



NEW PHYLOGENETIC INSIGHTS INTO HYDROCHARITACEAE

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ABSTRACT – Hydrocharitaceae is an aquatic monocots family, it has enormous importance for the equilibrium among ecosystems and as biological resource. Previous phylogenetic studies, on the family, did not receive strong nodes support and clear congruent resolution. This study revises Hydrocharitaceae based on the most comprehensive dataset possible. The dataset includes all the genera accepted and it is composed by molecular characters retrieved by GeneBank resource. Furthermore, only congruent DNA regions were considered and the resulting combined matrix was analysed with three different phylogenetic methods originating congruent topologies. Consequently, a new Hydrocharitaceae phylogeny was advanced in resolving taxa nodes position with high confidence hence new genera combinations needed to be proposed. As result, a more reliable Hydrocharitaceae phylogeny would be essential in order to improve the evolutionary model assessment of this poor size family, which can allow a high quality taxa management. The importance of delivering reproducible research was also stressed as it enhances the value of any analysis (investigation) and makes possible delivering answers to different issues with higher confidence.

KEYWORDS: CLADE, CONSERVATION, GENE REGIONS, HYDROCHARITACEAE, MOLECULAR CHARACTERS, PHYLOGENY

INTRODUCTION

The cosmopolitan Hydrocharitaceae are composed of diverse aquatic monocots that are adapted to both freshwater and marine habitats. The family is ascribed to the Alismatales, an early-divergent order of monocots, and it comprises 16 genera and approximately 148 species (The Plant List, 2015). The genera *Enhalus*, *Halophila* and *Thalassia* are placed in a seagrass subclade (Tanaka et al., 1997; Les et al., 2006; Chen et al., 2012; Ross et al., 2015), but more recently, the genera *Vallisneria*, *Nechamandra* and *Najas* as well as the fossil *Thalassites* were also suggested to be derived from the same seagrass clade (Benzecry & Brack-Hanes, 2016). Despite the family being relatively small, great variability among its species has been observed. The taxa are morphologically character-

ised by a strong adaptation to aquatic habitats and show significant convergence and character reductions both of which can complicate taxonomic species circumscription. Only DNA characters are used to recover potential relationships between Hydrocharitaceae taxa, but at the same time, the application of morphological features of taxa is not suggested for phylogenetic purposes. Consequently, the family phylogenetic reconstruction, is still considered controversial and additionally, different phyletic results (Tanaka, 1997; Les et al., 2006; Chen et al., 2012; Les & Tippery, 2013; Ross et al., 2015) offer potential for improving the evolutionary question. Many species of Hydrocharitaceae are either threatened (e.g., *Ottelia acuminata*, many seagrasses and several species of both

Halophila and *Blyxa*) or invasive (e.g., *Hydrilla verticillata*, *Egeria densa*, *Elodea canadensis* and *Elodea nuttallii*). Decreasing in seagrass species (numerous Hydrocharitaceae taxa are placed in this subclade) is causing large concerns, as these species are essential for maintaining high diversity among several marine biological forms of life; these species are involved in coastal ecosystem equilibrium and also constitute an important general resource, especially in tropical regions. Intensive human activities (i.e. land use action, coastal construction, land demand, waste and water pollution) and global climate change are highlighted as the major problems that negatively affect population sizes, causing fragmentation and reduced species richness (Beck, 2011; Collins et al., 2017). Consequently, a robust phylogenetic reconstruction of Hydrocharitaceae would represent a fundamental stage in the understanding of the evolutionary role of Hydrocharitaceae in order to improve the taxa management of the family.

MATERIALS AND METHODS

Sampling

In this study, the ingroup sampling included all 16 Hydrocharitaceae genera and 51 species (The Plant List, 2015; Table 1). Five outgroups from Alismatales were selected as specified by Chen et al., (2012): *Alisma plantago-aquatica* (Alismataceae), *Hydrocleys nymphoides* (Alismataceae), *Cymodocea rotundata* (Cymodoceaceae), *Potamogeton* sp. (Potamogetonaceae) and *Butomus umbellatus* (Butomaceae). The DNA sequences were downloaded from the GenBank website (<https://www.ncbi.nlm.nih.gov/genbank/>; the sample accessions are provided in Supplementary Material S1). The sequences of each DNA region were subsequently aligned using the software MAFFT (Katoh et al., 2002) implemented in GeneiousPro (Kearse et al., 2012), applying the default settings, and verified manually in accordance with the guidelines of Kelchner (Kelchner, 2000). A combined matrix of six genes was assembled (nuclear: ITS, plastid: *matK*, *rbcL*, *rpoB*, *rpoC* and *trnK*), totalling 5428 characters and composing 56 taxa (Table 2, the aligned matrix at Dryad Digital Repository).

In the present study, all the genera were considered except *Caulinia* Wild. (19 species), which was reported to be an unsure genus by the same website (The Plant List, 2015). The genus *Caulinia* is mainly described as synonymous with *Kennedyia* and *Glycine*, both of which were placed as genera inside the Fabales (in the Tropicos website; <http://www.tropicos.org/>). This genus was recently confirmed and reported to be a subgenus of *Najas* (Ito et al., 2017).

Table 1. List of genera in Hydrocharitaceae, number species for each genus, following The Plant List 2015. Number of species sampled in this study for each genus.

Genus	Number of species	Number of species sampled
<i>Blyxa</i> Noronha ex Thouars	10	3
<i>Caulinia</i> Wild.	19	0
<i>Egeria</i> Planch.	3	2
<i>Elodea</i> Michx.	6	3
<i>Enhalus</i> Rich.	1	1
<i>Halophila</i> Thouars	20	4
<i>Hydrilla</i> Rich.	1	1
<i>Hydrocharis</i> L.	3	2
<i>Lagarosiphon</i> Harv.	9	3
<i>Limnobium</i> Rich.	2	2
<i>Najas</i> L.	40	11
<i>Nechamandra</i> Planch.	1	1
<i>Ottelia</i> Pers.	17	5
<i>Stratiotes</i> L.	1	1
<i>Thalassia</i> Banks & Sol.	2	2
<i>Vallisneria</i> L.	13	10
Total	148	51

Analysis

The maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP) methods were used to develop evolutionary hypotheses of the family. The ML analysis was performed using RAXML v.8 (Stamatakis, 2014) through CIPRES portal (http://www.phylo.org/sub_sections/portal/). ML test was applied to the combined matrix with the GTRCAT model using a separate partition for each gene. The analysis was run for 1000 rapid bootstraps. BI (Huelsenbeck & Ronquist, 2001) was performed as implemented in MrBayes v. 3.2.6 (Ronquist et al., 2012) on CIPRES portal (http://www.phylo.org/sub_sections/portal/). The best-fitting nucleotide substitution model was estimated for each singular DNA region of the combined matrix using Modeltest v. 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004), under the Akaike information criterion (Akaike, 1973; Table 2). In order to approximate the posterior probability distribution, four independent Markov Chain Monte Carlo (MCMC) were run twice for 10 million generations and sampled every 1000th generations with 25% of the ‘burn-in’ discarded. Convergence between the runs and the length of the burn-in fraction were verified using the ‘‘sump’’ command in MrBayes and Tracer v.1.5 (Rambaut & Drummond, 2009).

Table 2. Statistic values of each gene selected.

	ITS	matk	rbcl	rpoB	rpoC	trnK	Combined matrix
Alignment Length	973	1432	1182	455	471	915	5428
C	267	713	850	274	302	429	2835
C(%)	27.44	49.79	71.91	60.22	64.12	46.89	52.23
V	706	719	332	181	169	486	2593
V(%)	72.56	50.21	28.09	39.78	35.88	53.11	47.77
Pi	585	528	233	129	106	303	1884
Pi(%)	60.12	36.87	19.71	28.35	22.51	33.11	34.71
AIC model	GTR+I+G	GTR+G	GTR+I+G	GTR+G	GTR+G	GTR+G	NA

NA= none, C= constant characters, V= variable characters, Pi=parsimony informative, GTR= generalised time reversible, rate variation among site: I= invariable site, G= gamma distribution.

Posterior distribution of trees was summarised using the halfcompat consensus tree from MrBayes. The consensus tree and Posterior Probability (PP) values were visualized with FigTree v.1.4 (Rambaut, 2012).

MP analysis criterion was applied using PAUP* v. 4.0b10 (Swofford, 2003). Each character was considered to have equal weight and considered unordered and independent. The most parsimonious tree with Bootstrap Support was obtained by establishing an heuristic search of 1000 replicates of random stepwise sequence addition, applying tree bisection reconnection (TBR) branch swapping and saving 10 trees per replication. The BS was evaluated using a simple sequence addition with TBR branch swapping and 1000 runs, saving 10 trees per replication (See Supplementary Material S2). In this study, node support was retrieved using the BS values in both the MP and ML analyses and also using the PP in the BI analysis. The BS values ranging from 85% to 100% were considered highly supported, a range between 75% and 84% was considered moderately confident and values ranging from 60% to 74% were considered low supported. At the same time, BS values <60% were not reported because judged not reliable. The PP support was considerably strong when it ranged from 0.95–1, moderate credibility was from 0.9 till 0.95 and low when it ranged from 0.8–0.9. PP values <0.8 were not showed.

Each DNA partition (ITS, *matK*, *rbcl*, *rpoB*, *rpoC* and *trnK*) was initially evaluated independently by examining every single tree topology that was recovered by ML analyses using RAXML v. 8 (Stamatakis, 2014). Moreover, to confirm the congruence among the regions used in this matrix, the incongruence length difference (ILD) test (Ferris et al., 1994) was run in PAUP* v.4.0b10 (Swofford, 2003) with the following settings: random stepwise sequence addition, TBR branch swapping and 100 replicates. The null hypothesis of congruence was discarded with a threshold of $p < 0.01$ (Li et al., 2015).

RESULTS

The ILD test results revealed no incongruence among the genes used in the combined matrix, as all the p-values were larger than 0.01 (all $p > 0.01$). Moreover, the comparison among tree topologies confirmed no conflict was present between the used genes (BS < 70%; see Supplementary Material S3).

Each of the three analyses (MP, ML and BI) resulted in highly congruent tree topologies (Fig. 1) that were well resolved and had mostly strong node support.

The PP and the BS values (from both the MP and ML analyses) were reported for the nodes on the final phylogenetic tree. The genera *Vallisneria* and *Nechamandra* were confirmed to be sister related with maximum probability (PP = 1, ML BS = 100% and MP BS 100%). The group formed by the genera *Vallisneria* and *Nechamandra* was hypothesised to be a sister of the *Hydrilla* genus (PP = 1, ML BS = 100% and MP BS = 99%). The clade comprising the genera *Halophila*, *Thalassia* and *Enhalus* (seagrasses group) was still recovered with maximum support (PP = 1, ML BS = 100% and MP BS = 100%). This clade consisting of seagrasses was suggested to be sister to the last one (*Vallisneria*, *Nechamandra* and *Hydrilla*) with a support of PP = 1, ML BS = 98% and MP BS = 66%. The *Najas* genus was still hypothesised to be monophyletic (PP = 1, ML BS = 100% and MP BS = 100%) but was suggested to be sister of the previously proposed clades (*Vallisneria*, *Nechamandra*, *Hydrilla*, *Halophila*, *Thalassia* and *Enhalus*). This last large group (*Vallisneria*, *Nechamandra*, *Hydrilla*, *Halophila*, *Thalassia*, *Enhalus* and *Najas*) was hypothesised with a probability of PP = 1, ML BS = 100% and MP BS = 62%. *Limnobium* and *Hydrocharis* were confirmed to be closely related (PP = 1, ML BS = 100%, PM BS = 100%). In addition, these genera (*Limnobium* and *Hydrocharis*) were suggested to be phylogenetically closely correlated with the larger

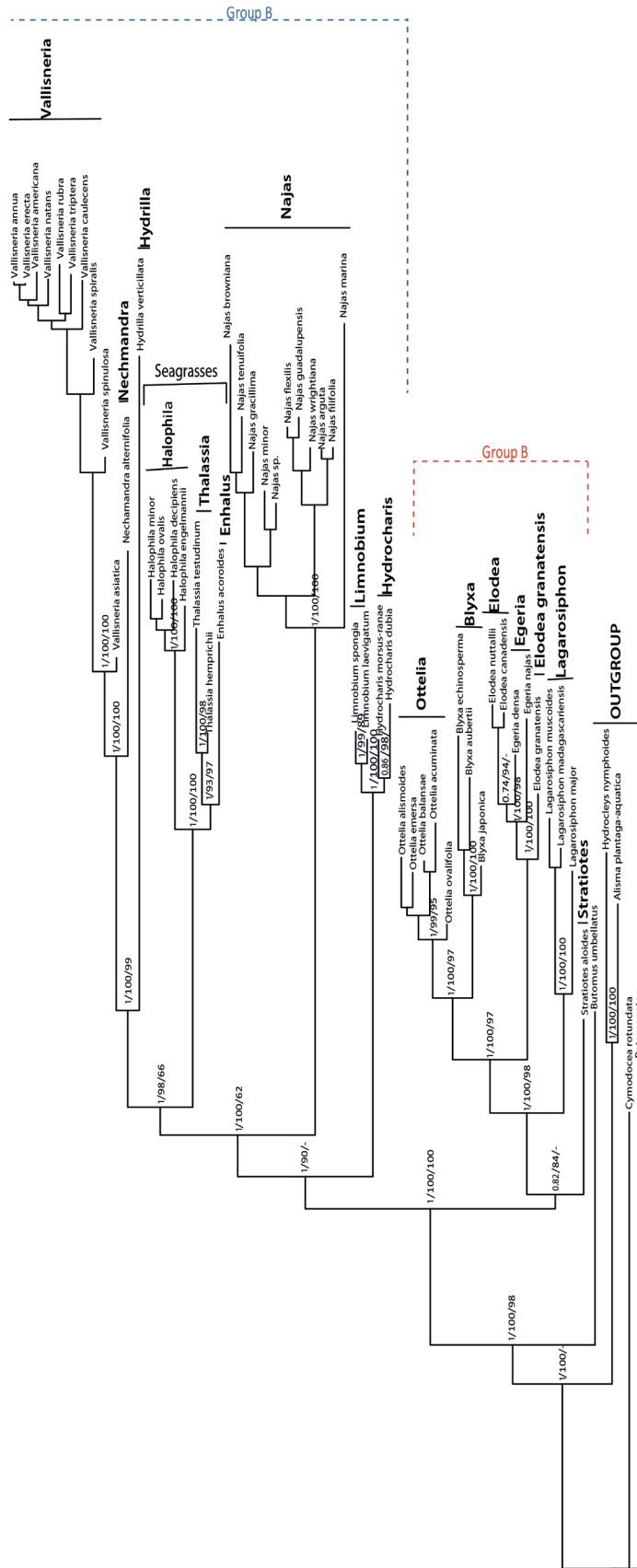


Figure 1. Bayesian 50% majority-rule consensus tree with supports. Bayesian Posterior Probability (PP) value on the left, Bootstrap Support confidence (with Maximum Likelihood analysis) in the middle, Bootstrap Support values (with Maximum Parsimony analysis) on the right. Bootstrap Support <60 are indicated by '-'. Scale bar: 0.04 substitution per site. Clade names are reported. Underlined Group A and Group B.

clade (*Vallisneria*, *Nechamandra*, *Hydrilla*, *Halophila*, *Thalassia*, *Enhalus* and *Najas*) with a PP = 1, ML BS = 90% and MP BS < 60%, attesting to the existence of the group A. In addition, a B group (comprising the clades *Ottelia*, *Blyxa*, *Egeria*, *Elodea* and *Lagarosiphon*) was also validated (PP = 0.82, ML BS = 84%, MP BS = 90%), and a close relationship existed between group A and group B (PP = 1, ML BS = 100%, MP BS = 100%). The clades hypothesised to exist within group B and their relationships were corroborated with maximum confidence (PP and BS values). Moreover, in this study, the phylogenetic tree did not recover the monophyly of *Elodea* and *Egeria*. However, *Egeria* and *Elodea* taxa were proposed to form a clade (PP = 1, ML BS = 100%, MP BS = 100%). *Stratiotes* was placed as sister clade only of all the genera in group B (PP = 0.82, ML BS = 84%, MP BS < 60%).

DISCUSSION

Hydrocharitaceae are characterised by reduced floral and vegetative structures, similar to other aquatic taxa, which results in a sensitive convergence of the forms and high homologies of character states (Sculthorpe, 1967; Les et al., 2006). Many similar studies have indicated strong incongruence between morphological and molecular data in this family (Les & Haynes, 1995; Tanaka et al., 1997; Tanaka et al., 2004; Les et al., 2006; Xiaoxian & Zhekun, 2009), likely due to the high variability of morphological and anatomical traits. Consequently, the complex features of this family do not easily allow the use of these characters either for phylogenetic proposes or for clearly describing the same species.

However, the effective species number of Hydrocharitaceae is still largely unclear, and different resources have reported different numbers of species (e.g., The Plant List, 2015, 2017; Christenhusz & Byng, 2016; WCPS, 2017; <http://delta-intkey.com/angio/www/hydrocha.htm>). The current data matrix presents a remarkably complete molecular character sampling, and the resulting phylogenetic tree exhibits good resolution and high support at the nodes recovered. Various results corroborate those of previous studies (Chen et al., 2012; Les & Tippery, 2013; Ross et al., 2015), and important differences are indicated regarding the evolutionary relationships between the Hydrocharitaceae taxa.

Six species of *Elodea* were recognised (The Plant List, 2015). However, only three species were analysed in the present matrix (*E. nuttallii*, *E. canadensis* and *E. granatensis*). In addition, two species of *Egeria* (*E. densa* and *E. najas*) were investigated out of three total (The Plant List, 2015). The output confirmed the lack of monophyly of the *Elodea*

group, which supports the necessity to restore the species name *Apalanthe granatensis* (Humb. & Bonpl.) Planch; however, this name is currently used as a synonym of *Elodea granatensis* Humb. & Bonpl. (The Plant List, 2015, 2017). Moreover, the results also suggested an uncertain monophyly for the *Egeria* taxa, despite the species of these two genera composing a strongly supported clade. As a result, including all of these taxa (*Elodea*, *Egeria* and *Apalanthe*) into one single new genus would be an interesting alternative.

These three genera are often described as morphologically very similar (Cook, 1985). St. John (St. John, 1962, 1965) proposed that *Apalanthe* should be considered a subgenus of *Elodea*. At the same time, Hauman-Merck (Hauman-Merck, 1912, 1915) combined the three genera (*Apalanthe*, *Egeria* and *Elodea*) because of their pollination system.

The genus *Hydrocharis* is characterised as having three species (The Plant List, 2015), but in this study, only two species (*Hydrocharis morsus-ranae* and *H. dubia*) were used, meanwhile the genus *Limnobium* was sampled completely (*L. spongia* and *L. laevigatum*).

The results confirm the genera *Limnobium* and *Hydrocharis*; this last clade was also not properly recovered in the MP analysis and received a weak PP value. However, all the *Limnobium* and *Hydrocharis* species were included in a higher clade detected with maximum credibility (PP=1, BS= 100 in both the ML and MP). Therefore, the *Limnobium* and *Hydrocharis* species could be combined into a single genus, which supports the previous proposal to merge these taxa on the basis of their very similar morphologies (Richard, 1812; Cook & Urmi-Konig, 1983a; Shaffer-Feher, 1991a, 1991b; Les et al., 2006).

The monotypic genus *Stratiotes* presents a complex lifecycle. The plants of this genus spend most of their vegetative time submerged but ascend to the surface of the water during their reproductive phase. Les (Les et al., 2006) recommended inserting *Stratiotes* into a new subfamily to reflect its particular morphological state within Hydrocharitaceae (Cook & Urmi-Konig, 1983b). Habitat loss is causing a dangerous reduction of this genus, which is mostly recognised for its important role in preserving ecosystem biodiversity (Suutari et al., 2009; Katzenberger & Zacharias, 2015).

The phylogenetic results showed that *Stratiotes* was not sister of the whole family as reported by Chen et al., (2012) and Ross et al., (2015) but only a sister group of the B clade, which was similar to that proposed by Tanaka (Tanaka et al., 1997). This genus therefore requires more study.

The position of *Najas* within the order Alismatales has been considered critical for a long time.

Najas was reported to be closely associated with *Potamogeton* (Sculthorpe, 1967). Moreover, *Najas* was placed in Zannichelliaceae by Singha (Singha, 1965) and Rendel (Rendel, 1901), who also noted a close morphological similarity between *Najas* and some Hydrocharitaceae genera

including *Elodea*, *Hydrilla* and *Lagarosiphon*. In addition, Miki (Miki, 1937) confirmed a close relationship between *Najas* and the same Hydrocharitaceae based on their features. Nevertheless, it was concluded that *Najas* was not derived from this family, and the genus was not considered to be within Potamogetonaceae. More recently, Les et al. (2006) supported the inclusion of *Najas* within Hydrocharitaceae on the basis of molecular and morphological data, despite moderate credibility values. The insertion of *Najas* was confirmed with high resolution only when the ML analysis was applied to molecular plastid characters (*matK* and *rbcL*); at the same time, the phylogenetic tree obtained using the nuclear gene (ITS) did not present strong support at the same nodes (Les et al., 2006). Moreover, the ambiguous genus was characterised by many long branches, indicating that the phylogenetic relationships could be influenced by a possible branch attraction effect.

Several recent studies (Les et al., 1993; Moody & Les, 2007; Les et al., 2010; Les et al., 2015) have described the occurrence of interspecific hybridisation in *Najas*. Often, the hybrid species do not present any distinct morphological evidence, and their morphology falls within the range of their parental species, unless those plants specifically are collected and analysed genetically (Moody & Les, 2002, 2007). As a result, the strong morphological analogy between hybrids and their maternal parents in Hydrocharitaceae remained unknown for a long time (Les et al., 2010). Hybridisation has been commonly described in many hydrophytic genera (Moody & Les, 2002; Les et al., 2004; Les et al., 2005; Les et al., 2009), which are also characterised by a large diversity of phenotypic traits (Sculthorpe, 1967). As a result, the morphological data of *Najas* were adjusted and completed on the basis of the hybrid populations. More generally, the hybrids of aquatic plants were hypothesised to be characterised by sequencing artefacts (Moody & Les, 2002; Les et al., 2004; Les et al., 2005; Les et al., 2009) which might have affected the phylogenetic conclusion. This phenomenon could justify the reason of many different phylogenetic results presented by several types of research. Furthermore, the current evolutionary hypothesis confirmed *Najas* within the family, but this genus was placed in a different position than that proposed by Chen (Chen et al., 2012). Likewise, a similar resolution was reported in several studies on Alismatales (Petersen et al., 2015; Ross et al., 2015) with low resolution and credibility.

However, Ito (Ito et al., 2017) suggested the need to re-evaluate the phylogenetic inferences reported by Chen (Chen et al., 2012).

Li (Li et al., 2015) emphasised the evidence that taxa hybridisation could also be provided through topological incongruence between phylogenies based on nuclear and plastid matrices. Since the plastid material is inherited maternally, the nuclear material is biparental.

As a result, the importance of simultaneously using congruent genes in order to reduce eventual hybridisation noise to obtain a potentially stronger phylogenetic signal should be highlighted (Zhang et al., 2015).

However, in the present work, it was possible to assemble the most comprehensive dataset of the Hydrocharitaceae family, using public records submitted by different researchers whose goals varied. Consequentially, the taxa available were arranged in a new combined matrix, adding new species and filling the empty DNA regions of the same taxa used in the most recent Hydrocharitaceae datasets (Chen et al., 2012). Therefore the combination of all these parameters, such as compiling a more complete dataset and applying more appropriated new analyses, might have been responsible for a considerable phylogenetic resolution and its higher node support.

CONCLUSIONS

The Hydrocharitaceae phylogenetic tree was recovered with high resolution and credibility.

The position of *Najas* was resolved with strong support as sister to the clade comprising *Vallisneria*, *Nechamandra* and *Hydrilla* as well as the seagrasses. However, Chen (Chen et al., 2012) proposed the seagrasses clade like sister group of *Najas* and the same group composed by *Vallisneria*, *Nechamandra* and *Hydrilla*, which was recovered with very low support.

The new position hypothesised for the monotypic genus *Stratiotes* may require additional analysis.

Elodea and *Egeria* were confirmed to be not monophyletic; consequently, new clades were suggested. Moreover, *Hydrocharis* and *Limnobium* species could be incorporated into just one larger genus.

In this review, it was possible to test and select the most appropriate genes among the DNA regions previously used in different studies to generate a new matrix unaffected by incongruence, which could influence phylogenetic results, especially with the presence of hybrid taxa.

Furthermore, the importance of a reproducible research has to be highlighted, which implies the necessity of storing new data in open public domains for facilitate the reuse. In addition, the production of 'reproducible research' is important with respect to checking the reliability of the whole research process, and reproducibility can specifically provide a useful jumping-off points for future research questions (<https://ropensci.org/blog/2014/06/09/reproducibility>), which can be achieved by ameliorating the pre-existing dataset with new inputs as well by investigating the data matrix using the newest analysis.

In the present study, major steps (e.g. the dataset was implemented and different new analysis were applied) were taken in order

to improve and clarify the phylogenetic relationships among Hydrocharitaceae genera. Various nodes of the tree were confirmed (but their confidence values increased), and new nodes were hypothesised with high support, placing and solving critical taxa within the family. In conclusion, distinct studies, based on different data types and analysis, were used to perform numerous phylogenetic reconstructions. In fact, by assembling all the results together, a clear and reliable phylogeny can be restored and used to solve additional important questions on the historical evolution of Hydrocharitaceae and to improve the conservation policies of this family.

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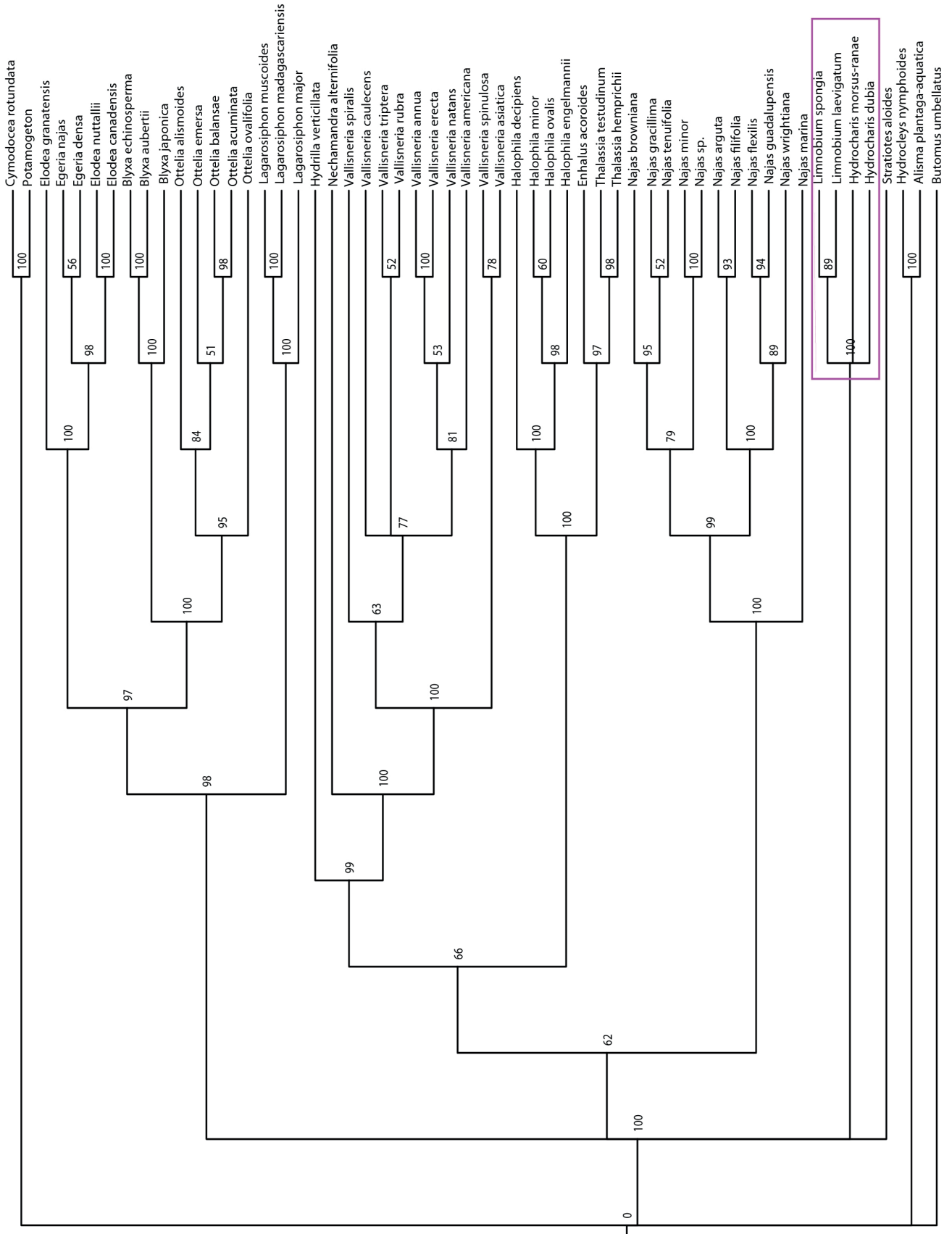
SUPPLEMENTARY MATERIAL

S1: List of taxa used in this study, GenBank accession numbers.

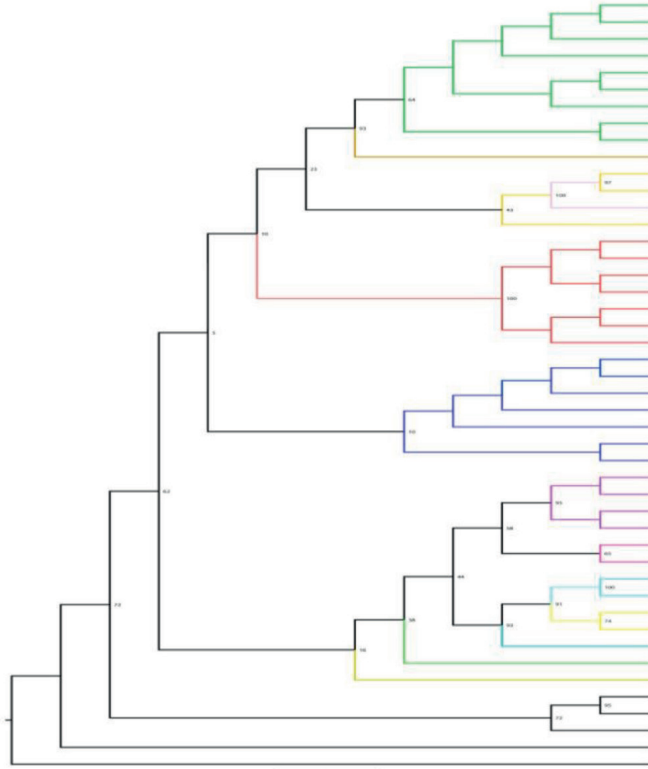
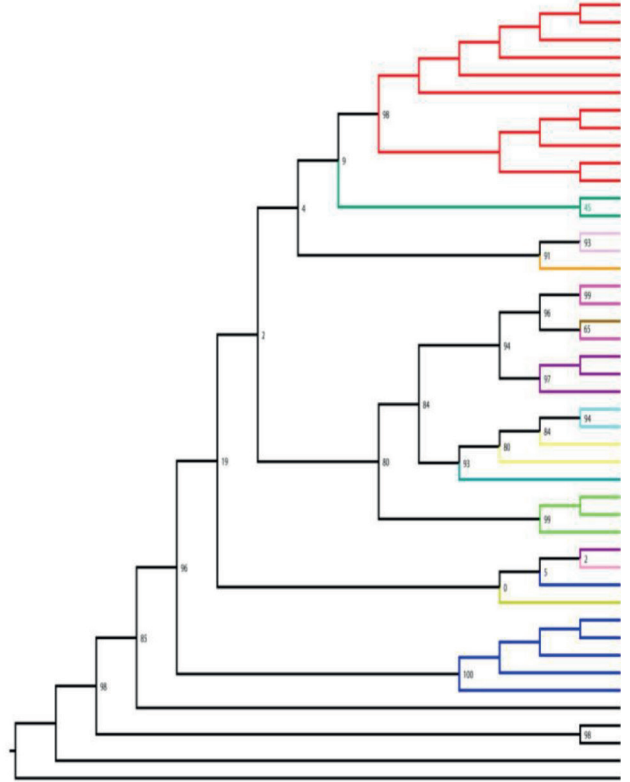
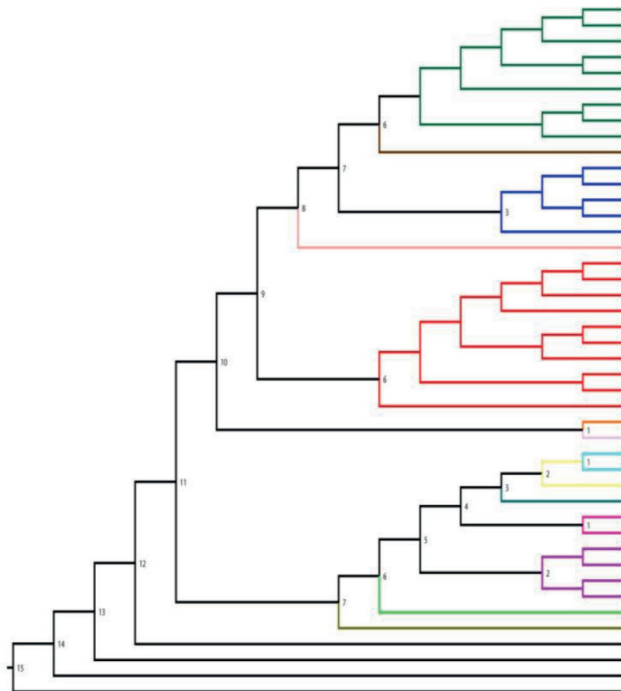
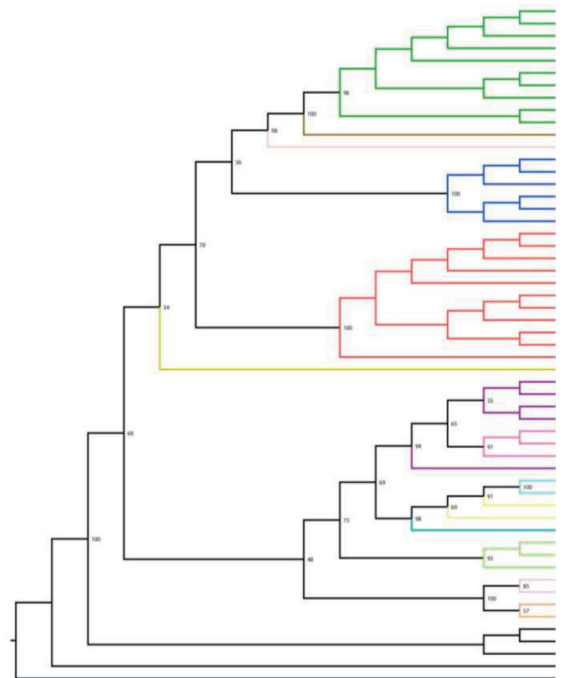
TAXA	ITS	matK	rbcL	rpoB	rpoC	trnK
<i>Alisma plantaga-aquatica</i> L.	0	AF542573	L08759	JF781089	JF781104	JF781025
<i>Blyxa aubertii</i> Rich.	AY870359	KF632789	U80694	0	0	AY870384
<i>Blyxa echinosperma</i> (C.B.Clarke) Hook.f.	JN578091	AB088781	AB088810	JF975513	JF975531	JF975509
<i>Blyxa japonica</i> (Miq.) Maxim.ex Asch. & Gürke	0	AB002566	AB004886	0	0	0
<i>Butomus umbellatus</i> L.	AY870346	DQ401367	AY149345	JF781091	JF781106	JF781027
<i>Cymodocea rotundata</i> Asch. & Schweinf.	KM609948	JQ031760	JQ031763	JF781091	JF781106	JQ031764
<i>Egeria densa</i> Planch.	JF703260	AB002567	AB004887	JF975514	JF975532	AY870385
<i>Egeria najas</i> Planch.	AY330708/	KF632795	DQ859166	0	0	0
<i>Elodea canadensis</i> Michx.	HQ456422	KF632796	DQ859167	JF975515	JF975533.	JF975505
<i>Elodea granatensis</i> Humb. & Bonpl.	AY870362	AY870367	U80693	0	0	AY870387
<i>Elodea nuttallii</i> (Planch.) H.St.John	AY330706.1	AB002568	AB004888	0	0	AY870386
<i>Enhalus acoroides</i> (L.f.) Royle	AY870347	AB002569	U80697	JF975516	JF975534	AY870372
<i>Halophila decipiens</i> Ostenf.	AF395673	JX457605	0	0	0	0
<i>Halophila engelmannii</i> Asch.	AY870349	0	U80699	0	0	AY870374
<i>Halophila minor</i> (Zoll.) Hartog	AF366406	JN225367.	JN225347	0	0	0
<i>Halophila ovalis</i> (R.Br.) Hook.f.	KM609942	AB002570	AB004890	JF975517	JF975535	JN003598
<i>Hydrilla verticillata</i> (L.f.) Royle	JF703269	AB002571	U80700	JF975518	JF975536	AY870378
<i>Hydrocharis dubia</i> (Blume) Backer	HQ687166	AB002572	JN578090	JF975519	JF975537	0
<i>Hydrocharis morsus-ranae</i> L.	AY870350	0	U80701	0	0	AY870375
<i>Hydrocleys nymphoides</i> (Humb. & Bonpl. ex Willd.) Buchenau	JF780985	AB002580	U80716	JF781096	JF781111	0
<i>Lagarosiphon madagascariensis</i> Casp.	0	AB002573	AB004893	0	0	0
<i>Lagarosiphon muscoides</i> Harv.	AY870363	AY870368	U80702	0	0	AY870388
<i>Lagarosiphon major</i> Moss	0	KF632803	KF632851	0	0	0
<i>Limnobium laevigatum</i> Humb. & Bonpl. ex Willd.) Heine	0	AB002574	AB004894	0	0	0
<i>Limnobium spongia</i> (Bosc) Steud.	AY870351	HQ456471	U80704	0	0	AY870376
<i>Najas arguta</i> Kunth	HM240420	HM240458	HM240485	0	0	0
<i>Najas browniana</i> Rendl.	HM240421	HM240459	HM240486	0	0	HM240459,
<i>Najas filifolia</i> R.R.Haynes	KM501786	KM501958	KM502150	0	KM373908.	KM501958
<i>Najas flexilis</i> (Willd.) Rostk. & W.L.E.Schmidt	HM240425	HM240463	HM240489.	0	0	HM240463

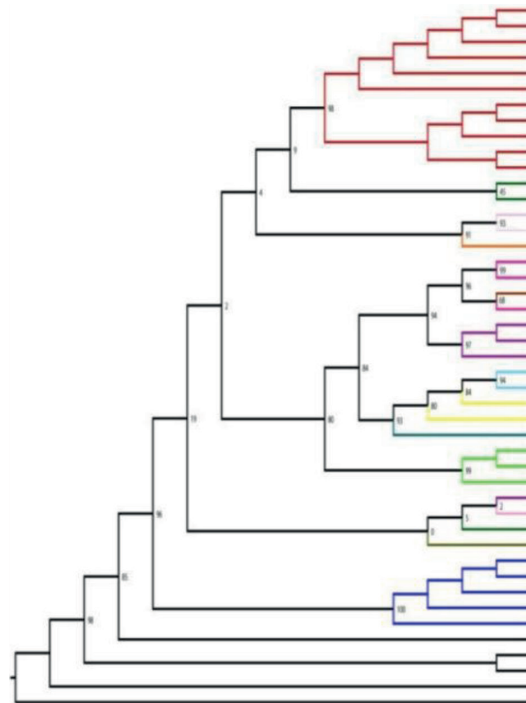
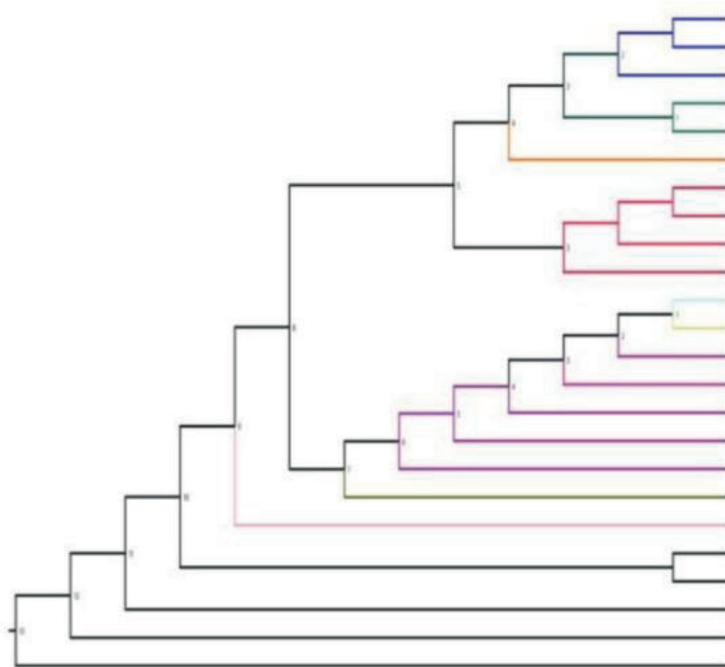
TAXA	ITS	matK	rbcL	rpoB	rpoC	trnK
<i>Najas gracillima</i> (A.Braun ex Engelm.) Magnus	HM240428	HM240464	HM240490	0	0	HM240464
<i>Najas guadalupensis</i> (Spreng.) Magnus	HM240433	HM240467	HM240493	0	0	HM240467
<i>Najas</i> sp.	0	KF632809	HM240509	JF975526.1	JF975540.1	HM240483
<i>Najas marina</i> L.	0	JN003597	U80705	JF975525	JF975539	AY870377
<i>Najas minor</i> All.	0	AB002579	AB004899	JF975524	JF975538	JF975512
<i>Najas tenuifolia</i> R.Br.	HM240451	HM240481	HM240507	0	0	HM240481
<i>Najas wrightiana</i> A.Braun	0	0	KM502157	0	0	KM501965
<i>Nechamandra alternifolia</i> (Roxb. ex Wight) Thwaites	AY870356.1	KF632810	U80706	0	0	AY870381
<i>Ottelia acuminata</i> (Gagnep.) Dandy	KP676565.1	0	AY952435	JF975521	JF975542	JF975507
TAXA	ITS	matK	rbcL	rpoB	rpoC	trnK
<i>Ottelia emersa</i> Z.C.Zhao & R.L.Luo	KP676566	JF975500	JF975498	JF975522	JF975543	JF975511
<i>Ottelia ovalifolia</i> (R.Br.) Rich.	0	KF632811	DQ859171	0	0	0
<i>Ottelia balansae</i> (Gagnep.) Dandy	JF975447	JF975501	JF975496	JF975523	JF975544	JF975508
<i>Potamogeton</i> sp.	HQ263548	AB088780	DQ859173	AB559936	JN034090	GQ247501
<i>Stratiotes aloides</i> L.	AY870357	AB002576	U80709	JF975527	JF975545	AY870382
<i>Thalassia hemprichii</i> (Ehrenb. ex Solms) Asch.	0	AB002577	U80710	JF975528	JF975546	JF975510
<i>Thalassia testudinum</i> Banks & Sol.	AY870348	0	U80711	0	0	AY870373
<i>Vallisneria americana</i> Michx.	EF142991	AY870366	U03726	0	0	AY870380
<i>Vallisneria asiatica</i> Miki	0	AY957575	EF155532	JF975529	JF975547	JF975503
<i>Vallisneria natans</i> (Lour.) H.Hara	EF142988.1	0	JF975497	JF975530	JF975548	JF975504
<i>Vallisneria annua</i> S.W.L.Jacobs & K.A.Frank	EF142962	0	EF143006	0	0	EF143028
<i>Vallisneria caulecens</i> F.M.Bailey F.Muell.	EF142975	0	EF143009	0	0	EF143039
<i>Vallisneria erecta</i> S.W.L. Jacobs	EF142980	0	EF143011	0	0	EF143044
<i>Vallisneria rubra</i> (Rendl) Les & S.W.L.Jacobs	AY870354	AY8703651	AY870370	0	0	AY870379
<i>Vallisneria spinulosa</i> S.Z. Yan	EF142993	0	EF143017	0	0	EF143056
<i>Vallisneria spiralis</i> L.	EF694962	0	EF694962	0	0	EF143066
<i>Vallisneria triptera</i> S.W.L.Jacobs & K.A.Frank	EF143003	0	EF143019	0	0	0

S2: Hydrocharitaceae MP Phylogenetic tree with Bootstrap Support (in MP analysis) at nodes. Pink square underlines the genera *Limnobium* and *Hydrocharis*.



S 3: Congruence among the gene used. For each genus a ML tree with bootstrap support at nodes for high taxonomical groups.

ITS gene**matK gene****trnK gene****rbcL gene**

***rpoC* gene****Legend colors**

Vallisneria = dark green
Nechamandra = brown

Hydrilla = light pink
Najas = red
 seagrasses = blue
Hydrocharis = orange

Limnobium = light violet
Ottelia = purple
Blyxa = fuchsia,
Elodea = light blue,

Egeria = yellow
Apalanthe = green water
Lagarosiphon = light green
Stratioites = mustard color