* sp. Owens Doyle s.n. (EF142992); *Vallisneria spinulosa* (1) voucher Chen 10 (WBG) (EF142993); *Vallisneria spinulosa* (2) voucher Chen 11 (WBG) (EF142994); *Vallisneria spiralis* (1) voucher Bogner 2910 (M) (EF694962); *Vallisneria spiralis* (2) voucher Karlicks.n. (CONN) (EF142995); *Vallisneria spiralis* (3) voucher Hussner s.n. (CONN) (EF142996); *Vallisneria spiralis* (4) voucher Weicherding s. n. (CONN) (EF142997); *Vallisneria spiralis* (5) voucher Weicherding s.n. (CONN) (EF142998); *Vallisneria spiralis* (6) voucher Wilstermann s.n. (CONN) (EF142999); *Vallisneria spiralis* (7) voucher Gabel s.n. (CONN) (EF143000); *Vallisneria spiralis* (8) voucher Owens Doyle (CONN) (EF143001); *Vallisneria spiralis* (9) voucher Jacobs 8555 Les 581 (CONN NSW) (EF143002); *Vallisneria triptera* voucher Wilson s.n. (NSW) (EF143003)

Appendix 2 List of taxa, used for our morphological study

Vallisneria americana USA: N. Ritter 04193 (W), F. Marie-Victorin, F. Rolland-German 12758, F. Warnock 14149 (W), J.K. Small, Ch.A. Mosier, P.A. Matthaues 17350 (W), H.E. Moore Jr. M.E. Ruff 655 (W), A. E. Radfend 18101 (W), C.J. Moser 274724 (W), L. McGuire 10327 (W), R.D. Henry 10328 (W), Canada: T.G. Lammers, R. Stuckey, D. Salamon 03630 (W), 13258, 13259 (W), J.M. Gillett 25957 (W), H.J. Scoggan 11689 (W)

Vallisneria neotropicalis

Cuba: J. M. Komlódi (s.n.) (BP), Russia: A.V. Scherbakov (s.n.) (IBIW, MW)

Vallisneria australis

Australia: S.W.L. Jacobs (s.n.) (B), D.B.G. Briggs, L.A.S. Johnson, C. L. Porter (s.n.) (B), Hungary: Simonkai (s.n.) (BP), Hűvös-Récsi A., Matus G. (s.n.) (BP),

Mesterházy A. (s.n.) (BP),

Vallisneria nana

Philippines: A.D.F. Elmer 10133 (W), R.C. McGregor (s.n.) (FI) Melanesia: L.N. Gray 13309 (W)

Vallisneria natans

Japan: M. Normua 33671 (QBG), M. Miyake 57316 (QBG), Dunn, S. T. (s.n.) (HK), K. Inami (s.n.) (VLA), K. Inagaki (s.n.) (VLA), China: Merrill, Elmer D. (s.n.) (HK), Matthew 5033 (HK), Lau, Y. S. (s.n.) (HK),

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