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Research article

Myriophyllum rubricaule sp. nov., a *M. aquaticum* look-alike only known in cultivation

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Abstract. A confusingly labeled water-milfoil of obscure status, known only in cultivation, is here formally described as a new species, *Myriophyllum rubricaule* Valk. & Duist. sp. nov. This species has fully replaced *M. aquaticum* in the horticultural trade in Europe since the addition of *M. aquaticum* to the list of invasive alien species of Union concern (EU regulation no. 1143/2014) in 2016. This manuscript provides a morphological description of *M. rubricaule* sp. nov., and its distinction from *M. aquaticum* is further supported by molecular data (chloroplast and nuclear loci).

Keywords. *Myriophyllum*, water-milfoil, EU regulation no. 1143/2014, invasive plant, horticulture.

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Introduction

Incorrect labeling of plants in trade and misidentification are widespread and may be caused by negligence or willful disrespect of regulations (Brunel 2009; Thum *et al.* 2012; Verbrugge *et al.* 2014; Hulme *et al.* 2018). Mislabeling may consist of simple misspelling of names, or treating a variety as a true species, using synonyms or just preferring a name that sounds nice or a name that customers are familiar with. The latter case actually equals misidentification of the plant in trade.

From observations of species of *Myriophyllum* Ponted. ex L. in trade in the Netherlands, it is clear that proper labeling is generally neglected, causing confusion about the proper identity of the species in trade (Van Valkenburg & Boer 2015; Van Valkenburg *et al.* 2015). Plants labelled as *Myriophyllum* '*brasiliensis*' or *M.* '*brasiliense*' have been in the horticultural trade in north-western Europe for several decades, though their taxonomy has caused some confusion among botanists, plant growers and regulators. When looking

at species of *Myriophyllum*, Moody & Les (2010) refer to sterile plants of unknown origin in horticulture as *Myriophyllum* 'red 1' and 'red 2', respectively. One of these taxa may well be identical to the taxon we found in trade in north-western Europe. The European material in trade was included in our previous study on barcoding invasive aquatic plants as *M.* '*brasiliense*' trade material and erroneously referred to as *M. robustum* Hook.f. (Ghahramanzadeh *et al.* 2013). After receiving the true *M. robustum* from New Zealand, and observing it flowering in 2013 (*Valkenburg 3739* WAGPD; only female flowers, however, *M. robustum* is described as monoecious by Orchard (1980)), the identification of the trade material was adjusted and the designation in Q-bank plants updated to *Myriophyllum* sp. trade name 'brasiliensis'. The name *Myriophyllum brasiliense* Cambess. is a synonym of *M. aquaticum* (Vell.) Verdc. In the absence of any knowledge of its occurrence in the wild, we refrained from formally describing it as a new species. In the meantime, plants have proved to be hardy in outdoor mesocosms at the NVWA (Nederlandse Voedsel en Warenautoriteit) in Wageningen (the Netherlands) since 2007. After *M. aquaticum* was included in the List of invasive alien species of Union concern ('the Union list') under EU regulation no. 1143/2014 in August 2016, it has been fully replaced by *Myriophyllum* sp. trade name 'brasiliensis' in the horticultural trade in Europe. Since then, there has been an increase in the number of recordings of *Myriophyllum* sp. trade name 'brasiliensis' in urban waters as well as in more natural habitats. This is likely a result of inappropriate disposal of garden waste or the deliberate planting of the species in urban waterways and residential areas. To-date, there are records of establishment for the Netherlands, Belgium and Hungary. The ongoing confusion with respect to the epithet 'brasiliensis' has prompted the authors to formally describe this taxon known only in cultivation.

Material and methods

Plant material of *Myriophyllum* has been collected since 2007, and deposited at the herbaria L, WAG and WAGPD (Appendix 1). Acronyms of herbaria follow Index Herbariorum (Thiers continuously updated).

In addition to the above mentioned specimens used for the present study, all *Myriophyllum* collections from South America at Naturalis (AMS, L, U, WAG) were consulted as well as most relevant revisions and new taxonomical publications on the genus *Myriophyllum* to ascertain the novelty of the new species (Meijden & Caspers 1971; Orchard 1980, 1981, 1986; Orchard & Kasselmann 1992; Wang *et al.* 2002; Yu *et al.* 2002; Wang & Yu 2007).

Morphological description is based on both living and herbarium material and follows Meijden & Caspers (1971) and Crow & Ritter (1999) for characters considered important in species recognition. No plant material was consulted of the *Myriophyllum* sp. 'red 1' and 'red 2' that were used in the molecular analysis as they were reported to be sterile (Moody & Les 2010).

An annotated collection of *M. aquaticum* (*Pedersen 3977* in Orchard 1981) from Argentina, as well as a diminutive collection of *M. aquaticum* from Brazil (*Lindeman 8405*) were included to ascertain the molecular novelty of the new species. A *Laurembergia tetrandra* (Schott) Kanitz collection (*van Proosdij 33*) was used as an outgroup (Appendix 1).

DNA extractions

Genomic DNA was isolated from approximately 100 mg of plant material with the DNeasy plant mini kit (Qiagen, Venlo, the Netherlands) using the TissueLyser procedure and eluted with 50 µl of prewarmed (65ºC) AE buffer. DNA was stored at -20°C until use.

PCR amplification and Sanger sequencing

PCR reactions for the chloroplast loci *rbcL* and *trnH-psbA* intergenic spacer and the nuclear locus ITS (partial 18S, ITS1, 5.8S, ITS2, partial 28S) were performed in 25 µl reaction mixes containing 1 × MyFi™ Mix (Bio-line, Taunton, USA), 2 µl of genomic DNA and 200 nM of either primer combinations rbcL-a F and rbcLa SI_Rev, trnH2 and psbAF, or ITS5 and ITS4 (Table 1), respectively.

Table 1. Primers used in this study.

Loci	Primer name	Primer sequence	Reference
rbcL	rbcL-a F	ATGTCACCACAAACAGAGACTAAAGC	Kress & Erickson 2007
	rbcLa SI_Rev	GTAAAATCAAGTCCACCRCG	Kress *et al.* 2009
trnH-psbA	trnH2	CGCGCATGGTGGATTCACAATCC	Tate 2002
	psbAF	GTTATGCATGAACGTAATGCTC	Sang *et al.* 1997
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* 1990
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* 1990

The cycle conditions for *rbcL* and *trnH-psbA* loci were as follows: 5 min at 95ºC, followed by 5 cycles of 30 s at 94ºC, 30 s at 45ºC, 30 s at 72ºC and 35 cycles of 30 s at 94ºC, 30 s at 50ºC, 30 s at 72ºC and a final extension for 10 min at 72ºC. For the ITS locus we used the following conditions: 5 min at 95ºC, followed by 40 cycles of 30 s at 94ºC, 30 s at 52ºC, 100 s at 72ºC and a final extension for 10 min at 72ºC.

The obtained PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, the Netherlands) preceding bidirectional cycle sequencing with the BigDye Terminator ver. 1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Bleiswijk, the Netherlands) using the aforementioned amplification primers as sequencing primers in separate reactions according to the manufacturer's instructions. Cycle sequence products were purified with the DyeEx 2.0 Spin Kit (Qiagen,Venlo, the Netherlands) and subsequently sequenced using a 3500 Genetic Analyzer (Thermo Fisher Scientific). Consensus sequences were generated from an assembly with trace files from both Sanger sequencing runs in Geneious Prime® 2021.1.1 (Biomatters Auckland, New Zealand). Amplification primer sequences were trimmed in the assembly, and when needed, additional trimming was performed to obtain high-quality (PHRED > 30) consensus sequences.

Illumina sequencing

DNA extracts were sent to GenomeScan (Leiden, the Netherlands) for Illumina 150PE (paired-end) sequencing using the NovaSeq 6000 (*Myriophyllum aquaticum* JL 8405, JvV 3494 and TP 3977, *Myriophyllum rubricaule* JvV 3298, 3342, 3495, 4118 and 4119) or 100PE sequencing with the HiSeq 2500 platform (*Laurembergia tetrandra* AP 33, *Myriophyllum heterophyllum* JvV 3651 and *M. rubricaule* JvV 3648) with at least 2 Gb output per sample.

Samples that were sequenced with the NovaSeq 6000 were processed using the NEBNext® Ultra II FS DNA module and the NEBNext® Ultra II Ligation module. Fragmentation, A-tailing and ligation of sequencing adapters and PCR using NEBNext® Ultra II Q5 master mix of the resulting product was performed according to the procedure described in the NEBNext Ultra II FS DNA module and NEBNext Ultra II Ligation module instruction manual. HiSeq 2500 samples were processed using the NEBNext® Ultra DNA Library Prep kit for Illumina (New England Biolabs, Ipswich, USA). Fragmentation of the DNA using the Covaris, ligation of the sequencing adapters and PCR amplification of the resulting product were performed according to the procedure described in the NEBNext Ultra DNA Library Prep kit for Illumina instruction manual.

After preparation, the quality and yield for all samples was measured with the Fragment Analyzer (Agilent Technologies, USA). The size of the resulting products was consistent with the expected size of approximately 500–700 bp.

De novo assembly and extraction of loci

Reads of samples were trimmed and de novo assembled in CLC Genomics Workbench 21.0.3 (Qiagen, Aarhus, Denmark) using default settings. Loci of interest were extracted with BLASTn (standard settings) from assembled contigs using a custom BLAST database in Geneious Prime® 2021.1.1 (Biomatters Auckland, New Zealand) containing the reference sequences of *M. aquaticum* ITS (GenBank no. KY767734; Lü *et al.* 2017), *Myriophyllum* sp. 'red 1' *matK* and *trnK3* (GenBank nos FJ870932 and FJ861339, respectively; Moody & Les 2010) and *M. rubricaule trnH-psbA* and *rbcL* (this paper, GenBank nos MZ399141 and MZ399133).

Phylogenetic analyses

Sequences were aligned using MUSCLE ver. 3.8.425 (Edgar 2004) within Geneious Prime® 2021.1.1 (Biomatters Auckland, New Zealand) with standard settings. We performed model testing on the alignments to select the most appropriate model for constructing a maximum likelihood tree using the model selection tool in MEGA X with standard settings (Kumar *et al.* 2018). Selected models were used to generate the phylogenetic trees in MEGA X.

Data accessibility

The annotated DNA sequences are available from the NCBI GenBank database under the following accession numbers (Supp. file 1): MZ401372–MZ401382 and OL806572–OL806573 (ITS), MZ399118–MZ399126 and OL827545–OL827546 (*matK*), MZ399127–MZ399134, MZ399152–MZ399156 and OL827547–OL827548 (*rbcL*), MZ399135–MZ399142, MZ399157–MZ399161 and OL827549–OL827550 (*trnH-psbA* intergenic spacer) and MZ399143–MZ399151 and OL827551–OL827552 (*trnK3*).

Illumina data are deposited in the Sequence Read Archive (SRA) under accession numbers ERR6000187–ERR6000195, ERR7645488 and ERR7645489. See Supp. file 1 for an overview.

Sequences are also available at https://qbank.eppo.int/plants/.

Results

Taxonomic treatment

Class Magnoliopsida Brongn.
Order Saxifragales Bercht. & J.Presl.
Family Haloragaceae R.Br.
Genus *Myriophyllum* Ponted. ex L.

Myriophyllum rubricaule Valk. & Duist. sp. nov.
urn:lsid:ipni.org:names:77299815-1
Fig. 1

Myriophyllum robustum auct. non Hook.f. *Molecular Ecology Resources* 13: 21–31 (Ghahramanzadeh *et al.* 2013).
Myriophyllum brasiliense auct. non Cambess.: plants in trade.
Myriophyllum sp. trade name 'brasiliensis': Beringen R. 2020. — Pot R. *et al.* 2021. — Q-bank Invasive Plants. 2019–2021. — EPPO-Q-bank. 2022.

Diagnosis

Herba perennis aquatica vel paludigena. Folia omnia verticillata pectinata. Flores unisexuales, solae plantae femininae culturaeque cognitus. Flores intra axillas foliorum solitarii. Planta valde similaris Myriophyllo aquatico, sed caules foliaque modice, caules purpurei et folia emergentia virida. Flores feminei subrosei.

Etymology

The species epithet is based on the purplish red color of the stem.

Type material

NETHERLANDS • Grashoek, Roomweg 85, 5985 NS, Golf course Kapelkeshof; 51°22.0′ N, 5°56.5′ E; herbarium specimen; 13 Jul. 2009; fl; *J.L.C.H. van Valkenburg & J. Hoogveld 3495*; holotype: L!; isotypes: WAGPD[WAG0453615]!, A!, BM!, BR!, MO!, NSW!; GenBank nos: MZ399148 (trnK3), MZ399140 (trnH-psbA), MZ399132 (rbcL), MZ399123 (matK), MZ401380 (ITS); SRA: ERR6000190.

Additional specimens examined

NETHERLANDS • Wageningen, open air pond at PD; herbarium specimen; originally from Wageningen, Garden Centre d'Oude Tol; 14 Jun. 2007; fl; *J.L.C.H. van Valkenburg 3298*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453612]; GenBank nos: MZ399146 (trnK3), MZ399138 (trnH-psbA), MZ399130 (rbcL), MZ399121 (matK), MZ401378 (ITS); SRA: ERR6000191 • Vinkeveen, Aquaflora Vinkeveen, Ter Aase Zuwe, near junction main road Vinkeveen to Wilnis; herbarium specimen; cultivated at Wageningen, open air pond at PD, originally from Vinkeveen, collected 5 Apr. 2007; 9 Jul. 2007; fl; *J.L.C.H. van Valkenburg 3314*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453613] • herbarium specimen; grown by 'Simon van der Velde Waterplanten BV', Albert van 't Hartweg 1, Bleiswijk; 30 Jul. 2007; *J.L.C.H. van Valkenburg 3342*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453614]; GenBank nos: MZ399147 (trnK3), MZ399139 (trnH-psbA), MZ399131 (rbcL), MZ399122 (matK), MZ401377 (ITS); SRA: ERR6000192 • Wageningen, Garden Centre d'Oude Tol; herbarium specimen; culta the Netherlands; 11 Apr. 2009; *J.L.C.H. van Valkenburg 3472*; J.L.C.H. van Valkenburg det.; L[L 0767219; L 0909277]; GenBank no.: JX100590 (trnH-psbA) • Wageningen, Geertjesweg 15, Plant Protection Service; herbarium specimen; culta, first received 1 May 2009 from Stoffels International BV, Maalbekerweg 14, 5951 NT, Belfeld, the Netherlands; 11 Sep. 2009; fl; *J.L.C.H. van Valkenburg 3510*; J.L.C.H. van Valkenburg det.; L[L 0767220; L 0909323]; GenBank nos: JX100591 (trnH-psbA), JX100753 (rbcL) • Wageningen, greenhouse Plant Protection Service; herbarium specimen; culta from plant obtained at garden centre; 28 Jul. 2011; *J.L.C.H. van Valkenburg 3648*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453609]; GenBank nos: MZ399149 (trnK3), MZ399158 (trnH-psbA), MZ399153 (rbcL), MZ399124 (matK), MZ401372 (ITS); SRA: ERR6000195 • Hoogeveen, Kinholt; 52°43.34′ N, 6°26.00′ E; herbarium specimen; 5 Nov. 2018; *J.L.C.H. van Valkenburg 3971*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453559]; GenBank nos: MZ399159 (trnH-psbA), MZ399154 (rbcL) • Steenwijk, Het Eemter; 52°47.415′ N, 6°08.840′ E; herbarium specimen; 8 Sep. 2020; *J.L.C.H. van Valkenburg 4116*; J.L.C.H. van Valkenburg det.; WAG[WAG.1971556], WAGPD[WAG0452369]; GenBank nos: MZ399160 (trnH-psbA), MZ399155 (rbcL), MZ401381 (ITS) • Hattem, Palmstraat; 52°27.768′ N, 6°04.428′ E; herbarium specimen; 8 Sep. 2020; *J.L.C.H. van Valkenburg 4117*; J.L.C.H. van Valkenburg det.; WAG[WAG.1971558], WAGPD[WAG0452378]; GenBank nos: MZ399161 (trnH-psbA), MZ399156 (rbcL), MZ401382 (ITS) • Klazienaveen; 52°44.580′ N, 7°00.615′ E; herbarium specimen; 7 Oct. 2020; *J.L.C.H. van Valkenburg 4118*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0452243]; GenBank nos: MZ399150 (trnK3), MZ399141 (trnH-psbA), MZ399133 (rbcL), MZ399125 (matK), MZ401375 (ITS); SRA: ERR6000193 • Wageningen, greenhouse NVWA; herbarium specimen; culta, origin garden center more than 8 years ago; 8 Oct. 2020; *J.L.C.H. van Valkenburg 4119*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0452240]; GenBank nos: MZ399151 (trnK3), MZ399142 (trnH-psbA), MZ399134 (rbcL), MZ399126 (matK), MZ401376 (ITS); SRA: ERR6000194.

HUNGARY • Borsod-Abaúj-Zemplén, Kács; 47°57.44′ N, 20°37.00′ E; herbarium specimen; 20 Sep. 2019; *A. Mesterházy s.n.*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0452409]; GenBank nos: MZ399157 (trnH-psbA), MZ399152 (rbcL).

Description

Dioecious amphibic or aquatic herb. Stem unbranched or with up to 6 branches per 20 cm, often rooting at submerged and lower emerged nodes; submerged part green to tinged red-brown and internodes 10–50 mm long; emerged part red or purplish red and internodes 3–25 mm long. Hydathodes few at base of submerged leaves, many at base of emerged leaves, ca 1 mm long, filiform, pale or reddish brown. Leaves in whorls of 4 or 5, pinnatifid with pinnae placed opposite and/or alternate. Submerged leaves 10–25(–50) × 3–10 mm, olive green or turning pale to dark reddish brown; pinnae 12–21, 3–14(–30) × 0.1–0.2 mm. Emerged leaves (7–)10–25 × 3–8 mm, bright green to bluish green, not glaucous, sometimes tinged red brown or pinnae red-tipped; pinnae (5–)7–21, 2.3–9 × 0.15–0.3(–0.5) mm. Flowers solitary in the axils of the emerged leaves, only female known, tinged pink. Pedicel 0.3–0.5 mm long. Bracteoles 2 at basis of pedicel, 0.5–1 × 0.1 mm, with 1–3 alternate and filiform lobes. Sepals 4, erecto-patent to reflexed, 0.5–0.7 × 0.2–0.3 mm, margin remotely fimbriate. Petals absent. Stamens absent. Ovary 4-sulcate, with 4 styles with feathery and more or less inrolled stigmas. Fruits unknown.

Amphibic herb with pinnate leaves in whorls of 4 or 5, only known from female plants and (escapes from) horticulture. Differs from *M. aquaticum* in its generally more modest dimensions, the stems being purplish red, the emergent leaves being green to bluish green and not glaucous (i.e., leaves without waxy coating) and the female flowers being pinkish (Fig. 1).

Fig. 1. Plants, prior to collection, of *M. rubricaule* Valk. & Duist. sp. nov. (*Valkenburg 3495*; red stem) growing amidst *M. aquaticum* (*Valkenburg 3494*; green stem) in an artificial pond at a golf course, illustrating the more modest dimensions and color differences (13 July 2009, Grashoek, the Netherlands).

Distribution

Origin unknown. Known from cultivation (Netherlands, Belgium) and as escapes from cultivation since 2018 in the Netherlands (Hoogeveen, Steenwijk, Hattem, Klazienaveen) and Belgium (Maasmechelen, Houthalen-Helchteren, Brugge, Gent, Beauraing, Waimes; all confirmed from photographs at waarnemingen.be), and at least since 2019 in Hungary (Kács). It is unclear whether the observations in Belgium before 2018 (in retrospect the first observation dates from 2012; see waarnemingen.be records 70176185 and 95286205) refer to escapes or planted material.

Note

If grown in particularly nutrient rich and/or high light conditions plants develop much bigger submerged leaves (e.g., *Valkenburg 3298* and *4116* WAGPD).

Similar species

Because both the submerged and emergent leaves are whorled, pectinate and not much different in length, and the inflorescence is emergent, the species fits in section *Pectinatum* M.L.Moody & D.H.Les. This section includes *M. aquaticum* and *M. mattogrossense* Hoehne (Moody & Les 2010). *Myriophyllum aquaticum* differs from *M. rubricaule* sp. nov. in having green stems only turning red when grown as a potted plant or in unfavorable conditions, but never purplish red, with leaves in whorls of 4–6, submerged leaves 25–45 mm long and green or red brown, emerged leaves 25–35 mm long and bluish green glaucous (i.e., with a waxy coating that can be rubbed of), and white flowers. *Myriophyllum mattogrossense* has green stems, small, globular, sessile glands on leaves and stems, submerged leaves 10–35 mm wide, and bisexual flowers (Crow & Ritter 1999).

While performing the DNA barcoding study of *M. aquaticum* (Ghahramanzadeh *et al.* 2013), we initially thought that the plants described here as a new species belonged to *M. robustum*. This is a species resembling *M. aquaticum* with similar emergent pectinate leaves and solitary axillary flowers. However, *M. robustum* is described as having hermaphrodite flowers whereas *M. aquaticum* is dioecious (Orchard 1980). Shortly after our paper was published, plants of *M. robustum* were received from New Zealand and were grown in a greenhouse. The cultivated plants had more robust erect emergent stems that were pink in the upper part, and subglaucous leaves that were oblong in outline with an acute tip. Surprisingly, the plants produced only female flowers in the greenhouse (*Valkenburg 3739* WAGPD). Grown outdoors in a mesocosm in later years, the plants first produced female flowers, a row of hermaphrodite flowers and then, distally, male flowers (*Valkenburg 3853* WAGPD).

Distinguishing molecular features of *Myriophyllum rubricaule* sp. nov.

As described above, *M. rubricaule* sp. nov. is morphologically distinguishable from *M. aquaticum*. Moody & Les (2010) described two specimens, *M.* sp. 'red 1' and *M.* sp. 'red 2', that were morphologically and genetically related to *M. aquaticum*. We made a molecular comparison of *M. rubricaule*, *M. aquaticum*, *M.* sp. 'red 1' and *M.* sp. 'red 2' using the same molecular markers (Moody & Les 2010), namely the nuclear locus ITS (partial 18S, ITS1, 5.8S, ITS2, partial 28S), and the chloroplast loci *trnK3* and *matK* to find out how *M.* sp. 'red 1' and *M.* sp. 'red 2' relate to *M. rubricaule* (Figs 2–3). We included *Myriophyllum heterophyllum* Michx. and *Laurembergia tetrandra* (Schott) Kanitz as more distantly related species within the *Haloragaceae* in this comparison.

Based on ITS (Fig. 2), all *M. rubricaule* sp. nov. specimens showed 100% sequence similarity. *Myriophyllum* sp. 'red 1' was most similar to *M. rubricaule* sharing 98.62% sequence identity. We only observed differences in the ITS1 sequence (38–42delCCCCG and 87delG). Interestingly, the specimens of *M. aquaticum* group with *M.* sp. 'red 2', albeit with a relatively low confidence value, sharing 96.27% and 93.65% identity with *M. rubricaule*, respectively.

The *trnK3* and *matK* loci were extracted from the sequence data and subsequently concatenated, before tree generation. Similarly to ITS, the *trnK3-matK* sequences were identical for all samples of *M. rubricaule* sp. nov. (Fig. 3). Intriguingly, the *M.* sp. 'red 2' and *M. aquaticum* JL 8405 *trnK3-matK* loci were identical to *M. rubricaule*. *Myriophyllum aquaticum* JvV 3494 and TP 3977 showed 99.88% similarity with *M. rubricaule* and *M.* sp. 'red 2' with only two single nucleotide polymorphisms (SNPs; T32A and T1243A). *Myriophyllum* sp. 'red 1' differs on 10 positions with *M. aquaticum*, *M. rubricaule* and *M.* sp. 'red 2' sharing 99.63% similarity.

Finally, we compared two other well-known chloroplast loci, *rbcL* and *trnH-psbA*, to identify more distinguishing molecular features between *M. aquaticum* and *M. rubricaule* sp. nov. We incorporated previously described sanger sequences (Ghahramanzadeh *et al.* 2013) as well as newly generated sanger and Illumina sequences in the comparison (Supp. file 1 and 2). For *trnH-psbA*, all 12 specimens of *M. rubricaule* were identical to each other. Seven out of eight *M. aquaticum* were identical, for *M. aquaticum* JL 8405 we observed one SNP (G230A) (Supp. file 3). However, for two samples of *M. aquaticum* not all sanger data could be resolved resulting in two ambiguities: M188A for *M. aquaticum* JvV 3329 and K255T for *M. aquaticum* HD 426. A clear distinction was observed towards the 5'-end of the *trnH-psbA* intergenic spacer (nucleotides #237–292) where 13 SNPs were identified between the specimens of *M. aquaticum* and *M. rubricaule* resulting in approximately 96% similarity. Interestingly, we did not find any differences between *M. aquaticum* and *M. rubricaule rbcL* sequences (Supp. file 4). Even the distantly related plant species *L. tetrandra* shared 97.29% of *rbcL* identity. In contrast, for *trnH-psbA*, only a ~ 69–73% identity score was observed between *L. tetrandra* and *M. rubricaule* and *M. aquaticum*.

Discussion

During our study of the confusingly labeled water-milfoil in trade, we consulted the most relevant revisions and new taxonomical publications on the genus *Myriophyllum* (Meijden & Caspers 1971; Orchard 1980, 1981, 1986; Orchard & Kasselmann 1992; Wang *et al.* 2002; Yu *et al.* 2002; Wang & Yu 2007). After attempting to identify the plants using the revised key for New Zealand (Orchard 1980), the two choices where *M. aquaticum* and *M. robustum*. Firstly, our taxon cannot be *M. aquaticum* because of the color of the leaves in the field, and secondly it is not *M. robustum* because the flowers are not hermaphroditic. The revision for South America (Orchard 1981) likewise does not yield a result as *M. aquaticum* has glaucous leaves and *M. mattogrossense* is disqualified by the plants being weak and having hermaphrodite flowers. In the revision for Australia (Orchard 1985), *M. aquaticum* is also the only realistic option. None of the species covered by the *Myriophylllum* treatment for Malesia (Meijden & Caspers 1971), nor recent publications for new species in Asia (Wang *et al.* 2002; Yu *et al.* 2002; Wang & Yu 2007) match our material as all of those species have dimorphic leaves.

The new species fits in section *Pectinatum* M.L.Moody & Les (2010: 136). They formally described the section, with *M. aquaticum* (Vell.) Verdc. as the type species, as follows:

"Folia omnia verticillata; difert a *M.* subsect. Myriophyllum folia omnia emersa pectinata et difert a *M.* subsect. Isophylleae folia emersa comparate non redacta de foliis submersis. Diagnosis: Submerged and emergent leaves whorled and pectinate. Emergent leaves pectinate and not highly reduced in relation to submerged leaves. Plants dioecious or flowers all hermaphrodite."

Species within this section have in common the emergent inflorescences and all leaves whorled and pectinate; the emergent leaves are not highly reduced.

The definition as such would also fit for *M. robustum*, that is placed in *M.* subsection *Myriophyllum* but is the exception in the subsection for having all pectinate emergent leaves that are not highly reduced in relation to submerged leaves.

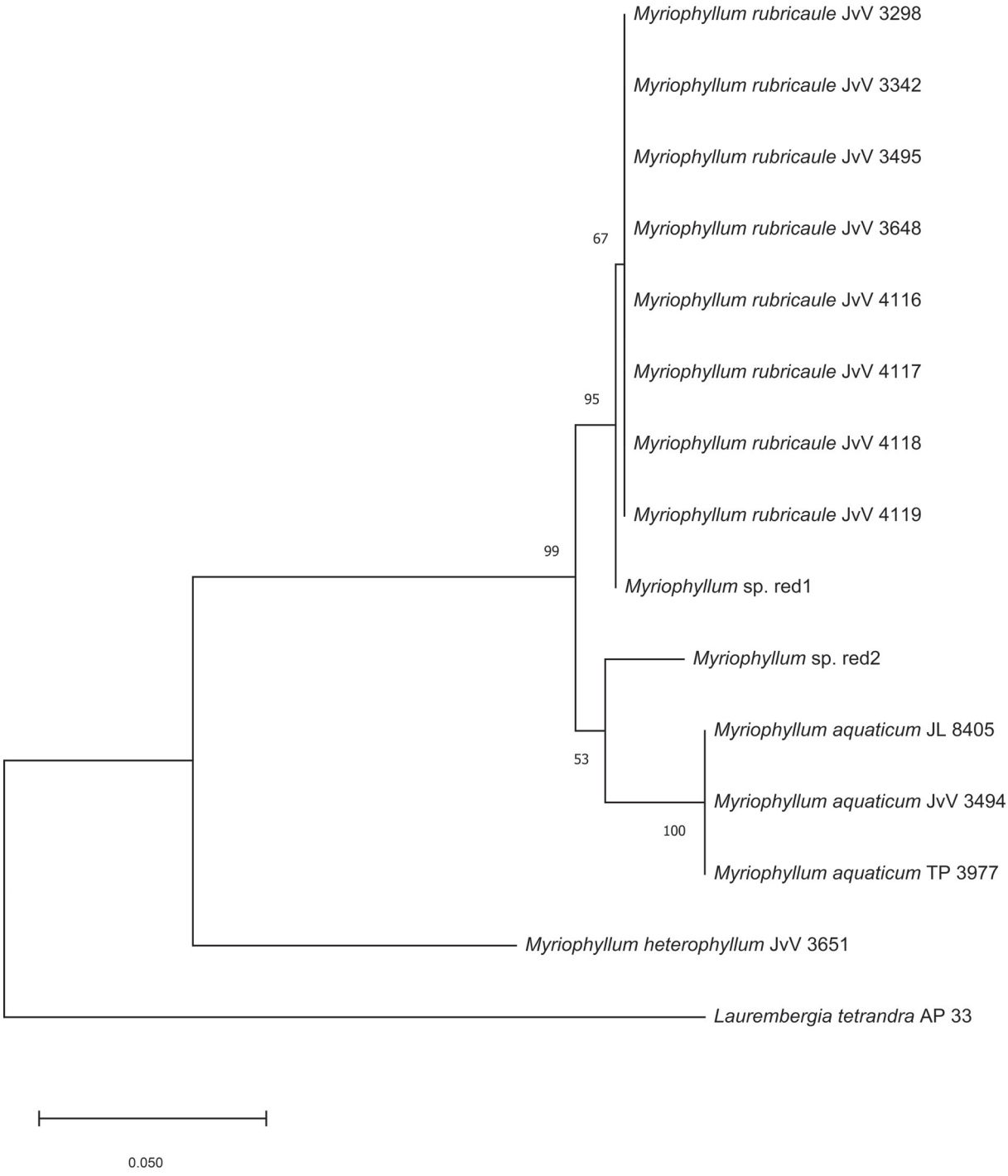

Fig. 2. Maximum likelihood tree based on ITS (partial 18S, ITS1, 5.8S, ITS2, partial 28S) sequences. The Tamura 3 parameter model was used (Tamura 1992). The tree with the highest log likelihood (-1398.71) is shown. Bootstrap values after 1000 replicates are expressed as percentages. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4031)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Laurembergia tetrandra* AP 33 was used as an outgroup.

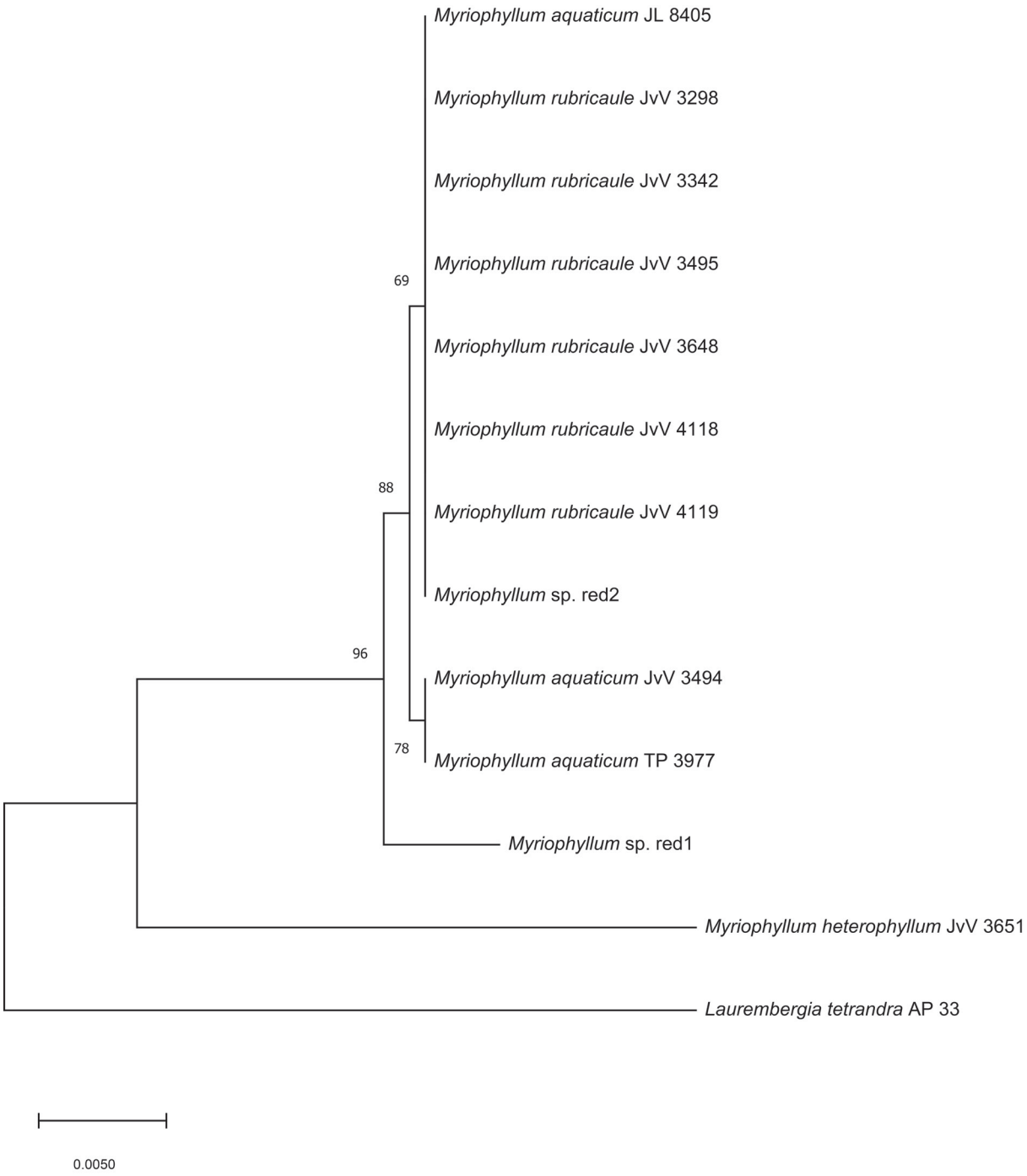

Fig. 3. Maximum likelihood tree based on concatenated *trnK3-matK* sequences. This tree was inferred by using the Maximum Likelihood method and Tamura 3 parameter model (Tamura 1992). The tree with the highest log likelihood (-2853.86) is shown. Bootstrap values after 1000 replicates are expressed as percentages. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Laurembergia tetrandra* AP 33 was used as an outgroup.

ITS, and the chloroplast locus *trnH-psbA* demonstrate that *M. aquaticum* markedly differs from *M. rubricaule* sp. nov. (Fig. 2 and Supp. file 3). The *rbcL* locus is not suitable to distinguish between the species, little variation was observed even with the more distantly related *L. tetrandra* (Supp. file 4). Intriguingly, we observed that the concatenated loci *trnK3-matK* of *M. aquaticum* JL 8405 were identical to *M. rubricaule*. However, only two SNPs were observed in comparison with the other *M. aquaticum* specimens (Fig. 3). Furthermore, stark genetic differences between *M. aquaticum* JL 8405 and *M. rubricaule* were found in comparison with the other loci.

Moody & Les (2010) described two specimens, *M.* sp. 'red 1' and 'red 2', that were closely related to *M. aquaticum* and showed intriguing morphological similarities with the specimens of *M. rubricaule* sp. nov. described here. Based on the chloroplast loci, *M.* sp. 'red 2' is identical to *M. rubricaule*. However, ITS sequences of *M. rubricaule* are more similar to *M.* sp. 'red 1'. Based on these data, we cannot conclude whether either *M.* sp. 'red 1', *M.* sp. 'red 2' or both are *M. rubricaule*. The elucidation of additional DNA sequences of the other loci described herein for *M.* sp. 'red 1' and 2 may help in resolving this question. Interestingly, our comparison between chloroplast loci yielded results that deviated from the analyses of Moody & Les (2010). In our hands, *M. aquaticum* seems to be more closely related to *M.* sp. 'red 2' than to *M.* sp. 'red 1', where the former only had 2 SNPs compared to *M. aquaticum*, whereas the latter showed 10. Possibly, the ITS or chloroplast sequences of *M.* sp. 'red 1' and *M.* sp. 'red 2' were switched when they were deposited into GenBank. If we assume this to be the case either *M.* sp. 'red 1' or *M.* sp. 'red 2' is likely a *M. rubricaule* specimen.

In addition to species in trade of unknown origin as further mentioned by Thum *et al.* (2012) incorrect labeling of plants is widespread and may be caused by negligence or willful disrespect of regulations. What we have noticed so far with respect to species of *Myriophyllum* in trade in the Netherlands is that, in general, negligence of proper labeling causes confusion in the proper identity of the species in trade (Van Valkenburg & Boer 2015; Van Valkenburg *et al.* 2015). Concerning the new species described, the only mislabeling observed was *M. proserpinacoides* Gillies ex Hook. & Arn. (*Valkenburg 3314*) a name that was also used for *M. aquaticum* in trade.

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Appendix 1

Specimens of other species used in the present study.

Myriophyllum aquaticum

ARGENTINA • Estancia 'Santa Teresa', Dep. Mburucuyá, Prov. Corrientes; herbarium specimen; 7 Sep. 1956; fl; *T.M. Pedersen 3977*; T.M. Pedersen det.; L[L.2571172]; GenBank nos: OL827552 (trnK3), OL827550 (trnH-psbA), OL827548 (rbcL), OL827546 (matK), OL806573 (ITS); SRA: ERR7645488.

BRAZIL • Rio Grande do Sul, 16 km N da BR 290 km 380, NE de Rosario; herbarium specimen; 16 Oct. 1971; *J.C. Lindeman, B.E. Irgang & J.F.M. Valls 8405*; J.C. Lindeman det.; U[U.1329313]; GenBank nos: OL827551 (trnK3), OL827549 (trnH-psbA), OL827547 (rbcL), OL827545 (matK), OL806572 (ITS); SRA: ERR7645489.

NETHERLANDS • Eindhoven, Jan van Eijckgracht; 51°25.73′ N, 5°30.80′ E; herbarium specimen; 2 Jul. 2007; *H. Duistermaat 418*; H. Duistermaat det.; L[L 0768064]; GenBank nos: JX100595 (trnH-psbA), JX100747 (rbcL) • Wageningen, open air pond at Plant Protection Service; herbarium specimen; plants raised in pond, obtained as potplant (cultivated above ground) on 15 May 2007 from Wageningen, Garden Centre d'Oude Tol.; 8 Jun. 2007; fl; *J.L.C.H. van Valkenburg 3294*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453553]; GenBank nos: JX100592 (trnH-psbA), JX100749 (rbcL) • Emmer - Erfscheidenveen, Kanaal E, Middenweg ZW; 52°48.30′ N, 6°59.38′ E; herbarium specimen; 20 Jul. 2007; fl; *J.L.C.H. van Valkenburg 3329*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453554]; GenBank nos: JX100589 (trnH-psbA), JX100752 (rbcL) • Grashoek, Roomweg 85, 5985 NS, Golf course Kapelkeshof; 51°22.0′ N, 5°56.5′ E; herbarium specimen; 13 Jul. 2009; fl; *J.L.C.H. van Valkenburg 3494*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453552]; GenBank nos: MZ399144 (trnK3), MZ399136 (trnH-psbA), MZ399128 (rbcL), MZ399119 (matK), MZ401379 (ITS); SRA: ERR6000188 • Wageningen, Geertjesweg 15, Plant Protection Service; herbarium specimen; culta, first received 1 May 2009 from Stoffels Internationaal BV, Maalbekerweg 14, 5951 NT, Belfeld, the Netherlands; 11 Sep. 2009; fl; *J.L.C.H. van Valkenburg 3511*; J.L.C.H. van Valkenburg det.; L[L 0909322]; GenBank nos: JX100594 (trnH-psbA), JX100754 (rbcL).

FRANCE • Bretagne, Plouay; 47°55′ N, 3°20′ E; herbarium specimen; 25 Jul. 2007; *H. Duistermaat 426*; H. Duistermaat det.; L[L 0768057]; GenBank nos: JX100596 (trnH-psbA), JX100748 (rbcL).

Myriophyllum heterophyllum

NETHERLANDS • Wageningen, greenhouse Plant Protection Service; herbarium specimen; culta; 28 Jul. 2011; *J.L.C.H. van Valkenburg 3651*; J.L.C.H. van Valkenburg det.; WAGPD[WAG0453530]; GenBank nos: MZ399145 (trnK3), MZ399137 (trnH-psbA), MZ399129 (rbcL), MZ399120 (matK), MZ401373 (ITS); SRA: ERR6000189.

Laurembergia tetrandra

GABON • Ogooué-Maritime, Gamba, just W of the SHELL terminal; 2.47300° S, 10.00600° E; herbarium specimen; 10 Jan. 1998; *A.S.J. van Proosdij 33*; J.J. Wieringa det.; WAG[WAG.1550989]; GenBank nos: MZ399143 (trnK3), MZ399135 (trnH-psbA), MZ399127 (rbcL), MZ399118 (matK), MZ401374 (ITS); SRA: ERR6000187.

Supplementary material

Supp. **file 1**. SRA and GenBank accession numbers of sequences used in this study.
https://doi.org/10.5852/ejt.2022.828.1847.7211

Supp. **file 2**. Length of sequences in nucleotides that were used in this study.
https://doi.org/10.5852/ejt.2022.828.1847.7213

Supp. **file 3**. Maximum likelihood tree based on *trnH-psbA* sequences. The Tamura 3 parameter model was used (Tamura 1992). The tree with the highest log likelihood (-898.13) is shown. Bootstrap values after 1000 replicates are expressed as percentages. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Laurembergia tetrandra* AP 33 was used as an outgroup.
https://doi.org/10.5852/ejt.2022.828.1847.7215

Supp. **file 4**. Maximum likelihood tree based on *rbcL* sequences. This tree was inferred by using the Maximum Likelihood method and Jukes-Cantor model (Jukes & Cantor 1969).The tree with the highest log likelihood (-883.52) is shown. Bootstrap values after 1000 replicates are expressed as percentages. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Laurembergia tetrandra* AP 33 was used as an outgroup. https://doi.org/10.5852/ejt.2022.828.1847.7217