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Evolution of the aquatic habit in *Ludwigia* (Onagraceae): Morpho-anatomical adaptive strategies in the Neotropics

Ana M. Bedoya*, Santiago Madriñán

Laboratorio de Botánica y Sistemática, Departamento de Ciencias Biológicas, Universidad de los Andes, KR 1 18A-12 Bogotá, Colombia

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ABSTRACT

Ludwigia displays a great variety of life forms adapted to aquatic habitats ranging from humid to water saturated. Morpho-anatomical characters were used to characterize and discriminate among selected Neotropical species of *Ludwigia* in terms of their root and shoot adaptations. A partial molecular phylogenetic hypothesis was reconstructed using the nrDNA ITS and cpDNA *rpl32–trnL*^{UAG} regions for Neotropical species. ITS accessions of North temperate species were also included to provide a framework for the placement of our sampled taxa. Mapping of morpho-anatomical characters was performed on the resulting phylogenetic tree. We were able to separate the species in three groups according to their morpho-anatomical adaptations to aquatic habitats. Our results strengthen the support for at least two separate origins for the strictly aquatic habit in Neotropical representatives, in line with previous taxonomic classifications. We also suggest that these species experienced convergence of characters, but also diversified into various life forms adequate to live strictly in aquatic environments. We would like to highlight the great value of *Ludwigia* as a model system for the study of the evolution of characters that respond to pressures imposed by aquatic ecosystems within the dicotyledons, as well as within aquatic plants in general.

1. Introduction

Plants that have recolonized the aquatic environment face numerous constraints including an inadequate supply of oxygen, restrictions to photosynthesis, and phytotoxin accumulation (Jackson, 1985; Ernst, 1990; Armstrong et al., 1991; Jackson and Colmer, 2005). To overcome such constraints, aquatic plants possess a well-expressed ability to alter morphological, anatomical and physiological functional traits (Niklas, 2009).

Morpho-anatomical adaptations to aquatic conditions appear to have evolved in line with the acquisition of herbaceous habits, and the extent to which they are expressed depends on the moisture conditions to which the plants are exposed (Arber, 1920; Rial, 2006; Scremin-Dias et al., 2011). These adaptations include, among others, the presence of aerenchyma, reduction of lignification, and the appearance of a wide variety of growth forms that range from rooted-emergent shrubs to submersed or floating herbs. These adaptations enable plants to facilitate gas exchange and transport throughout the plant body, reduce the amount of tissue requiring oxygen, promote radial oxygen loss to reduce phytotoxin accumulation, withstand water currents, take advantage of water as an external support mechanism, and enhance photosynthesis (Justin and Armstrong, 1987; Ernst, 1990; Jackson and Armstrong, 1999; Somavilla and Graciano-Ribeiro, 2012).

The aquatic angiosperm species, which make up approximately 2% of the clade, are phylogenetically scattered and include proportionally more representatives in the monocotyledons than in the dicotyledons (Cook, 1999). Within the dicotyledons, some of the families that include aquatic representatives are predominantly herbaceous including the Menyanthaceae, Nymphaeaceae, Podostemaceae and Ranunculaceae, which modify primary tissues to adapt to water life (Rutishauser, 1997; Seago et al., 2000; Borsch et al., 2008; Richards et al., 2010; Seago and Fernando, 2013). However, others tend to be more woody, such as aquatic members of the Fabaceae, Lythraceae, Melastomataceae and Onagraceae, which in addition to the alteration of primary tissues, have modified tissues produced after secondary growth and lignification (Angeles, 1992; Stevens et al., 2002; Shimamura et al., 2010; Somavilla and Graciano-Ribeiro, 2012).

Within the Onagraceae, *Ludwigia* L. are herbs or rarely shrubs whose aquatic habit is reflected in their association with humid soils, the presence of primary and secondary aerenchyma, and possession of the lowest degree of vessel grouping in the family

* Corresponding author. Tel.: +57 1 3394949x2745; fax: +57 3394448.
E-mail addresses: am.bedoya250@uniandes.edu.co (A.M. Bedoya), samadrin@uniandes.edu.co (S. Madriñán).

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(Schenck, 1889; Carlquist, 1987; Angeles, 1992, Wagner et al., 2007). They include a variety of growth forms adapted to live in habitats that range from humid to water saturated, and from seasonally to permanently flooded. *Ludwigia* is a fairly well-studied genus with a large amount of research regarding its cytology, palynology, systematics, morphology, reproductive features, wood and foliar anatomy, and molecular data that support its monophyly (Keating, 1982; Zardini and Raven, 1992; Conti et al., 1993; Hoch et al., 1993; Levin et al., 2003, 2004; Ford and Gottlieb, 2007).

Given the prior knowledge of *Ludwigia*, its strong association to aquatic habitats, and the diversity of forms it displays in such places, we selected it as the model system to describe herbaceous and woody adaptations to aquatic life. Specifically, we aimed to characterize and discriminate among selected woody and herbaceous aquatic species in terms of the morpho-anatomical adaptations of their roots and shoots, and to provide preliminary insight into the evolution of these adaptations. To do so, we generated quantitative and qualitative measurements of morpho-anatomical characters and used them to statistically determine the similarity among species. Finally, we constructed a molecular phylogenetic hypothesis including the species at hand, and used it to map selected morpho-anatomical characters considered to be adaptations to the aquatic habit.

2. Materials and methods

2.1. Study sites

Plants of *Ludwigia* were collected throughout the Orinoco basin and in Bogotá, Colombia (Fig. 1). Sampling effort was distributed over a variety of aquatic environments, under varying degrees of flooding stress. The principal habitats included flooded savannas, *Mauritia* palm swamps, streams, rivers, wetlands and lakes.

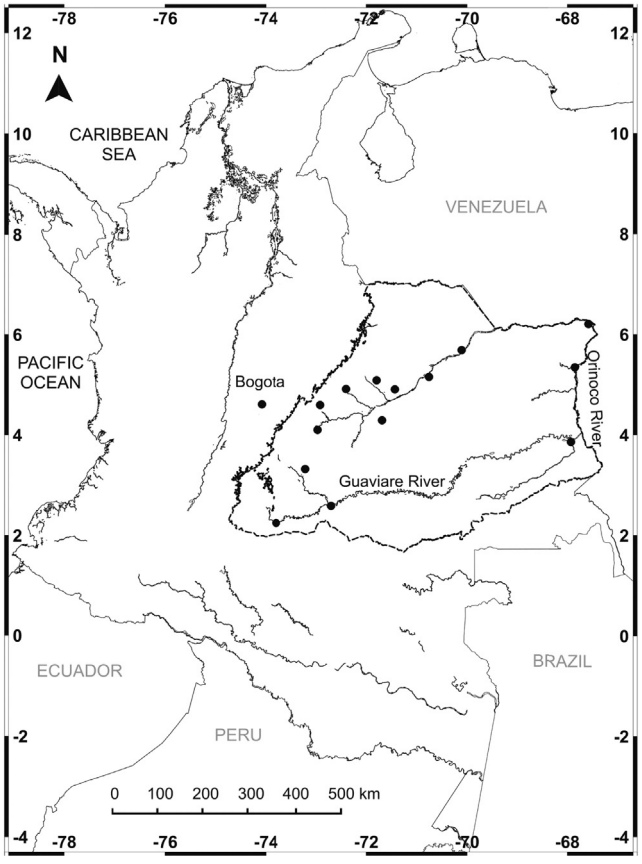

Fig. 1. Map of Colombia and adjacent countries with major watersheds, illustrating the Orinoco basin of Colombia (dashed line), and the localities where *Ludwigia* specimens were sampled (points). Each locality included various aquatic habitats.

2.2. Taxon sampling

Ten species of Neotropical *Ludwigia* were sampled, representing seven of the ten recognized sections that have Neotropical representatives and approximately 20% of the species diversity in the region. Silica-dried leaf material, dried specimens and liquid-preserved material were deposited at the ANDES herbarium, Universidad de los Andes, Bogotá, Colombia. Up to three individuals per species were included for both morpho-anatomical characterization and DNA extraction. To provide a framework for the placement of the Neotropical species in the phylogenetic tree, accessions of nine north temperate *Ludwigia* species were also incorporated. *Hauya* was chosen as outgroup based on previous molecular studies of the family Onagraceae (Conti et al., 1993; Ford and Gottlieb, 2007; Levin et al., 2003, 2004). The incorporated accessions of north temperate species, the outgroup and one accession of the ITS region for *Ludwigia peploides* (Kunth) P.H. Raven, were available in GenBank. Accessions and voucher numbers of the material used in both the morpho-anatomical and phylogenetic analyses are presented in Table 1.

2.3. Microtechniques

To visualize and analyze the morpho-anatomical adaptations of the species included in the study, submersed rhizomes and adventitious roots – which are the prevalent aerial and radicular systems in aquatic plants, and are exposed to flooded conditions in aquatic plants – were collected and stored in 70% alcohol. Samples were processed by standard histological methods and free-hand transverse sections were carried out with a razor blade. Anatomical images of rhizomes and roots of *L. peploides* were taken from previous studies (Ellmore, 1981a,b; Boeger and Adis, 2007).

Anatomical slides of 220 samples were analyzed as follows: up to three mature roots of approximately the same length were excised at three positions from 2 cm behind the root tip to assure that all sections were performed on fully mature tissues and to control for the variation that could exist within each root; three sections of the submersed rhizome at various positions per individual were examined to control for the variation within each organ. The histochemical test used for lignin was phloroglucinol-HCl. Toluidine blue O was used to visualize all tissues by enhancing their contrast (Soukup et al., 2002). Sections were mounted on glass slides with a calibrated reference and photographed under a Carl Zeiss Axiostar Plus light microscope using an Olympus Stylus 600 digital camera. Further adjustment and measurements were performed in ImageJ (Rieger and Litvin, 1999).

2.4. Morpho-anatomical characterization

Each species was characterized in terms of the morpho-anatomy of its roots and shoots, based on characters involved in the adaptation of plants to the aquatic habit. Those characters were incorporated into a matrix that included both qualitative and quantitative data. Not all the characters measured were incorporated into the analyses due to redundancy (see Table 2 for characters included in the analyses; for the full list of characters analyzed, character descriptions and character state assignments see Appendix A in the Supplementary material). For quantitative data, most of the characters measured were expressed as a proportion of the total area of each section. Only lacunae

Table 1
Voucher information and GenBank accessions for both the ITS and *rpl32-trnl* markers for each taxon or individual included in the morpho-anatomical and phylogenetic analyses.

Taxon/Individual	Voucher	GenBank accession	
		ITS	*rpl32-trnL*
Hauya heydeana	-	AY357768	-
Hauya heydeana	-	GQ232550	-
*Ludwigia affinis*01	MFL 300	KP026969	KP026988
*Ludwigia affinis*02	MFL 666	KP026970	KP026989
Ludwigia alata	-	HE585696	-
Ludwigia arcuata	-	FN263227	
Ludwigia brevipes	-	FN263239	-
Ludwigia curtisii	-	HE585701	-
*Ludwigia decurrens*01	AMB 468	KP026978	KP027000
*Ludwigia helminthorrhiza*01	AMB 481	KP026983	KP026995
*Ludwigia helminthorrhiza*02	MFL 307	KP026981	KP026996
*Ludwigia helminthorrhiza*03	MFL 657	KP026982	KP026997
*Ludwigia hyssopifolia*01	AMB 192	KP026966	KP026990
*Ludwigia hyssopifolia*02	MFL 671	KP026967	KP026991
*Ludwigia hyssopifolia*03	SM 2012100	KP026968	KP026992
*Ludwigia inclinata*01	AMB 282	KP026972	KP026985
*Ludwigia inclinata*02	MFL 615	KP026974	KP026987
*Ludwigia inclinata*03	MFL 673	KP026973	KP026986
Ludwigia linifolia	-	FN263216	-
Ludwigia microcarpa	-	FN263217	-
*Ludwigia nervosa*01	AMB 394	KP026979	KP026998
*Ludwigia nervosa*02	MFL 417	KP026980	KP026999
Ludwigia ovalis	-	FN263221	-
Ludwigia palustris	-	FN263233	-
Ludwigia peploides	-	AY271517	-
Ludwigia ravenii	-	AY271518	-
Ludwigia repens	-	FN263229	-
*Ludwigia rigida*01	MFL 641	KP026971	KP026993
Ludwigia spathulata	-	FN263232	-
*Ludwigia sedioides*01	AMB 270	KP026975	KP027001
*Ludwigia sedioides*02	AMB 396	KP026976	KP027003
*Ludwigia sedioides*03	MFL 607	KP026977	KP027002
*Ludwigia torulosa*01	AMB 407	KP026984	KP026994

area was measured without considering the total area of the section.

To determine whether there were differences in the observed and measured morpho-anatomical characters among species, the mean value of all the measured characters for each root and rhizome was calculated. The mean value of all the roots per individual was also estimated. Finally, a Multiple Factorial Analysis (MFA) was conducted using FactoMineR package in R (Lê et al., 2008). Continuous characters were compared among the included species. ANOVA was used to test if there were significant differences within the woody and herbaceous groups, followed by a Tukey's HSD test to allow for comparisons between species.

2.5. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica-dried material using the DNeasy Plant Mini Kit (Quiagen Corporation, Valencia, California, USA) and following a protocol for silica columns by Alexander et al. (2007). The nrDNA ITS region, and cpDNA *rpl32–trnL*^{UAG} regions were amplified. The polymerase chain reaction (PCR) was performed with 25 μL reactions containing 1× Taq buffer with 2 mM $MgCl_2$, 1 mM dNTP mix (2.5 mM each), 0.4 μM of each primer, 1.5 U *Taq* DNA polymerase (Thermo Scientific) and 2 μL of DNA extract. To improve amplification, bovine serum albumin (0.25 μg μL^{-1}), and dimthyl sulphoxide (8%) were added for

Table 2
Discrete and continuous characters and character states included in the analyses. Continuous characters were measured as proportions of the total area of the rhizome or root. Only lacunae areas were measured without considering the total area of the section.

Character	State 0	State 1
Habit	No	
Rhizome trichomes	No	Yes
Rhizome secondary aerenchyma	Yes	No
Rhizome primary aerenchyma pattern	Honeycomb expansigeny	Schizo-lysigeny
Rhizome endodermis developmental stage	II	III
Rhizome secondary aerenchyma pattern	Expansigeny	Schizo-lysigeny
Rhizome lacunae area	–	–
Rhizome lignified area proportion	–	–
Rhizome aerenchyma area proportion	–	–
Root pneumatophores	No	Yes
Root secondary aerenchyma	No	Yes
Root primary aerenchyma pattern	Expansigeny	Schizo-lysigeny
Root lignified endodermis	No	Yes
Root aerenchyma area proportion	–	–
Root lignified area proportion	–	–
Root lacunae area	–	–

the ITS region. The same PCR cocktail was used to amplify the cpDNA region but 0.2 μM of each primer was used and no dimthyl sulphoxide was added. Amplifications were run in a C100 Thermal Cycler (Bio-Rad). Amplification and sequencing of the ITS region was carried out using primers ITS4 and ITS5 of White et al. (1990), and for *rpl32–trnL*^{UAG}, primers rpl32F and trnL$^{(UAG)}$ of Shaw et al. (2007) were used. The PCR conditions for the amplifications of the ITS region were: one cycle at 94 °C for 5 min; 26 cycles each at 94 °C for 30 sec, 52 °C for 1 min and 72 °C for 2 min; and one cycle at 72 °C for 7 min, hold at 4 °C. The temperature profiles for *rpl32–trnL*^{UAG} followed Shaw et al. (2007). Amplified products were purified with ExoI-FastAP (Thermo Scientific) following the manufacturer's protocol, and sequenced by the sequencing laboratory at Universidad de Los Andes (Bogotá, Colombia), using the same primers as in the PCRs in a Bio-Rad C1000^{TM} Thermal Cycler.

2.6. Phylogenetic reconstruction and character mapping

Both the ITS and *rpl32–trnL*^{UAG} regions were analyzed separately, as well as in a combined matrix. Two species of *Hauya* Donn. Sm. were included as outgroups in the phylogenetic analysis of the ITS region alone because no accessions of the chloroplast region were available for any genera close to *Ludwigia*. The rooting of this analysis was used as a reference to root the trees of the other phylogenetic analyses. Complementary strands were edited and assembled in Geneious Pro 6.0 (Drummond et al., 2011), which was also used to produce ClustalW initial alignments for the two markers. Subsequent alignment by eye was performed in Mesquite v.2.75 (Maddison and Maddison, 2011). The best-fit model for nucleotide substitution for Bayesian analysis (BA) was chosen for each individual marker using the Akaike Information Criterion (AIC), as implemented in jModelTest v. 2.1.4. (Posada, 2008). Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian analysis (BA) with the software programs RaxML hosted by the CIPRES Science Gateway portal v. 3.3. at the San Diego Supercomputer Center (Miller et al., 2010), and MrBayes v.3.2.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), respectively. Phylogenetic incongruence among data partitions was investigated using the MP incongruence-length difference (ILD) test (Farris et al., 1994). Two simultaneous simulations were run for BA over 10×10^6 generations. Resulting topologies of both ML and BA were examined using FigTree v1.4.0 (Rambaut, 2008). We used Mesquite v.2.75 (Maddison and Maddison, 2011) to map selected characters onto the strict consensus tree of the combined analysis obtained from BA. Continuous characters were transformed into discrete characters under the parsimony reconstruction method in Mesquite v.2.75.

3. Results

3.1. Morpho-anatomical characterization

Three morpho-anatomically differentiated groups were obtained from the MFA in two dimensions that together explain 65% of the variance in the data. Fig. 2 shows the spatial separation of the groups with respect to the first two dimensions. The first group (I) comprises the species *Ludwigia affinis* (DC.) H.Hara, *L. decurrens* Walter, *L. hyssopifolia* (G.Don) Exell, *L. nervosa* (Poir.) H.Hara, *L. rigida* (Miq.) Sandwith and *L. torulosa* (Arn.) H.Harahi. The second group (II) includes the species *L. helminthorrhiza* (Mart.) H.Hara and *L. peploides*. *Ludwigia inclinata* (L.f.) M.Gómez and *L. sedioides* (Humb. & Bonpl.) H.Hara form the third group (III).

Plants within group I are all rooted emergent and more or less woody. The morphology of these species is very similar, only differing by height. The shoot system is made up of a rhizome that

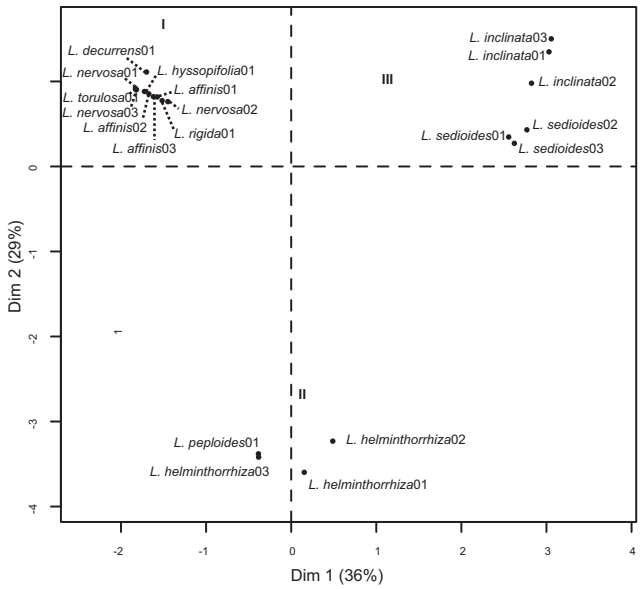

Fig. 2. Ordination of variables and individuals using multiple factor analysis (MFA). Individuals and species were separated in three groups, labeled as I, II, and III.

grows in muddy to completely flooded soils, or along the edges of water bodies. Aerial stems and adventitious roots – which comprise the root system – arise from the rhizome. Within group II, *L. helminthorrhiza* and *L. peploides* are highly plastic species that live as free-floating or sometimes "creeping on mud" herbs (sensu Wagner and Hoch, 2005). The shoot system is made up of a rhizome that either floats freely on the water, is partially submersed, or both. Roots and pneumatophores are produced in nodes along the rhizome. Finally, within group III *L. inclinata* and *L. sedioides* are submersed and floating herbs, respectively, and both are rooted in the sediment. These plants have submersed reddish rhizomes that produce homorrhizic roots in nodes along its length. Leaves are also produced along the stem axis.

With respect to their anatomy, species within group I have rhizomes that are characterized by extensive secondary growth. The stem pith is made up of thin-walled parenchyma cells with small intercellular spaces. The periderm is swollen and produces secondary aerenchyma derived from the phellogen (Fig. 3A–D). The prevalence of secondary aerenchyma differs among species, being less prominent in *L. hyssopifolia* and *L. affinis* (Fig. 3A) and most prominent in *L. torulosa* (Fig. 3D). Secondary aerenchyma shows an expansigenous developmental pattern in most of the species. *Ludwigia decurrens* is the only exception with secondary aerenchyma produced by schizo-lysigeny (Fig. 3C). All roots are homorrhizic and when mature, have extensive secondary growth and expansigenous secondary aerenchyma derived from the phellogen (Fig. 3E–G).

Plants in group II have rhizomes with a single-celled epidermal layer, and a thin cuticle that surrounds an aerenchymatous cortex with hexagonal cell packing (Boeger and Adis, 2007). The aerenchyma has a honeycomb expansigenous pattern (Seago et al., 2005). An endodermis with lignified cell walls surrounds the inconspicuous secondary vascular tissues delimiting the pith, which is similar in appearance to that present in the plants of the first group (Fig. 4A).

Roots of both species are dimorphic with downward-growing roots (DGR) and upward-growing roots (UGR). The DGRs possess one layer of epidermis followed by a hypodermis with one layer of exodermis and one non-exodermal layer internal to it (Seago pers. comm.). These structures surround an aerenchymatous cortex with cubical packing. The aerenchyma develops by expansigeny.

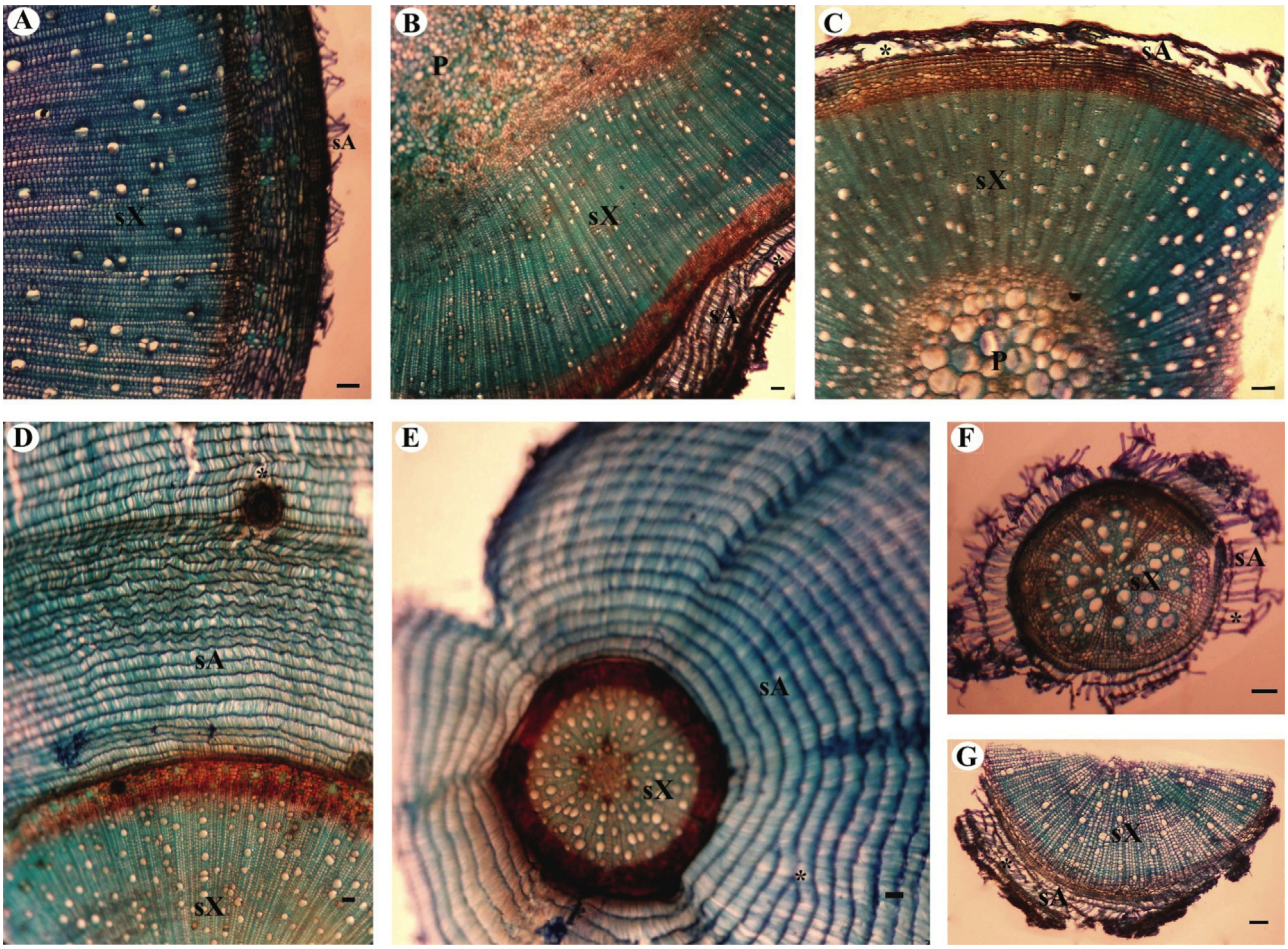

Fig. 3. Transverse sections of rhizomes and roots of species of *Ludwigia* in group I; Toluidine blue O stain, light microscopy. P—pith; sX—secondary xylem; sA—secondary aerenchyma; Lacunae (*). Scale bar = 100 μm. (A) *L. affinis* rhizome; (B) *L. nervosa* rhizome; (C) *L. decurrens* rhizome; (D) *L. torulosa* rhizome; (E) *L. torulosa* root; (F) *L. affinis* root; (G) *L. nervosa* root. sA in (C) has schizo-lysigenous developmental pattern as opposed to the expansigenous pattern in all the other sections.

The UGR consists of an epidermis that is indistinguishable from the well-developed aerenchymatous cortex produced by expansigeny. These structures surround a minute stele (Ellmore, 1981b) (Fig. 4B and C).

Finally, within group III *L. inclinata* has a thin cuticle with a single-cell layered epidermis, and *L. sedioides* has a thin cuticle and an epidermis that appears to be re-divided into two layers. However, this second epidermal layer could also be a result of periclinal divisions of the outermost cortex. The aerenchymatous cortex in both species has hexagonal cell packing and appears to combine honeycomb expansigeny and schizo-lysigeny (Seago pers. comm.). An endodermis with suberin lamellae surrounds inconspicuous secondary vascular tissues delimiting the pith similar to the former groups (Fig. 4D). Roots consist of one layer of epidermis followed by a hypodermis. The aerenchymatous cortex with hexagonal packing originates from schizo-lysigeny and surrounds an endodermis with suberin lamellae and primary vascular tissues interior to it (Fig. 4E).

When comparing measurements in both rhizomes and roots, no significant differences were found among woody species (group I). However, herbaceous species (groups II and III) did differ in the proportion of lignified tissues, aerenchyma and lacunae areas in their rhizomes and the lignified tissue area in their roots (Table 3). Lignified tissue area proportion in rhizomes is greater in plants of group II, and aerenchyma and lacunae area in rhizomes is greater in plants of group III. All measurements and ANOVA results are shown in Appendices B and C, respectively, in Supplementary material.

3.2. Phylogenetic reconstruction and character mapping

For ITS and *rpl32–trnL*^{UAG}, GTR + G and GTR turned out to be the best-fitting models, respectively. In all analyses, the topologies resulting from ML and BA were consistent and differed in few support values for certain branches. Fig. 5 shows the phylogenetic tree deduced from BA using only ITS data, and including north temperate taxa. This topology strongly supports a split between the north temperate and the Neotropical clades. The main difference between ML and BA is that BA is more resolved, having two strongly supported nodes that are not present in the strict consensus ML trees. These include the support for the north temperate clade of *Ludwigia*.

When analyzed individually, the phylogenetic hypothesis deduced from the *rpl32–trnL*^{UAG} marker shows low support for deep nodes compared to the topology resulted from the analysis of the ITS region (Supplementary Fig. S1). However, some derived portions of the tree are better resolved in the *rpl32–trnL*^{UAG} topology. The main difference between the topologies deduced from the individual markers is in the placement of *L. rigida*, which is grouped with *L. affinis* in the ITS topology (PP = 1) and with *L. nervosa* in the *rpl32–trnL*^{UAG} phylogenetic reconstruction (PP = 0.95). The possible reasons for these differences are discussed later.

With all taxa included, the ILD test revealed significant differences between the topologies resulting from the analysis of individual markers ($P < 0.05$). However, when excluding *L. rigida* from the analyzed topologies, the test revealed no significant

Table 3
Results of Tukey test for the measurements of rhizome and root characters in herbaceous species.

Species	Rhizome			Root
	Lignified tissue area proportion	Aerenchyma area proportion	Lacunae area	Lignified tissue area proportion
L. sedioides–L. inclinata	0.996	0.919	0.009*	0.024*
L. peploides–L. inclinata	0.029*	0.034*	–	–
L. helminthorrhiza–L. inclinata	0.040*	0.019*	0.001*	0.158
*L. peploides–*L. *sedioides*	0.035*	0.056	–	–
L. helminthorrhiza–L. sedioides	0.051	0.037*	0.156	0.442
L. helminthorrhiza–L. peploides	0.559	0.880	–	–

* Mean difference is statistically significant.

incongruence between the two phylogenetic trees ($P > 0.05$). We opted to combine the sequences to maximize the data included in the analysis. The combined data consisted of sequences of 1883 nucleotides in length. *Ludwigia rigida* and *L. peploides*, which had missing data for one of the two combined markers, were included given that it has been shown that adding taxa, even if incomplete, can improve phylogenetic accuracy when there is limited taxon sampling (Wiens, 2003, 2006; Wiens and Tiu, 2012). We excluded the ITS sequence from *L. rigida*, based on previous taxonomic work that suggests it should be included with *L. nervosa* and others in section *Myrtocarpus* (Ramamoorthy and Zardini, 1987; Wagner and Hoch, 2005; Wagner et al., 2007). The previous taxonomic work provides support for the evidence provided by the *rpl32–trnL*^{UAG} marker, and suggests that the signal given by the ITS marker could be conflicting. Given that the ITS sequence for *L. peploides* was obtained from NCBI and not from our own specimens, we were not able to include an *rpl32–trnL*^{UAG} sequence for *L. peploides* in the combined analysis. The ML and BA are congruent, only differing in the support for one of the nodes (see single asterisks, Fig. 6). Despite the placement of *L. rigida*, all results strongly support groups II and III from the MFA as monophyletic clades.

Rhizome trait mapping is shown in Fig. 7A. All plants in group I display a rooted emergent habit. Within groups II and III (shaded clades), clade II is characterized by a free-floating or "creeping on mud" habit. Clade III displays the rooted floating and rooted emergent habits. The possession of trichomes is a sinapomorphy of clade II. The presence of endodermis and primary aerenchyma are characteristic of clades II and III. However, these clades differ in the

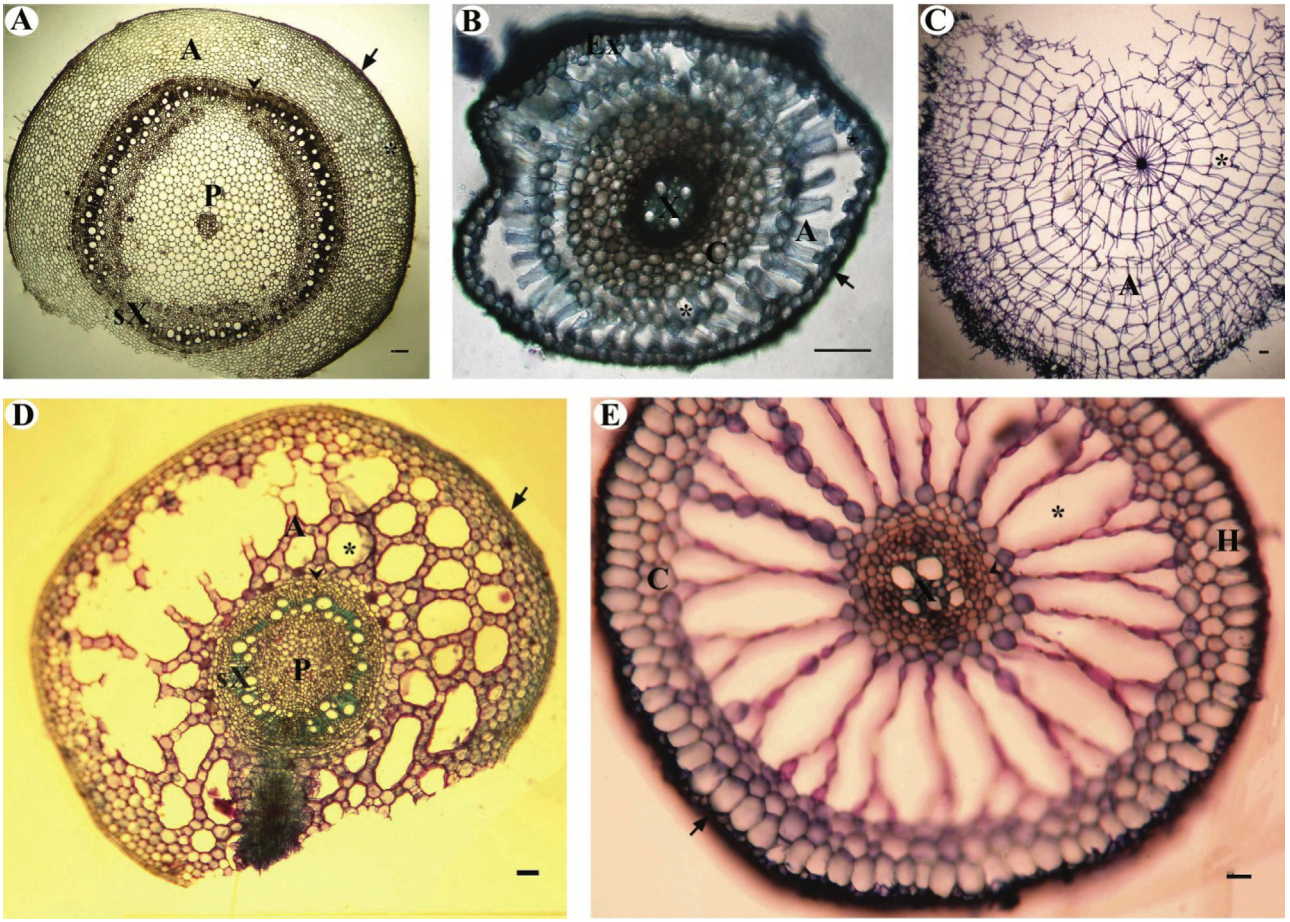

Fig. 4. Transverse sections of rhizomes and roots of species of *Ludwigia* in groups II and III; Toluidine blue O and Phloroglucinol stains, light microscopy. P—pith; X—xylem; C—cortex; A—aerenchyma; Ex—exodermis; H—hypodermis; sX—secondary xylem; sA—secondary aerenchyma; Epidermis (arrow); Lacunae (*); Endodermis (arrowhead); non-exodermal layer of the hypodermis (star). (A) *L. helminthorrhiza* rhizome; (B) *L. helminthorrhiza* DGR. (C) *L. helminthorrhiza* UGR; (D) *L. sedioides* rhizome; (E) *L. sedioides* root.

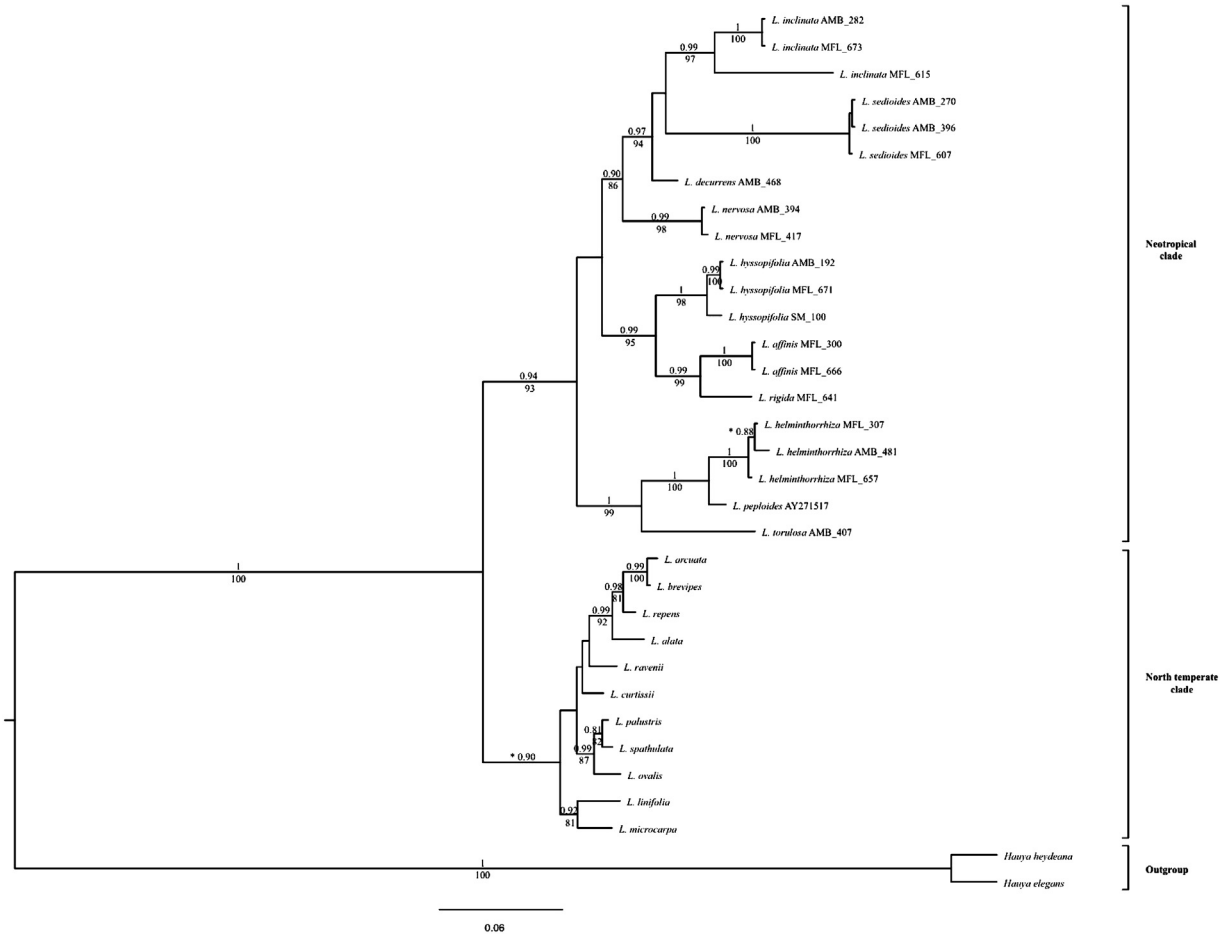

Fig. 5. Strict consensus tree of *Ludwigia* deduced from Bayesian analysis (BA) of ITS data. Tree rooted with two individuals of *Hauya heydeana* (Lythraceae). Numbers above and below branches indicate Bayesian posterior probabilities >0.75, and Bootstrap values >75, respectively. Single asterisks, indicate branches that were not present in either the ML or BA reconstruction.

developmental stage of the endodermis, with lignin in clade II and suberin lamellae in clade III. The primary aerenchyma pattern is expansigenous in clade II and schizo-lysigenous in clade III. Secondary aerenchyma is expansigenous in all plants of clade I, except for *L. decurrens* which has a schizo-lysigenous pattern.

Continuous characters show a clear trend across the tree with a greater mean aerenchyma proportion in clade III than found in clade II. Although intraspecific variation is observed, the differences between the species in groups II and III are statistically significant (Table 3). Values for the mean proportion of aerenchyma do not apply to clade I because those species have secondary aerenchyma that sloughs off as secondary growth progresses. Mean lignified area proportion is greater in clade I than in clades II and III. In addition, significant differences were found between the species in the latter two groups (Table 3).

Mapping of root traits is shown in Fig. 7B. Clades I and II have pneumatophores. These structures were not present in clade III, nor did we record them in *L. decurrens*. As opposed to secondary growth in rhizomes, root secondary growth is restricted to plants in group I. On the other hand, primary aerenchyma is characteristic of clades II and III, exhibiting expansigenous and schizo-lysigenous patterns, respectively. The presence of a lignified endodermis is a sinapomorphy of clade II. No suberin lamellae was recorded in clade III. Mean lignified area proportion is greater in clade I than in clades II and III. We did not map lacunae area because this character could not be measured in many of the species where the developmental pattern of aerenchyma was expansigenous.

4. Discussion

4.1. Morpho-anatomical characterization

Our results support a separation of the species of *Ludwigia* into one group of more or less woody plants (I), and two groups of herbaceous species (II and III). The species in group I are mostly found along the edges of water bodies or in flooded plains, so they have retained some terrestrial characters such as a rooted-emergent habit and extensive secondary growth in both shoots and roots. The secondary aerenchyma of expansigenous origin would provide the mechanism for gas transport throughout the plant body. This pattern of aerenchyma origin has been previously reported for several aquatic species including *Ludwigia* (Schenck, 1889; Angeles, 1992; Stevens et al., 2002; Little and Stockey, 2006; Somavilla and Graciano-Ribeiro, 2012). We are still uncertain of the significance of the schizo-lysigenous pattern of secondary aerenchyma in *L. decurrens*, which has been determined by Seago et al. (2005) to be the formation of intercellular spaces or lacunae by cell separation and subsequent death. The development of ascending pneumatophores in most of the plants in group I may be explained as an adaptation to enhance gas exchange in order to overcome the strong lignification they display.

The herbaceous groups II and III both include forms that live strictly in the water. However, they differ both morphologically and anatomically. The main difference between the two groups

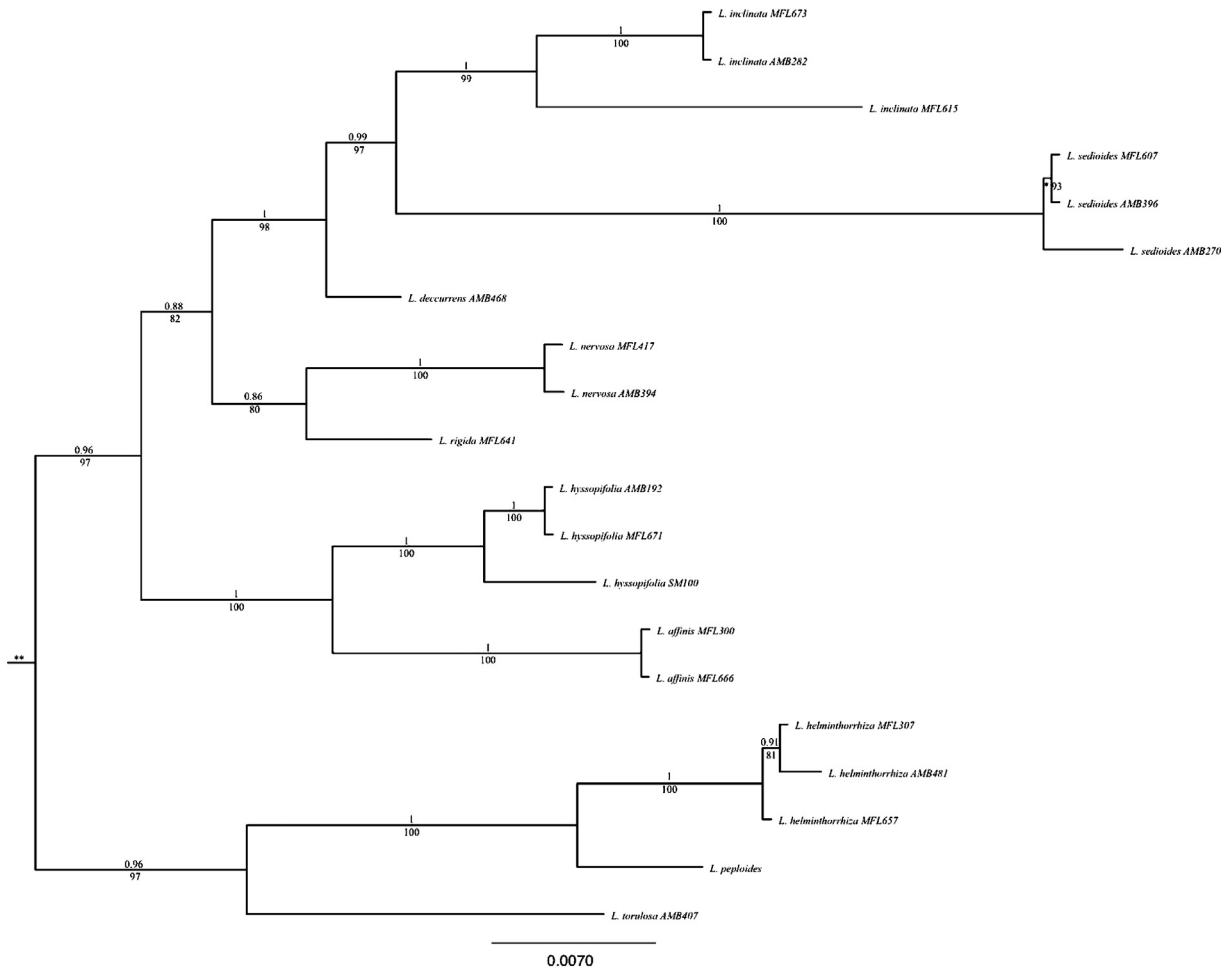

Fig. 6. Strict consensus tree of *Ludwigia* from Bayesian analysis (BA) deduced from the combined analyses of ITS and *rpl32–trnL*$^{(AUG)}$. Tree rooted with two individuals of *Hauya heydeana* (Lythraceae) where the double asterisks are. Numbers above and below branches indicate Bayesian posterior probabilities >0.75, and Bootstrap values >75, respectively. Single asterisks indicate branches that were not present in the ML reconstruction.

is their habit. The occasional presence of the "creeping on mud habit" in group II, suggests that these plants still retain characters from a terrestrial ancestor, although they are adapted to live strictly in the water. The manner in which plants have adapted to life in the water has driven anatomical differences. These include the presence of trichomes as protection from solar radiation in the free-floating and "creeping on mud" species in group II, and their absence in the rooted-floating and rooted-submerged species in group III. Smaller aerenchyma area in group II in relation to group III, could be explained by the fact that floating herbs are not exposed to anoxic conditions as much as rooted-floating and rooted-emergent herbs are, since their photosynthetic organs are above water. However, in terms of lacunae area, no differences were found between *L. helminthorrhiza* and *L. sedioides*. This could be resulting from the presence of rhizomes that reach the water surface and would therefore experience conditions similar to those of plants in group II. However more sampling and comparisons are needed to support this supposition. Finally, the smaller lignified tissue area in group III could be explained by the fact that these organs are completely submerged and water lends external support to the plant body parts.

Root structural differences are also associated with the habit of the plants. Group II has pneumatophores that function as floatation devices and serve to enhance gas exchange throughout the plant body (Ellmore, 1981b). In contrast, group III, which has roots attached to the substrate, do not have pneumatophores. Transverse sections of the rhizomes and roots of *L. peploides* are almost identical to those of *L. helminthorrhiza* (Ellmore, 1981a,b; Boeger and Adis, 2007).

4.2. Phylogenetic reconstruction and character mapping

The separation of a north temperate clade from the Neotropical representatives of *Ludwigia* as indicated by our partial phylogenetic analysis, has been documented in previous molecular and taxonomic studies (Hoch and Barber, 2007; Barber et al., 2008; Hoch et al., 2011; Liu et al., 2013, Hoch pers. comm.). The differing placement of *L. rigida* in the phylogenetic hypotheses deduced from the cpDNA (*rpl32–trnL*^{UAG}) and nrDNA (ITS), may be the result of 'anomalous gene trees' produced during tree analysis (Degnan and Rosenberg, 2006, 2009; Rosenberg and Tao, 2008). Furthermore, hybridization, the presence of paralogs, or the presence of pseudogenes might be driving the occurrence of multiple ITS types in the same genome (Baldwin et al., 1995; Álvarez and Wendel, 2003; Bailey et al., 2003; Soltis et al., 2008). Natural hybridization is considered to be fairly common among *Ludwigia* species, and some species are even thought to have originated from hybridization (Raven and Tai, 1979; Peng, 1989; Zardini et al., 1991; Zardini and Raven, 1992; Nesom and Kartesz, 2000; Peng et al., 2005; Hung et al., 2009; Ghahramanzadeh et al., 2013). However, additional molecular data are needed to clarify whether the conflicting signal comes from the nuclear or the chloroplast markers.

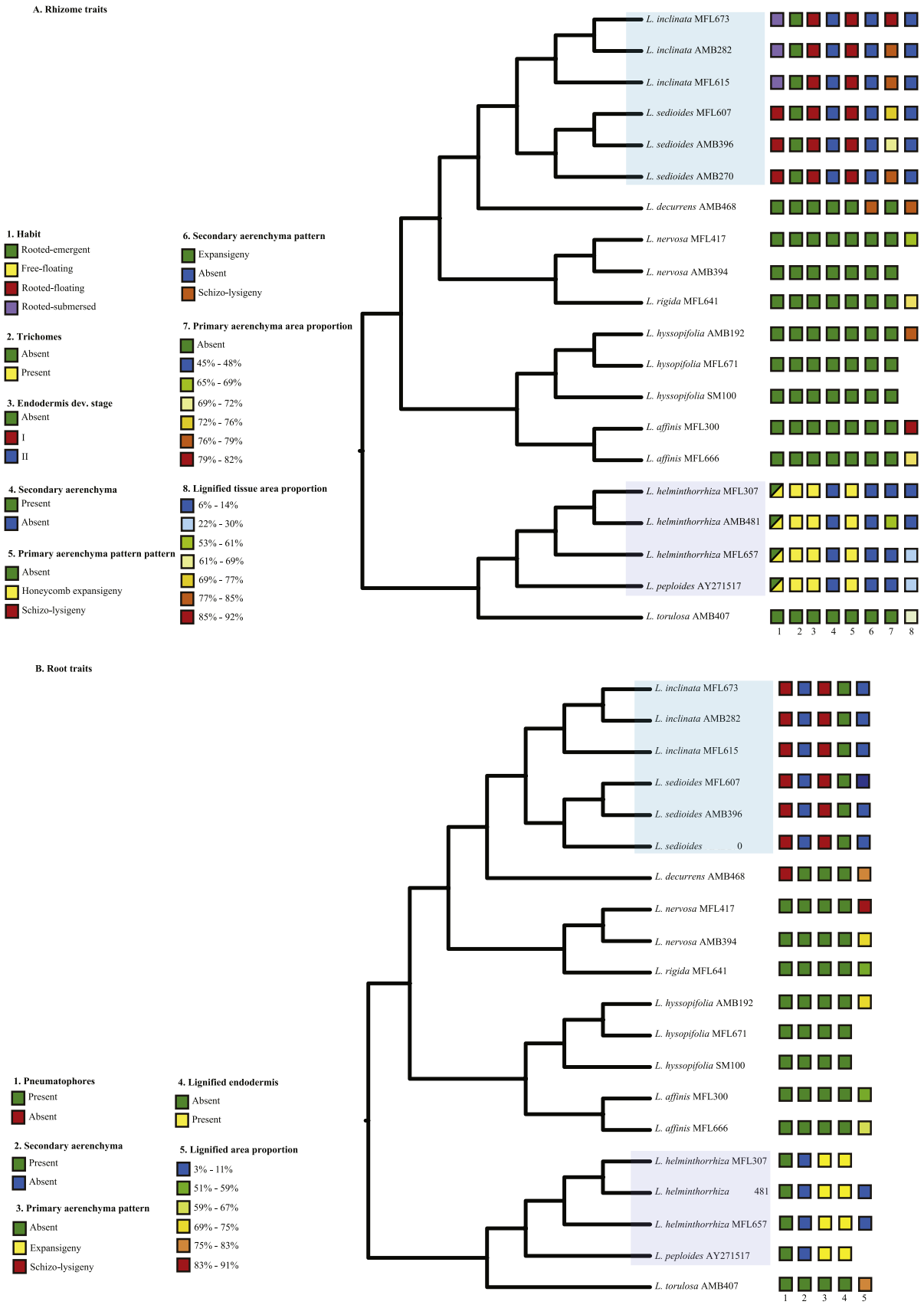

Fig. 7. Trait mapping of morpho-anatomical characters. (A) Rhizome trait mapping. (B) Root trait mapping. Character states were mapped to *Ludwigia* combined phylogentic tree with Mesquite v. 2.75. For comprehensive representation, characters of each organ are combinatorially presented on a single tree. Shaded areas highlight the clades that correspond to groups II and III. The code key for the character states is shown on the left side of the figure. Lacunae area was not mapped in neither rhizomes nor roots, because it could not be measured in most of the taxa. Aerenchyma area proportion in roots was not mapped, for most of the roots have secondary aerenchyma that sloughs off.

Only the two monophyletic clades corresponding to groups II and III have growth forms whose body is almost entirely in contact with water, providing evidence that the acquisition of that aquatic habit evolved at least two times in Neotropical *Ludwigia*. The same results are also supported by the systematic treatments of *Ludwigia* in which strictly aquatic species are included in more than one section (Raven, 1963; Ramamoorthy and Zardini, 1987; Zardini and Raven, 1992). However, given that the phylogenetic hypothesis presented here is based on incomplete sampling, it is also possible that this event evolved only once with multiple reversals toward inhabiting in less flooded conditions. We found the last hypothesis to be the least parsimonious, given that abundant previous work on the genus assigned growth forms such as those found in groups II and III to only 19 out of the 82 *Ludwigia* species (Wagner and Hoch, 2005). Of these, only *L. sedioides* and *L. inclinata* are rooted-floating and rooted-submerged herbs.

Character mapping highlights the considerable convergent evolution between the herbaceous groups II and III (Fig. 7A and B). The convergent characters include the absence of secondary growth in roots, presence of primary aerenchyma, and relatively smaller lignified tissue area proportion in both rhizomes and roots; these characters are all basic adaptations to the aquatic habit. This results complement Cook (1999), who recognized that given that there are apparently few morphological and physiological solutions to the problem of becoming secondarily aquatic, the event of re-colonization of water has led to considerable convergent and parallel evolution. Furthermore, the occurrence of autapomorphies in both groups II and III is evidence that in addition to the convergence of characters to adapt to aquatic life, Neotropical *Ludwigia* also diversified into various life forms suitable to live strictly in the water. We would like to highlight the great value of *Ludwigia* as a model system for the study of the evolution of characters that respond to pressures imposed by aquatic ecosystems within the dicotyledons, as well as within aquatic plants in general.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2014.10.005.

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