Genomic relationships among diploid and polyploid species of the genus Ludwigia L. section *Jussiaea* using a combination of cytogenetic, morphological, and crossing investigations

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ABSTRACT

The genus Ludwigia L. section *Jussiaea* is composed of a polyploid species complex with 2x, 4x, 6x and 10x ploidy levels, suggesting possible hybrid origins. The aim of the present study is to understand the genomic relationships among diploid and polyploid species in the section Jussiaea. Morphological and cytogenetic observations, controlled crosses, genomic *in situ* hybridization (GISH), and flow cytometry were used to characterize species, ploidy levels, ploidy patterns, and genomic composition across taxa. Genome sizes obtained were in agreement with the diploid, tetraploid, hexaploid, and decaploid ploidy levels. Results of GISH showed that progenitors of *Ludwigia stolonifera* (4x) were *Ludwigia peploides* subsp. *montevidensis* (2x) and *Ludwigia helminthorrhiza* (2x), which also participated for one part

(2x) to the *Ludwigia ascendens* genome (4x). *Ludwigia grandiflora* subsp. *hexapetala* (10x) resulted from the hybridization between *L. stolonifera* (4x) and *Ludwigia grandiflora* subsp. *grandiflora* (6x). One progenitor of *L. grandiflora* subsp. *grandiflora* was identified as *L. peploides* (2x). Our results suggest the existence of several processes of hybridization, leading to polyploidy, and possibly allopolyploidy, in the section Jussiaea due to the diversity of ploidy levels. The success of GISH opens up the potential for future studies to identify other missing progenitors in *Ludwigia* L. as well as other taxa.

Keywords: GISH, invasive plant, *Ludwigia* L., Onagraceae, polyploidy, phylogenetics

INTRODUCTION

Polyploidization is widespread in plants and is considered as a major driving force in plant speciation and evolution (Otto and Whitton, 2000; Husband et al., 2013; Alix et al., 2017). Autopolyploid plants arise from the duplication of one genome within one species and allopolyploid plants result from the association of two or more divergent genomes through interspecific hybridization and subsequent genome duplication (Alix et al., 2017; Soltis et al., 2015). Furthermore, some polyploids can arise from both auto- and allopolyploidy events because of their evolutionary histories and are called auto-allo-polyploid. Genomic analyses have revealed that all angiosperms have been subjected to at least one round of polyploidy in their evolutionary history and are thus considered paleopolyploids (Garsmeur et al., 2014). Thus, understanding the origins of polyploid taxa is integral to understanding angiosperm evolution.

Polyploid plants are often thought to be more resilient to extreme environments than diploids because of their increased genetic variation (Husband et al., 2013). Their duplicated genes act as a buffer and can include gene conversion events, activation of transposable

elements, chromatin remodelling, and DNA methylation changes (Hollister, 2015). Polyploidy might confer an advantage with both abiotic and biotic stress by increasing tolerance to salt or drought stress or by improving resistance to bioagressors (Van de Peer et al., 2021). Thus, polyploids are able to occupy new ecological niches (Stebbins, 1985; Blaine Marchant et al., 2016) and often show greater adaptability than their progenitors (McIntyre, 2012; Allario et al., 2013; Baniaga et al., 2020; Akiyama et al., 2021; Van de Peer et al., 2021). Van de Peer et al. (2021) suggested that as in a constant environment, polyploidization may play an important role in response to habitat disturbance, nutritional stress, physical stress, and climate change (Wei et al., 2019). For example, Baniaga et al. (2020) showed that ecological niches of polyploid plants differentiated often faster than found in their diploid relatives. A polyploid advantage has also been reported in invasive plants and their success in non-native habitats (Te Beest et al., 2012). However, Lobato-de Magalhães et al. (2021) observed little difference in the incidence of each ploidy state within a set of 49 of the world's most invasive aquatic weeds and concluded there is no consistent evidence of polyploid advantage in invasiveness. Nevertheless, *Spartina anglica*, an invasive neoallopolyploid weed species that appeared around 1890, has increased fitness with its prolific seed production, fertility, and extensive clonal growth as compared to its progenitors (Baumel et al., 2002). A recent study including 50 alien non-invasive aquatic plant species and 68 alien invasive species across various aquatic habitats in the Kashmir Himalayas found that invasive species are largely polyploids whereas non-invasive species tend to diploids (Wani et al., 2018).

Ludwigia L., a worldwide wetland genus of 83 species, forms a strongly monophyletic lineage sister to the rest of the Onagraceae. It is currently classified as members of 23 sections (Levin et al., 2003, 2004). Sections were clustered into three main groups by Raven (1963). The first group concerned the Myrtocarpus complex, comprising 14 sections (Raven, 1963; Eyde, 1977; Ramamoorthy, 1979; Zardini and Raven, 1992). The second group included

species in the section *Eujussiaea* Munz (Munz, 1942), also referred to as a sect. *Oligospermum* (Raven, 1963) but now correctly called sect. *Jussiaea* (Hoch et al., 1993). The third group combined species in sect. *Isnardia*, sect. *Ludwigia*, sect. *Microcarpium*, and sect. *Miquelia* P.H. Raven (Raven, 1963; Wagner et al., 2007). Liu et al. (2017) provided the first comprehensive molecular phylogeny of *Ludwigia* genus using both nuclear and chloroplast DNA regions. Sixty of 83 species in the *Ludwigia* genus were distributed in the two clades A and B, with the sub-clade B1 which consisted of only sect. *Jussiaea*. This section included seven species: three diploid species (2n=2x=16) (*Ludwigia torulosa* (Arn.) H. Hara, *Ludwigia helminthorrhiza* (Mart.) H. Hara, *Ludwigia peploides* (Kunth) P.H. Raven); two tetraploid species (2n=4x=32) (*Ludwigia adscendens* (L.) H. Hara, *Ludwigia stolonifera* (Guill. &Perr.) P.H. Raven); one hexaploid species (2n=6x=48) (*Ludwigia grandiflora* subsp. *grandiflora*); and one decaploid species (2n=10x=80) (*Ludwigia grandiflora* subsp. *hexapetala*). It is not easy to distinguish between the hexaploid and decaploid species morphologically and both have previously been treated as a single species (*Ludwigia uruguayensis* (Cambess.) H. Hara; Zardini et al., 1991).

In this study, we focused on species belonging to the second group, sect. *Jussiaea*. Most species of the section grow in warm temperate to subtropical moist or wet habitats worldwide. Some of these species, such as *Ludwigia peploides* subsp. *montevidensis* (Kunth) P.H. Raven, *Ludwigia grandiflora* (syn. *L. grandiflora* subsp. *grandiflora*), *Ludwigia hexapetala* (Hook. & Arn.) Zardini, H.Y. Gu & P.H. Raven (syn. *L. grandiflora* subsp. *hexapetala*) (Hook. & Arn.) Zardini, H. Y. Gu & P. H. Raven, can be invasive weeds in wetlands and other wet areas in the USA (Grewell et al., 2016), Europe (Portillo-Lemus et al., 2021), Japan (Hieda et al., 2020), and Korea (Kim et al, 2019). Recently, Méndez Santos and González-Sivilla (2020) revealed that *L. helminthorrhiza* (Mart.) H. Hara must be treated and managed as an invasive alien species in Cuba. Reproductive systems in Ludwigia L. are both clonal with production of asexual fragments and sexual with seeds production. Okada et al. (2009) showed that clonal

101 spread through asexual reproduction is the primary regeneration mode of *L. grandiflora* subsp.
102 *grandiflora* and *L. grandiflora* subsp. *hexapetala* in California. Furthermore, Dandelot (2004)
103 reports that all the populations of *L. grandiflora* subsp. *hexapetala* in the French Mediterranean
104 area could have originated from a single clone. Similarly, Reddy et al. (2021) observed low
105 genotypic diversity in both *L. grandiflora* subsp. *grandiflora* and *L. grandiflora* subsp.
106 *hexapetala* in the United State with as example an analysis of multiple invasive populations of
107 *L. grandiflora* subsp. *hexapetala* in Alabama, California, Oregon, Washington, and Florida
108 identified a single genotype.

109 The aim of this study is to characterize the complicated evolutionary history of genus
110 Ludwigia L. section Jussiaea using a combination of cytogenetic, morphological, and crossing
111 investigations. This is a difficult puzzle to elucidate, with taxa ranging from diploid to decaploid
112 and with both allo- and autopolyploidy involved in the history of these taxa. The occurrence of
113 different ploidy levels of *Ludwigia* species belonging to the same clade might indicate that a
114 diploid species in this clade could be the progenitor of the polyploids analysed. We
115 characterized the different species by analysis of their genome size using flow cytometry, their
116 ploidy level by cytogenetic observations and some morphological traits. We identified the
117 'phylogenic origin' by Genomic *in situ* Hybridization (GISH) and evaluated the ability of inter-
118 species hybridization after controlled pollination. The genomic relationships between diploid
119 and polyploid species are reported for the first time in sect. *Jussiaea*.
120

121 **MATERIAL AND METHODS**

122 ***Plant material***

123 Two diploid, two tetraploid, one hexaploid, and one decaploid *Ludwigia* species were
124 analysed. Fifteen plants of *Ludwigia peploides* subsp. *montevidensis* (2x) (hereafter, *Lpm*) and

125 of *L. grandiflora* subsp. *hexapetala* (hereafter, *Lgh*) (10x) were collected in France at the
126 marshes of la Musse (47°14’27.5”N, 1°47’21.3”W) and Mazerolles (47°23’16.3”N,
127 1°28’09.7”W), respectively. Ten plants of the diploid species *L. helminthorrhiza* (hereafter, *Lh)*
128 was purchased in aquarium store (provider Ruinemans Aquarium B.V. Netherland). Five plants
129 of *Ludwigia adscendens* (L.) H. HARA (4x) (hereafter, *La*), and of *L. stolonifera* (4x)
130 (hereafter, *Ls*) and ten of *L. grandiflora* subsp. *grandiflora* (6x) (hereafter, *Lgg*) were collected
131 in Flores island, Indonesia (Pulau Flores; 8°49'40.8"S, 120°48'39.0"E), Lebanon (Hekr al
132 Dahri; 34°37'54.5"N, 36°01'28.9"E), and the USA (Co. Rd 73, outside Greensboro, AL;
133 32°61’51.41”N, 87°68’65.4”W), respectively. All Ludwigia species growth preferentially by
134 clonal reproduction (Okada et al., 2009; Glover et al., 2015) and the plants were easily
135 maintained in the greenhouse at Institut Agro Rennes - Angers before analysis (Portillo-Lemus
136 et al, 2021).
137
138 ***Morphology***
139 We recorded the colour of the flowers and roots and the pneumatophore form to
140 characterize collected *Ludwigia* species, following the morphological traits considered in the
141 *Ludwigia* sp. description (Armitage et al., 2013; Thouvenot et al., 2013). Morphological
142 observations for each species were randomly made on at least 30 plants in the greenhouse and
143 confirmed in natura on 15 plants in 15 and 36 populations of *Lpm* and *Lgh* in France,
144 respectively.
145
146 ***Chromosome counting***
147 Root tips of 0.5 - 1.5 cm in length were taken for each *Ludwigia* sp. as follows from 15
148 *Lpm*; ten *Lh*; five *La*; five *Ls*; ten *Lgg* and 15 *Lgh* different plants and were incubated in 0.04%
149 8-hydroxiquinoline for 2 hours at room temperature in the dark, followed by 2h at 4°C to

150 accumulate metaphases. Tips were then fixed in 3:1 ethanol-glacial acetic acid for 48 hours at
151 4°C and stored in ethanol 70 % at -20 °C. Before use, tips were washed in 0.01 M citric acid-
152 sodium citrate buffer (pH 4.5) for 15 min and then digested in a solution of 5% Onozuka R-10
153 cellulase (Cat No. C1794, Sigma), 1% Y23 pectolyase (Cat No. P5936, Sigma) at 37 °C for 45
154 min. The digested root tips were then carefully washed with distilled water for 30 min. One root
155 tip was transferred to a slide and macerated with a drop of 3:1 fixation solution. The slides were
156 dried at room temperature and stored at -20°C until 4',6-diamidino-2-phenylindole (DAPI)
157 staining. At least four roots per species were observed. The chromosome counts per species
158 were estimated on a total of 20 cells at the mitotic metaphase stage using the visualization
159 software Zen 2 PRO (Carl Zeiss, Germany).
160
161 ***Genome size estimation by flow cytometry***
162 To explore the genome size among the different *Ludwigia* spp., we used flow cytometry.
163 Approximately 4 mg of fresh roots or leaves from five plants of *Ludwigia* spp. and of fresh
164 leaves from five plants of *Trifolium rupens or Zea mays* (used as an internal reference standard
165 for *Lpm*, *Lh* and *Lgh* species and *Ls*, *La*, *Lgg* and *Lgh* species, respectively) were harvested and
166 transferred to a Petri dish. This material was finely chopped using a sharp razor blade in 500 µl
167 of staining buffer (from Cystain PI OxProtect, Cat No. 05-5027) and incubated at room
168 temperature for 30 sec to 90 sec. The solution was then filtered through a 50 µm nylon mesh
169 and 1.5 ml of solution (0.0166 mg of RNase A and 10 µl of Propidium Iodide) was added per
170 sample. Incubation at room temperature was made for 30 min to 60 min, protected from light.
171 Estimation of genome size for each species was obtained using a CyFlow space cytometer
172 (Sysmex Corp., Kobe, Japan). This instrument was equipped with a 488 nm blue laser 50 mW
173 and a band-pass filter LP590 used as an emission filter. Prior to running the samples, gain and
174 linearity of the instrument were adjusted by using DNA control PI from Sysmex. Finally, G1

175 peaks in *Ludwigia* spp. and *Trifolium rupens* or *Zea mays* were collected for each sample to
176 calculate nuclear DNA content (1C) and haploid genome size (Mbp). For the different Ludwigia
177 spp., two or three measures of genome size were made, excepted for *Ls* (only one measure).
178 From each species, the mean ratio of DNA content was calculated (mean + CI (Confidence
179 Interval), P= 0.05)). Genome sizes were converted from picograms (pg) to Megabases (Mb)
180 using 1 pg = 978 Mbp (Dolezel et al., 2003).
181
182 ***Genomic in situ hybridization (GISH)***
183 DNA was extracted from 30 mg of freeze-dried buds taken from 15 *Lpm*, ten *Lh*, five
184 *Ls*, five *La*, ten *Lgg,* and 15 *Lgh* plants, using the Macherey-Nagel extraction kit NucleoSpin®
185 Food to which we have made following modifications to obtain a polysaccharide free DNA: (1)
186 after lysis step with Buffer CF, we mixed freeze-dried buds with an equivalent volume of PCIA
187 25:24:1 (parts of phenol, chloroform, isoamyl alcohol) for 5 minutes ; (2) then we transferred
188 the whole in a tube containing phase-look gel and centrifuged at 800rpm for 5 minutes
189 (Quantabio, Massachusetts, USA); (3) then the DNA was precipitated using absolute ethanol at
190 -18°C instead of QW and C5 buffers. Finally, the DNA was resuspended after an incubation of
191 5 min in 100 ml elution buffer with 5 mM TRIS at pH 8.5 at 65°C. Total genomic DNA were
192 used as probes and labelled by random priming with biotin-14-dCTP (Invitrogen by Thermo
193 Fisher Scientific).
194 Total genomic DNA used as a blocking DNA was autoclaved to yield fragments of 100-
195 300 bp. The ratio DNA probe / blocking DNA was 1:50. The hybridized probes correspond to
196 the chromosomes present on the slide (i.e., same species) and genomic DNA (blocking DNA)
197 from different species were used as competitors in to block the common sequences at both
198 species.

199 Chromosome preparations were incubated in RNase A (100ng/µL) (Cat. No R4642,
200 Sigma) for 1 h then in pepsin (0.05%) in 10 mmol HCL for 15 min, fixed with paraformaldehyde
201 (4%) for 10 min, dehydrated in an ethanol series (70%, 90% and 100%) for 3 min, and finally,
202 air-dried. The hybridization mixture consisted of 50% deionized formamide, 10% dextran
203 sulfate, 2 X SSC, 1% SDS, 100 ng of probe labelled probe, and a 50-fold excess of blocking
204 DNA and was denatured at 92°C for 6 min, before being transferred to ice. Chromosomes were
205 denatured in a solution of 70% formamide in 2X SSC at 70°C for 2 min. The denatured probe
206 was placed on the slide and *in situ* hybridization was carried out overnight in a moist chamber
207 at 37°C. After hybridization, slides were washed for 5 min in 50% formamide in 2 X SSC at
208 42°C, followed by several washes in 4 X SSC-Tween. Biotinylated probes were
209 immunodetected by Texas Red avidin DCS (Vector Laboratories, Burlingame, CA, USA) and
210 the signal was amplified with biotinylated anti-avidin D (Vector Laboratories). The
211 chromosomes were mounted and counterstained in Vectashield (Vector Laboratories)
212 containing 2.5µg/mL 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were
213 captured using an ORCA-Flash4 (Hamamatsu, Japan) on an Axioplan 2 microscope (Zeiss,
214 Oberkochen, Germany) and analysed using Zen 2 PRO software (Zeiss, Oberkochen,
215 Germany). For each *Ludwigia* species, at least three independent slides were made with a total
216 of 20 cells observed per species. The images were processed using Photoshop v.8.0.1 (Adobe
217 Systems Inc., San Jose, CA, USA). Original images put in supplementary data (Appendix S1;
218 see Supplemental Data with this article).
219
220 ***Controlled interspecific crosses***
221 Controlled interspecific pollinations were carried out in the greenhouse between
222 *Ludwigia* species which putatively shared the same parental genome. Thus, interspecific
223 hybridizations were made between *L. peploides* subsp. *montevidensis*, *L. stolonifera* and/or *L.*

224 *grandiflora* subsp. *hexapetala* used as male or as female. Ten plants of each species were used
225 for crosses. Ludwigia spp. produced flowers in continuous on a shoot until July to October,
226 with at one time only one flower per shoot at the good stage of mature for pollination. Flowers
227 used as 'female' were emasculated before anthesis. A mix of pollen from flowers of 5 different
228 plants for each of other species was used to pollinate emasculated flowers. Between two to 25
229 interspecific crosses were made according to the availability of flowers. To control efficiency
230 of pollination in greenhouse, we also conducted at the same time 45, 75 and 50 intraspecific
231 crosses for *Lpm, Lgh* and *Ls*, respectively.

232 Pollination success was estimated by the number of fruits, fruit size and weight, the
233 number of seeds, viable plantlets, and the number of plants ultimately produced.

234

235 **RESULTS**

236 ***Morphological traits of Ludwigia species***

237 For the diploid species, red roots, yellow flowers, and rare cylindric pneumatophores
238 were observed in *Lpm*. In contrast, in *Lh*, we observed red roots, creamy white petals with
239 narrow yellow base, and abundant, clustered conical pneumatophores (around ten per node;
240 Figure 1). For the tetraploid species, *La* had pink roots, white petals with yellow base, and had
241 few conical pneumatophores (approx. six). *Ls* had white roots, petal color light yellow f and
242 similar form of pneumatophores as those of *La*. For the hexaploid species *Lgg*, only roots were
243 observed and were pink. The decaploid species *Lgh* had white roots, flowers with yellow petals,
244 and few, long cylindrical pneumatophores per node (one to two). Color of roots and
245 pneumatophore number and form were confirmed in natura for the different populations of *Lpm*
246 and *Lgh* observed (Figure 1).

247

248 ***Genome size and ploidy level***

249 The chromosome numbers were as excepted: for both diploids, *Lpm* and *Lh* 2n = 16, for
250 both tetraploids *Ls* and *La* 2n =32, for hexaploid *Lgg* 2n=48 and for decaploid, *Lgh* 2n=80
251 (Table 1, Appendix S2). *Ludwigia* spp. exhibited an ~0.77-fold range of C-values. The lowest
252 value, 0.53 pg/2C, was found in *Lpm* and the highest, 2.9pg/2C, in *Lgh* (Table 1, Appendix S3).
253 The tetraploid species *Ls* (1.07pg/2C) and *La* (1.06pg/2C) have C-values that were twice that
254 the value for the diploid *Lpm* (0.53pg/2C) and *Lh* (0.55pg/2C). The hexaploid species *Lgg* had
255 C-value 1.77pg/2C. Thus, the genome size by ploidy level revealed that the monoploid genome
256 sizes (1Cx-value, 0.133-0.147 pg) of the tetraploid, hexaploid, and decaploid species are the
257 same (0.34-0.49 pg/1Cx). The difference is accounted for by the higher ploidy levels.
258 *Ludwigia* genome sizes of diploid and tetraploid species were similar between species
259 with the same ploidy level and varied proportionally with ploidy levels (i.e., 2x≈260 Mb, 4x≈
260 500 Mb; Table 1, Appendix S3). The genome size of hexaploid and decaploid species were
261 closer than those expected with regard to ploidy level (i.e., ratio (6x/2x) = 1.07; ratio (10x/2x)
262 = 1.06; Table 1) with 864 Mb and 1419 Mb, respectively.
263
264 ***Genomic relationships using the GISH technique***
265 For the diploid species, when we hybridized slides of *Lpm* with a *Lpm* probe (red) and
266 *Lh* blocking DNA (grey), 16 chromosomes were tagged in red signals and zero chromosome
267 showed a grey signal (Figure 2A). Thus, the *Lh* blocking DNA did not block any sequence
268 present in the *Lpm* probe. But, when slides of *Lh* were hybridized with a *Lh* probe and *Lpm*
269 blocking DNA, ten chromosomes of *Lh* showed grey signal corresponding to *Lpm*
270 chromosomes (Figure 2B). These two diploid species seem to be genetically close to each other.
271 Due to the absence of chromosomes marked by *Lh* blocking DNA in *Lpm*, we can suggest that
272 *Lpm* and *Lh* correspond to different genomes, arbitrarily noted A for *Lpm* and B for *Lh*.
273

274 For the tetraploid species *Ls* and *La*, we hybridized *Ls* slides with a *Ls* probe and three
275 blocking DNA from species having different ploidy levels – *Lpm* (2x), *Lh* (2x) and *La* (4x) –
276 and for *La* slides, with a *La* probe and *Lh* blocking DNA (Table 2, Figure 3). When *Lpm* DNA
277 was hybridized over *Ls*, the blocking DNA *Lpm* blocked 16 chromosomes (grey) and the other
278 16 chromosomes tagged in red by the *Ls* probe (Figure 3A). A similar result was obtained with
279 the blocking DNA of *Lh*, with 16 chromosomes showing red signals and 16 grey (Figure 3B).
280 Thus, the tetraploid *Ls* would be the result of an interspecific hybridization between the two
281 diploid species *Lpm* and *Lh.* Based on the genome naming proposed here, the genomic
282 composition of *L. stolonifera* could be AABB.

283 After use of *La* blocking DNA over *Ls* chromosomes, we observed 16 chromosomes
284 tagged in red and 16 chromosomes tagged in grey (Figure 3C). The hybridization performed
285 with *Lh* blocking DNA on the second tetraploid, *La*, identified 16 red chromosomes and 16
286 grey chromosomes (Figure 3D). Both results suggested that the two tetraploid species *La* and
287 *Ls* shared a same genome coming from *Lh* (BB component). Thus, *Lh* would also be one of the
288 components of the tetraploid *La*, with a XXBB putative genome composition, where the XX
289 genome corresponds to an unknown *Ludwigia* diploid species.

290 For the hexaploid species *Lgg*, slides of *Lgg* were hybridized with a *Lgg* probe and four
291 blocking DNA of different ploidy levels – *Lpm* (2x), *Lh* (2x), *Ls* (4x), *La* (4x), and *Lgh* (10;
292 Table 2). The *Lpm* competitor DNA blocked 16 chromosomes (tagged in grey) and 32
293 chromosomes showing red signals were hybridized with the *Lgg* probe DNA (Figure 4 A). A
294 similar hybridization was obtained with the *Ls* blocking DNA in which slides of *Lgg* had 16
295 grey chromosomes and 32 chromosomes with red signals (Figure 4 B). Thus, the hexaploid
296 species *Lgg* contains an identical genomic component found in *Ls* (4x) and in *Lpm* (2x; i.e., AA
297 genomic part).

298 Hybridizations performed on slides of *Lgg* with *Lh* (2x) and *La* (4x) blocking DNA
299 exhibited hybridization profiles that were more challenging to interpret with 48 red
300 chromosomes, but with different hybridization intensities (with 16 more intense signals with
301 *La* blocking DNA and 8 less intense signals with *Lh* blocking DNA; Table 2, Figures 4C, 4D).
302 The 16 more intense signals could correspond to a 2x component (16 chromosomes) specific
303 to *Lgg*. However, the concentration of the *La* blocking DNA (10 µg) was probably not sufficient
304 to completely block the common sequences in the 6x compared to the 4x which would explain
305 the 48 red chromosomes with 16 chromosomes more intense compared to 32 chromosomes.
306 For the decaploid species, *Lgh*, slides were hybridized with a *Lgh* probe and five
307 blocking DNA of different ploidy levels, including *Lpm*, *Lh*, *Ls*, *La* and *Lgg*, respectively (Table
308 2). The *Lpm* DNA competitor blocked 32 chromosomes whereby 48 chromosomes showing red
309 signals and 32 grey chromosomes (Figure 5A). An identical hybridization result was obtained
310 with the *Ls* blocking DNA with 48 chromosomes with red signals and 32 grey chromosomes
311 (Figure 5C). Thus, the 2x component, *Lp*, also present in *Ls* (4x), is found in a double dose (32
312 chromosomes) in *Lgh* (10x). The results obtained with the *Lh* and *La* DNA blocking showed
313 80 red chromosomes but 16 with lower intensity (Table 2, Figures 5B, 5D). After GISH
314 hybridization of *Lgg* (6x) DNA on *Lgh* (10x) chromosomes, 32 of 80 *Lgh* chromosomes showed
315 a red signal (Figure 5E). This result revealed that *Lgg* was probably one of progenitors of *Lgh*.
316
317 ***Interspecific hybridization***
318 Interspecific hybridization between species sharing the AA genome were carried out
319 and reproductive success was observed by fruit production when the species used as female
320 possessed the lower ploidy level (Table 3). No fruits were obtained after crosses between *Ls*
321 (4x) used as female and *Lp* (2x) used as male or between *Lgh* (10x) used as female and *Lpm*
322 (2x) or *Ls* (4x) used as male. Thus, all interspecific crosses with the diploid species *Lpm* (2x)

323 used as female and *Ls* (2x) or *Lgh* (10x) used as male gave fruits showing similar weight and
324 length (Table 3). The fruits obtained from the *Lpm* (2x) x *Lgh* (10x) crosses had very large
325 seeds whose development led to the bursting of the fruit walls (Figure S5). However, only
326 53.4% and 3.9% of seeds from *Lpm* (2x) x *Ls* (4x) and *Lpm* (2x) x *Lgh* (10x) crosses germinated.
327 If all germinated seeds gave plantlets for *Lpm* (2x) x *Ls* (2x) crosses, only three plants developed
328 for *Lpm* (2x) x *Lgh* (10x). Finally, no plants survived 90 days after seedling, as all plants showed
329 chlorotic signs and at the end of the observation period, they were not able to survive (Table 3,
330 Appendix S4). Similarly, fruits were produced after *Ls* (4x) x *Lgh* (10x) crosses with a mean
331 number of seeds per fruit of 23.5 (Table 3) but no seed has germinated. Unfortunately, chlorotic
332 plants from *Lpm* (2x) x *Ls* (4x) and *Lpm* (2x) x *Lgh* (10x) crosses did not develop enough roots
333 for chromosome observations. For control intraspecific crosses *Lpm* x *Lpm*, *Lgh* x *Lgh* and *Ls*
334 x *Ls*, all crosses produced fruits revealing the fertility of *Lgh*, *Lpm*, and *Ls* species and
335 effectiveness of the greenhouse pollination conditions.
336

337 **DISCUSSION**

338 ***Ludwigia* species in section *Jussieae***

339 Wagner et al. (2007) summarized the complex history of the Onagraceae. The genus
340 *Ludwigia* forms a lineage separate from the rest of the Onagraceae family (Eyde, 1981, 1982)
341 and is characterized by the absence of the floral tube, pollen in tetrads (lost in some sections,
342 occasionally polyads), and nectaries at the base of stamen (Wagner et al., 2007). *Ludwigia*
343 species of section *Jussiaea* (old section name *Oligospermum*) are described as perennial herbs
344 with terete stems, floating or emergent, rooting at nodes or often forming spongy white
345 pneumatophores from roots, and flowers 5(6)-merous with yellow petals (Hoch et al., 2015).
346 The long-standing taxonomic confusion surround aquatic *Ludwigia* species required a
347 comprehensive approach combining morphometric and cytogenetic evaluations to differentiate

348 the species and improve taxonomic identification (Grewell et al., 2016). In this study,
349 morphological traits were observed for the 6 Lg ssp. here altogether in same conditions.

350 Our morphological observations complement the cytological observations to
351 differentiate species studied here in the field. For the diploid species, rare pneumatophores and
352 yellow flowers in *Lpm* were previously observed (Dandelot, 2004; Armitage et al., 2013).
353 However, the red roots of *Lpm* were never described before, but are visible on the seedlings as
354 soon as the seeds germinate until the plant reaches maturity in natura (Appendix S5). Recently,
355 the morphological traits for *Lh* were reported and emphasized the existence of white spongy
356 pneumatophores emerging from each branch knot and white petals, with basal yellow spot that
357 are obovate and unguiculated, with rounded apex (Rocha and Melo, 2020). *Lh* plants studied
358 had these same characteristics, but the petals were more creamy-white than white and were
359 sharply narrow at the petiole. The existence of abundant clustered conical pneumatophores at a
360 node, petal and root coloration could differentiate these both species (Figure 1).

361 For the tetraploid species, flowers of *La* are described as creamy white petals with
362 yellow at the base (Wagner et al., 2007). We observed white petals similar to *Lh* (Appendix
363 S6). *Ls* had light yellow petals and exhibited a greater number of pneumatophores clustered at
364 a node than was observed for the other tetraploid *La*. However, variation in the production of
365 the pneumatophores from abundant (up to ten pneumatophores/cluster) to occasional
366 pneumatophores (up to three pneumatophores/cluster) at the nodes of floating stems has been
367 reported in different morphotypes of this species. Likewise, the conical or cylindrical form of
368 the pneumatophores has been documented, where both forms are present in the same node
369 (Soliman et al., 2018). Nevertheless, the floral color may a good characteristic with which to
370 distinguish these two tetraploid species (Appendix S4). For the hexaploid species *Lgg*, we only
371 saw pink roots and more morphological investigations are required. Finally, the decaploid

372 species *Lgh* had white roots, one to two pneumatophores per node, and bright yellow petals
373 (Figure 1).
374 Distinguishing *Ludwigia* species in field presents a real challenge. Nesom and Kartesz
375 (2000) suggested that few morphological distinctions between *Lgh* and *Lgg* exist and broadly
376 overlapping: plants with larger leaves and flowers and less dense vestiture characterize *Lgh*,
377 whereas smaller leaves and flowers and denser vestiture would describe *Lgg*. However,
378 comparing flower morphology in sterile and fertile French *Lgh* populations, two flower sizes
379 were observed which may call into question the criterion for distinguishing flower size between
380 *Lgh* and *Lgg* (Appendix S5, Portillo-Lemus et al., 2021). Furthermore, Dandelot (2004) and
381 Armitage et al. (2013) summarized the principal morphological traits that distinguish both
382 species as emergent leaves, leaf surface, venation, stipules, sepals, and pneumatophores. The
383 presence of pneumatophores observable on summer when populations were largely developed,
384 appears as a late criterion to distinguish *Lpm* and *Lgh* and not adapted at early stage of growing.
385 Likewise, the stipule is reniform for *Lpm* and oblong and acuminate for *Lgh* (Thouvenot et al.,
386 2013), but this character is also not easily used. For all these reasons, we propose new criteria
387 to help field managers: the color of roots. *Lpm* has red roots, whereas *Lgh* has white roots.
388 Importantly, this character can be observed at different stages of plant development (Appendix
389 S5). *Lgg* seems to have pink roots at a young plant stage. Whether this characteristic is also true
390 at all stages of *Lgg* development, it could also be a promising way to distinguish *Lgg* and *Lgh*.
391
392 ***Phylogenetic relationships in section Jussieae***
393 We propose the first phylogenetic history of *Ludwigia* diploid to decaploid species
394 belonging to the section *Jussiaea* (Figure 6). The diploid species studied here were composed
395 of two different genomes, we have called AA and BB for *Lpm* and *Lh*, respectively. Both
396 diploid *Lpm* and *Lh* were the progenitors of *Ls,* with the latter composed of AABB (Figure 3).
397 We also found that *Lh* was a progenitor of *La*. Our results are in agreement with phylogenetic

398 analysis of Liu et al. (2017) which suggested that *Lp* or a close relative contributed to the origin
399 of *Ls*. *Lh* and *Ls* shared at least one genome. Furthermore, considering the genome sizes of both
400 diploid species *Lpm* and *Lh* and assuming additivity, our genome size data fit perfectly with our
401 scenarios of tetraploid *Ls* and *La* origin. On the other hand, we showed that *Lpm* also
402 participated for one part (2x) to the origin of the hexaploidy *Lgg* genome. The decaploid species
403 *Lgh* seems to have emerged from interspecific hybridization and allopolyploidization events
404 between the tetraploid species *Ls* (4x) and the hexaploidy species *Lgg*. Liu et al. (2017) also
405 demonstrated a close relationship between *Lgg* and *Lgh* using nuclear and chloroplast DNA
406 regions as molecular markers. In addition, *Lgh* shares the same pneumatophore form as *Lpm*
407 and the same root colour as *Ls*, which may provide further evidence that both species are
408 progenitors of *Lgh*.

409 In addition to these results, interspecific crosses between *Ludwigia* species sharing the
410 A genome produced fruits only when female parent possessed lower ploidy level suggesting
411 that efficiency of pollination was possible through the presence of the same genome in both
412 species. In interspecific crosses differences also exist according to the ploidy level of the female
413 parent. For example in *Brassica ssp.*, more hybrids formed when allotetraploid species,
414 *Brassica napus* is used as female in crosses with diploid species used as male (Kerlan et al.,
415 1992). In contrary, several crosses between *Triticum aestivum* L. and diploid wild relatives were
416 successful provided when female parent had the lower chromosome number (Sharma, 1995).

417 All chromosomes of *Lgg* and *Lgh* were tagged by *Lh* blocking DNA, but had strong or
418 light hybridization intensities for 16 chromosomes respectively. These results may suggest
419 genome divergence between *Lgg* or *Lgh* and *Lh*. The intensity level of the signal over the
420 majority of the chromosomes likely indicates a mixing of genomic sequences between parental
421 genomes, in particular for the *Lh* genome (BB), in the hexaploidy and decaploid formation. The
422 effectiveness of GISH is much reduced, with clear evidence of considerable mixing of genomic

423 sequence between parental DNA. Lim et al. (2007) have shown that within 1 million years of
424 allopolyploid *Nicotiana* divergence, there is considerable exchange of repeats between parental
425 chromosome sets. After *c.*5 million years of divergence GISH fails. Repetitive sequences,
426 including dispersed repeats, such as transposable elements (Tes), or tandem repeats such as
427 satellite DNAs, represent an important fraction of plant genomes that impact evolutionary
428 dynamics (Vicient and Casacuberta, 2017; Giraud et al., 2021). Yet, no exhaustive
429 investigations have been undertaken to evaluate the nature and dynamics of repetitive sequences
430 between different species of *Ludwigia* that probably diversified since hexapolyploid and
431 decapolyploid events when the *Ludwigia* family originated at least 50 m.y. ago (Raven and Tai,
432 1979).

433 Natural hybrids within section *Jussieae* have been reported between *La* (2n = 32) and
434 *L. peploides* subsp. *stipulacea* (2n=16), with production of a triploid sterile hybrid (2n = 3x=
435 24) named *L. x taiwanensis* (Peng, 1990). Between *Lgg* (2n = 48) and *Lgh* (2n = 80), an
436 octoploid hybrid was produced (2n = 8x = 64) and between *Lgg* (2n = 48) and *L. hookeri* (2n =
437 32), a pentaploid hybrid was produced (2n = 5x = 40) (Zardini et al., 1991; Zardini and Raven,
438 1992). For our *Lpm* x *Lgh* crosses, we obtained fruit production after each pollination. Despite
439 the production of a significant seed number, very low germination was found, with no viable
440 plants. Dandelot (2004) reported that in France, hybrids between *Lpm* and *Lgh* have never been
441 recorded in nature, whereas hybrids have been created under experimental conditions. But if
442 Dandelot (2004) obtained fruit from *Lpm* x *Lgh* crosses, the ability of seeds to germinate and
443 viability of plantlets were not analyzed. As found by Dandelot (2004), we found zero fruit
444 production when *Lgh* was used as female.

445 All interspecific crosses using the lower ploidy of *Ludwigia* ssp. as female were
446 functional and fruits were produced. But depending on the type of interspecific crosses, no
447 viable seeds or necrotic plants were obtained. Crosses between related species or parents with

448 different ploidy are often impossible due to post-zygotic reproductive barriers in which the
449 hybrid progeny fails to develop or becomes sterile. Thus, in crosses between *B. napus* and a
450 more distant species such as *Sinapis alba*, the interspecific hybridization efficiency is also
451 extremely low and embryos need to be rescued using fertilized ovary culture (Chèvre et al.,
452 1994). This indicated an early abortion of seeds after fertilization and the parental genome
453 dosage in the endosperm plays an important role for seed collapse.

454 Interspecific hybrids between *Ludwigia* spp. in section Jussieae seem possible only if
455 interspecific crosses occur between a female plant with lower ploidy level than male plant, and
456 probably at a very low success rate in natura. However, observing fruit production is not
457 enough, thus, we recommend observing seed germination, plantlet viability, plant survival, and
458 chromosome counts.

459

460 ***Combination of different data to identify phylogenetic relationships within a polyploid***
461 ***complex.***

462 In this study we demonstrated the interest of a truly novel combination of data to rebuilt
463 the phylogeny within a polyploid complex. One way to investigate phylogenetic relationship in
464 a polyploid complex is to use of flow cytometric analyses complemented with chromosome
465 counts, as recently described for the analysis of polyploid complex *Linum suffruticosum s.l.*
466 (Linaceae) (Afonso et al., 2021). The authors concluded that if genome size and/or chromosome
467 counts might be useful tools for identifying polyploid complex *L. suffruticosum s.l.*, further
468 studies were necessary to identify origin of the not easy disentangle polyploid complex. Another
469 way but more expensive approach to phylogenetic studies involves (i) the use of organellar
470 DNA (chloroplast or nuclear regions) as molecular markers as it was described for phylogenetic
471 analysis of the genus *Isoëtes* (Pereira et al., 2019) or the diploid and autohexaploid cytotypes
472 of *Aster amellus* (Mairal et al., 2018); or (ii) OMICS-data tools as RAD-Seq (restriction site-

associated DNA sequencing) as described in the evolutionary processes of apomictic polyploid complexes on the model system *Ranunculus* (Karbstein et al., 2022).

Thus, the different approaches used in this study, combining morphological, cytogenetic and in situ hybridization analyses and interspecific crosses, could serve as a less expensive model to approach phylogenetic studies of species belonging to poorly known species complexes for which few genomic resources exist in the future.

CONCLUSION

In conclusion, our results suggest allopolyploidy played an important role in the evolutionary history of the *Ludwigia* L., section *Jussieae*, giving rise to complex relationships among species. However, some species are missing in our analyses as well as in Liu et al. (2017). The missing species of section *Jussiaea* are the four diploid species following, *Ludwigia peploides* (Kunth) P.H.Raven subsp. *glabrescens* (O. Kuntze) P.H.Raven, *Ludwigia peploides* subsp. *peploides*, *Ludwigia peploides* subsp. *stipulacea* (Ohwi) P.H.Raven, *Ludwigia torulosa* (Arn.) H.Hara. and the two tetraploid species, *Ludwigia hookeri* (Micheli) H.Hara, *Ludwigia peduncularis* (C.Wright ex Griseb.) M.Gómez (Hoch et al., 2015). As one part of the phylogenetic relationships remains unresolved, new GISH experiments must be done with these species, especially to identify the progenitor of the unknown 2x and 4x genome of *Lgg* and *Lgh*, respectively. Furthermore, as based on morphological observations, Zardini et al. (1991) suggested that *Lgh* may be result of interspecific hybridization between *Lgg* and *L. hookeri*, the tetraploid species *L. hookeri* could be one of progenitor of missing genomes of *Lgg* and *Lgh* species.

Conflict of interest

The authors declare they have no conflict of interest relating to the content of this article.

498

499 *Acknowledgments*

500 This research was supported by FEDER funds from Région Centre-Val de Loire and by Agence
501 de l'eau Loire-Bretagne (grant Nature 2045, programme 9025 (AP 2015 9025). FEDER also
502 financed the doctoral grant of L. Portillo – Lemus. Collections in Alabama were supported by
503 the Department of Biology at the University of Alabama at Birmingham with field help from
504 S. Heiser and S. Shainker-Connelly.

505

506 *Author Contributions*

507 DB, LMP and OC contributed to conception and design of experiments. LPM and DB provide
508 roots and DNA of all Lg species for *in situ* hybridization and ploidy level analysis. SAKH
509 collected Lgg samples from USA. VH and OC acquired GISH and cytological data. BD, LMP
510 and OC carried out analysis and interpretation of data. DB write the draft of this manuscript
511 and DB, LPM, SAKH and OC revised the manuscript. All authors gave a final approval of the
512 version to be published.

513

514 *Supporting Information*

515 Appendix S1: Original images of In situ genomic hybridation analyses of somatic metaphase
516 chromosomes, a) of *L. peploides* subsp. *montevidensis* 2(n=2x=16). corresponding to figure 2 ,
517 b) ; of the tetraploid species, *L. stolonifera* and *L. adscendens* (2n=4x=32) corresponding to
518 figure 3 ; of *L. grandiflora subsp. grandiflora* (2n=6x=48) corresponding to figure 4 ; from *L.*
519 *grandiflora* subsp. *hexapetala* (2n=10X=80) corresponding to figure 5.

520

521 Appendix S2: Polyploidy levels of different species of ludwigia sp section Jussieae. (**A**)
522 *Ludwigia peploides* subsp. *montevidensis* chromosomes (2n=2x=16), (**B**) *Ludwigia*

523 *helminthorrhiza* chromosomes (2n=2x=16); (**C**) *Ludwigia stolonifera* chromosomes
524 (2n=4x=32); (**D**) *Ludwigia adscendens* chromosomes (2n=4x=32); (**E**) *Ludwigia grandiflora*
525 subsp. *grandiflora* (2n=6x=48); *Ludwigia grandiflora* subsp. *hexapetala* (2n=10x=80).
526 Chromosome number correspond to ploidy level: 16 chromosomes for diploid species (**A**) and
527 (**B**); 32 chromosomes for tetraploid species (**C**) and (**D**); 48 chromosomes for hexaploid species
528 (**E**) and 80 chromosomes for decaploid species (F)
529 Appendix S3: Flow Cytometry results (**A**) and examples of peak profiles (logarithmic) in the
530 flow cytometer of nuclei stained from roots with propidium iodide (PI) (**B**). The 'trifolium
531 repens' peak (1C=1,12 pg) or "Zea mays" peak (1C=2,77 pg) is used as internal standard to
532 determinate the DNA contents of the sample nuclei (*). (1) *Ludwigia peploides* subsp.
533 *montevidensis*; (2) *L. helminthorrhiza;* (3) *L. adscendens*; (4) *L. grandiflora* subsp. *grandiflora*
534 and (5) *L. grandiflora* sp. *hexapetala*
535
536 Appendix S4: Fruit production and seedling from interspecific hybridization between ludwigia
537 species possessing A genome ; *Lpm* = Ludwigia peploides subsp. montevidensis (2n=16, AA)
538 ; *Ls = Ludwigia stolonifera* (2n=32, AABB); *Lgh = Ludwigia grandiflora* subsp. *hexapetala*
539 (2n=80, AAAABBXXXX/XXYY). (a) the seeds produced from *Lpm* x *Lgh* interspecific cross
540 are large, which has led to the fruit bursting. (b) 30 days after seedling, green plantlets from
541 *Lpm* x *Ls* interspecific cross were obtained. But, 60 days later, plants showed chlorotic
542 development, stopped growing and died. Das : Number of day after seedling
543
544 Appendix S5: Morphological traits to distinguish *Ludwigia peploides* subsp. *montevidensis* and
545 *Ludwigia grandiflora* subsp. *hexapetala,* (**a**) roots at seedling stage ; (**b**) adult roots in natura ;
546 (**c**) pneumatophores in natura ; (**d**) flowers.
547

Appendix S6: Size and color of Ludwigia sp. flowers. **a**: Flower of *L. grandiflora* subsp. *hexapetala* in sterile population (10x), **b**: Flower of *L. grandiflora* subsp. *hexapetala* in fertile population (10x), **c**: Flower of *L. peploides* subsp. *montevidensis* (2x), **d**: Flower of *L. adscendens* (4x) and **e**: Flower of *L. stolonifera* (4x)

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714 Tables

715

716 Table 1: Ploidy levels, chromosome numbers and genome sizes estimated by flow cytometry

717 in Ludwigia L. spp. sect. *Jussiaea*.

718 Species names are mentioned according to the revised nomenclature by Hoch et al. (2015).

719 Genome sizes were converted from picograms (pg) to Megabases (Mb) using 1 pg = 978 Mbp.

720

Species name	**Ploidy and chromosome numbers**	**Genome size (pg/2C)**	**Genome size (Mb)**
Ludwigia peploides subsp. *montevidensis (Lpm)*	2n= 2x= 16	0.53	262
Ludwigia helminthorrhiza (Lh)	2n= 2x= 16	0.55	268
Ludwigia adscendens (La)	2n= 4x= 32	1.06	520
Ludwigia stolonifera (Ls)	2n= 4x= 32	1.07	522
Ludwigia grandiflora subsp. *grandiflora (Lgg)*	2n= 6x= 48	1.77	864
Ludwigia grandiflora subsp. *hexapetala (Lgh)*	2n= 10x= 80	2.09	1419

721

Table 2: Results of GISH with different Ludwigia L. probes (red) combined with blocking DNA (grey) on *L. peploides* subsp. *montevidensis* (Lpm), *L. helminthorrhiza* (Lh), *L. adscendens* (La), *L. grandiflora* subsp. *grandiflora* (Lgg) and *L. grandiflora* subsp. *hexapetala* (Lgh) chromosomes.

Chromosomes of one species tagged in red correspond to DNA of this species and chromosomes tagged in grey are blocked by DNA of others species.

Chromosomes / Blocking DNA	***Lpm*** **(2n = 16)**	***Lh*** **(2n = 16)**	***Ls*** **(2n = 32)**	***La*** **(2n= 32)**	***Lgg*** **(2n= 48)**	***Lgh*** **(2n= 80)**
Lpm **(2n = 16)**		4 red signals + 10 grey signals	16 red signals + 16 grey signals		32 red signals + 16 grey signals	48 red signals + 32 grey signals
Lh **(2n = 16)**	16 red signals		16 red signals + 16 grey signals	16 red signals + 16 grey signals	48 red signals (8 less intense)	80 red signals (16 less intense)
Ls **(2n = 32)**					32 red signals + 16 grey signals	48 red signals + 32 grey signals
La **(2n = 32)**			16 red signals + 16 grey signals		48 red signals (16 more intense)	80 red signals (16 less intense)
Lgg **(2n = 48)**						32 red signals + 48 grey signals

742 Table 3: Reproductive success after controlled interspecific crosses between different Ludwigia
743 L. spp. belonging to the section *Jussiaea*.
744 Interspecific hybridization (female x male) between the three species, *Ludwigia peploides*
745 subsp. *montevidensis* (Lpm), *Ludwigia stolonifera* (Ls) and/or *Ludwigia grandiflora* subsp.
746 *hexapetala* (Lgh, AAAA XXXX/XXYY) used as female or male. All species possess same
747 genome A: Lpm (2x, AA); Ls (4x, AABB); Lgh (10x, AAAA XXXX or XXYY). Number of
748 plantlets and plants were counted three (21 days) and 8 weeks (56 days) after seed germination,
749 respectively. NA: data not available. (+/-= confidence interval, α=0.05). For control
750 interspecific crosses *Lgh* x *Lgh* and *Lpm* x *Lpm*, a set of randomly selected plantlets were
751 followed until 56 days after seed germination.

Controlled interspecific crosses	*Lpm* x *Ls*	*Lpm* x *Lgh*	*Ls* x *Lpm*	*Ls* x *Lgh*	*Lgh* x *Lpm*	*Lgh* x *Ls*	*Lgh x Lgh*	*Lpm x Lpm*
Number of cross pollination	8	25	10	2	10	10	75	45
Number of fruits	8	25	0	2	0	0	75	45
Mean length of fruit (mm)	15.08 (+/- 0.78)	16.64 (+/- 0.82)	/	NA	/	/	7	NA
Mean fruit weight (g)	62.04 (+/- 6.46)	64.64 (+/- 6.02)	/	NA	/	/	NA	NA
Number of total seed	221	1101	/	47	/	/	3750	1980
Number of germinated seeds	118	34	/	0	/	/	3375	1881
Number of plantlets 21 days	118	3	/	0	/	/	3750	1881

Number of plants 56 days	0	0	/	0	/	/	100 from a set of 100	50 from a set of 50

755 **Legends of figures:**

756 Figure 1: Morphological traits of Ludwigia L. species in section *Jussiaea*.
757 Ludwigia L. species are classified in a phylogenic tree as proposed by Liu et al (2017). Three
758 morphological traits were observed (color of roots, pneumatophore form, color of flower).

759

760 Figure 2: In situ genomic hybridation analyses of somatic metaphase chromosomes from
761 *Ludwiga peploides* subsp. *montevidensis* (2n=2x=16) using *Ludwigia peploides* subsp.
762 *montevidensis* probe (2x) (red) and *Ludwigia helminthorrhiza* (2x) (10µg) as blocking DNA
763 (A) and from *L. helminthorrhiza* (2n=2x=16) using *L. helminthorrhiza* probe (2x) and *L.*
764 *peploides* subsp. *montevidensis* (2x) (10µg) as blocking DNA (B).
765 Thus, GISH reveals specifically 16 red signals (white stars) and 0 *L. peploides* subsp.
766 *montevidensis* chromosomes (grey) (A) and 4 red signals (white stars) and 10 *L.*
767 *helminthorrhiza* chromosomes (grey) (B). Chromosomes were counterstained with DAPI
768 (grey). Bar represents 5 µm.

769

770 Figure 3: In situ genomic hybridation analyses of somatic metaphase chromosomes of the
771 tetraploid species, *Ludwigia stolonifera* and *Ludwigia adscendens* (2n=4x=32).
772 GISH was carried out for *L. stolonifera* using *L. stolonifera* probe (4x) (red) and *Ludwigia*
773 *peploides* subsp. *montevidensis* (2x) (10µg) as DNA blocking (A), *Ludwigia helminthorrhiza*
774 (2x) as block (B) and *L. adscendens* (4x) as block (C) and for *L. adscendens* (4x) using *L.*
775 *adscendens* probe (4x) (red) and *L. helminthorrhiza* (2X) (10µg) as block (D). Thus, GISH
776 revealed for *L. stolonifera* specifically 16 red signals (white stars) and 16 *L. peploides* subsp.
777 *montevidensis* chromosomes (grey) (A), 16 red signals (white stars) and 16 *L. helminthorrhiza*
778 chromosomes (grey) (B), 16 red signals (white stars) and 16 *L. adscendens* chromosomes (grey)

779 (C) and for *L. adscendens* 16 red signals (white stars) and 16 *L. helminthorrhiza* chromosomes
780 (grey) (D). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.
781
782 Figure 4: In situ genomic hybridation analyses of somatic metaphase chromosomes from *L.*
783 *grandiflora* subsp. *grandiflora* (2n=6x=48) using *Ludwigia grandiflora* subsp. *grandiflora*
784 probe (6x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (A), *Ludwigia*
785 *helminthorriza* (2x) (10µg) as block (B), *Ludwigia stolonifera* (4x) (10µg) as block (10µg) as
786 block (C), *Ludwigia adscendens* (4x) (10µg) as block (D), *Ludwigia grandiflora* subsp.
787 *hexapetala* (10x) as block (E).
788 Thus, GISH reveals specifically 32 red signals (white star) and 16 *L. peploides* chromosomes
789 (grey) (A), 48 red signals with 8 present less intensity (white star) (B), 32 red signals (white
790 star) and 16 *L. stolonifera* chromosomes (grey) (C) and 48 red signals with 16 present more
791 intensity (white star) (D). Chromosomes were counterstained with DAPI (grey). Bar represents
792 5 µm.
793
794 Figure 5: In situ genomic hybridation analyses of somatic metaphase chromosomes from
795 *Ludwigia grandiflora* subsp. *hexapetala* (2n=10X=80) using *L. grandiflora* subsp. *hexapetala*
796 probe (10x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (10µg) as block (A),
797 *Ludwigia helminthorrhiza* (2x) as block (B), *Ludwigia stolonifera* (4x) (10µg) as block (C),
798 *Ludwigia adscendens* (4x) as block (D) and *L. grandiflora* subsp. *grandiflora* (6x) as block (E).
799 Thus, GISH reveals specifically 48 red signals and 32 *L. peploides* chromosomes (grey) (A),
800 80 red signals and 16 present less intensity (white stars) (B), 48 red signals and 32 *L. stolonifera*
801 chromosomes (grey) (C), 80 red signals and 16 present less intensity (white stars) (D) and 32
802 red signals and 48 *L. grandiflora* subsp. *grandiflora* (grey) (E). Chromosomes were
803 counterstained with DAPI (grey). Bar represents 5 µm.

Figure 6: Hypothetical phylogenetic history of Ludwigia L. species of section Jussieae

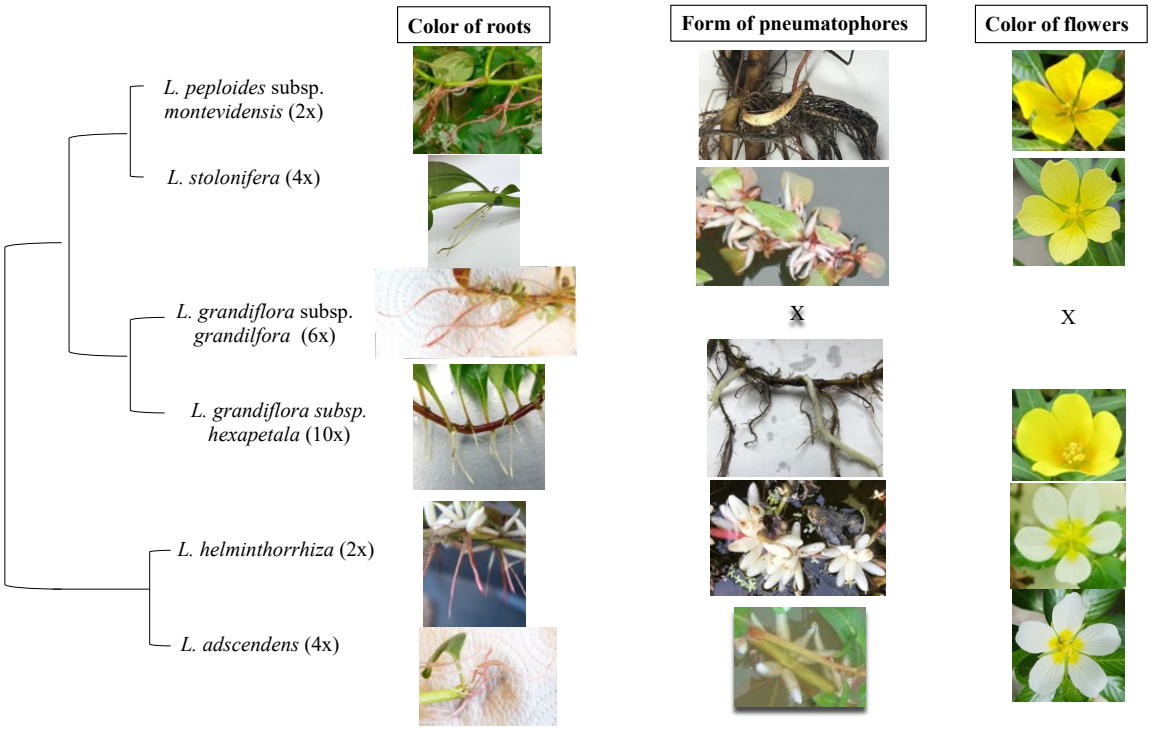

Figure 1: Morphological traits of Ludwigia L. species in section *Jussiaea*. Ludwigia L. species are classified in a phylogenic tree as proposed by Liu et al (2017). Three morphological traits were observed (color of roots, pneumatophore form, color of flower).

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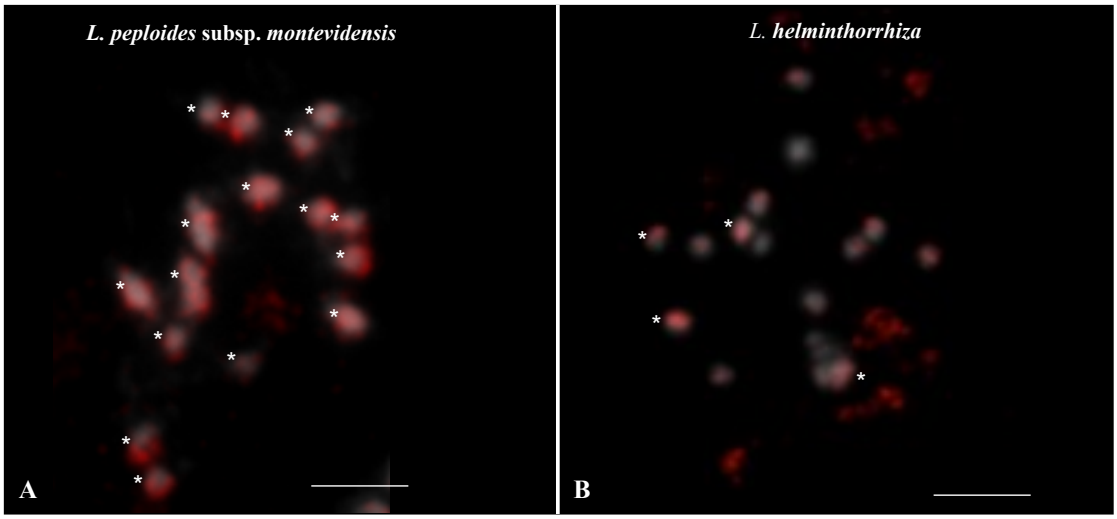

818
819

820 Figure 2: In situ genomic hybridation analyses of somatic metaphase chromosomes from
821 *Ludwigia peploides* subsp. *montevidensis* (2n=2x=16) using *L. peploides* subsp. *montevidensis*
822 probe (2x) (red) and *Ludwigia helminthorrhiza* (2x) (10µg) as blocking DNA (A) and from *L.*
823 *helminthorrhiza* (2n=2x=16) using *L. helminthorrhiza* probe (2x) and *L. peploides* subsp.
824 *montevidensis* (2x) (10µg) as blocking DNA (B).
825 Thus, GISH reveals specifically 16 red signals (white stars) and 0 *L. peploides* subsp.
826 *montevidensis* chromosomes (grey) (A) and 4 red signals (white stars) and 10 *L.*
827 *helminthorrhiza* chromosomes (grey) (B). Chromosomes were counterstained with DAPI
828 (grey). Bar represents 5 µm.

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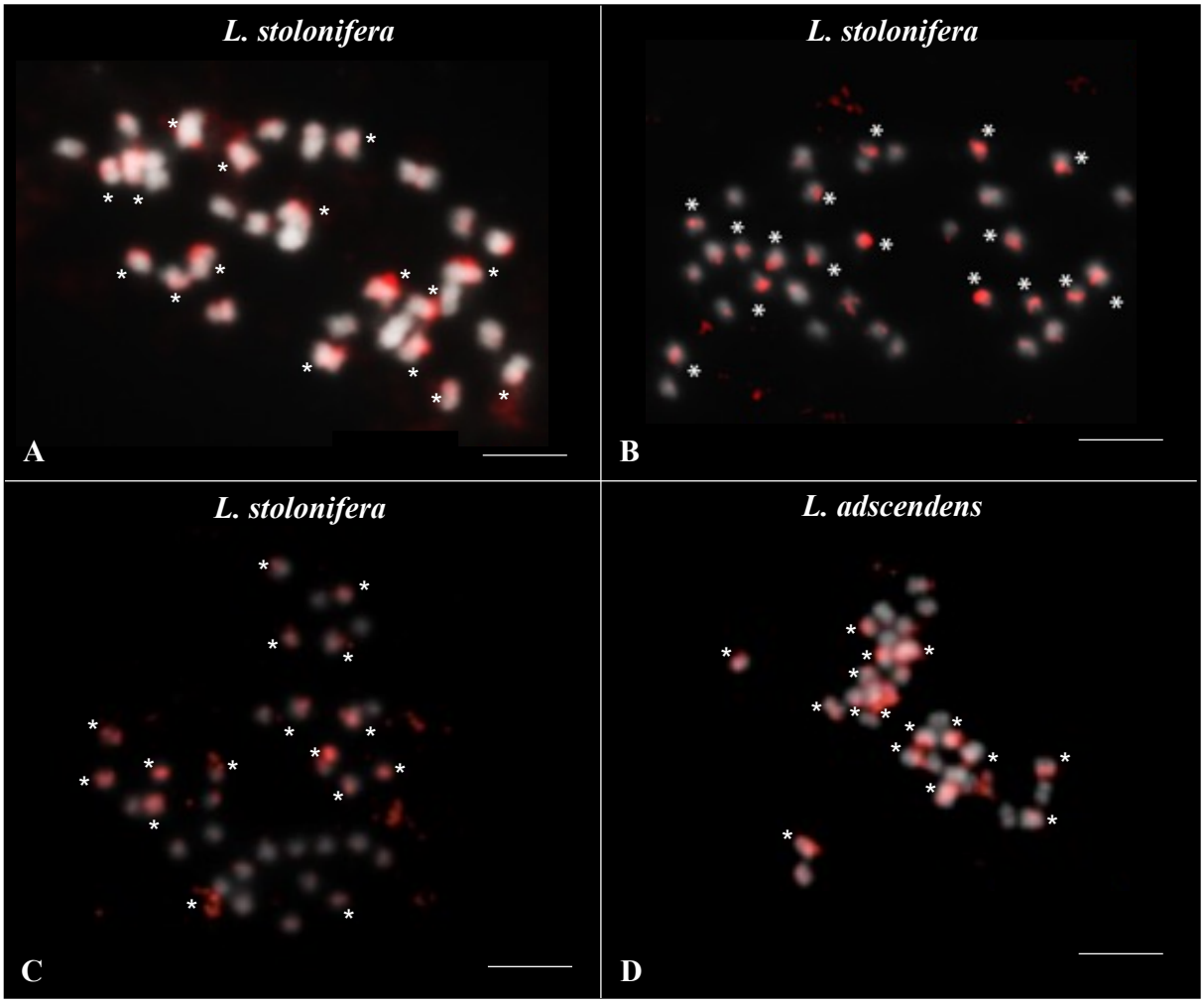

Figure 3: In situ genomic hybridation analyses of somatic metaphase chromosomes of the tetraploid species, *Ludwigia stolonifera* and *Ludwigia adscendens* (2n=4x=32).

GISH was carried out for *L. stolonifera* using *L. stolonifera* probe (4x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (10µg) as DNA blocking (A), *Ludwigia helminthorrhiza* (2x) as block (B) and *L. adscendens* (4x) as block (C) and for *L. adscendens* (4x) using *L. adscendens* probe (4x) (red) and *L. helminthorrhiza* (2X) (10µg) as block (D) . Thus, GISH revealed for *L. stolonifera* specifically 16 red signals (white stars) and 16 *L. peploides* subsp. *montevidensis* chromosomes (grey) (A), 16 red signals (white stars) and 16 *L. helminthorrhiza* chromosomes (grey) (B), 16 red signals (white stars) and 16 *L. adscendens* chromosomes (grey) (C) and for *L. adscendens* 16 red signals (white stars) and 16 *L. helminthorrhiza* chromosomes (grey) (D). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

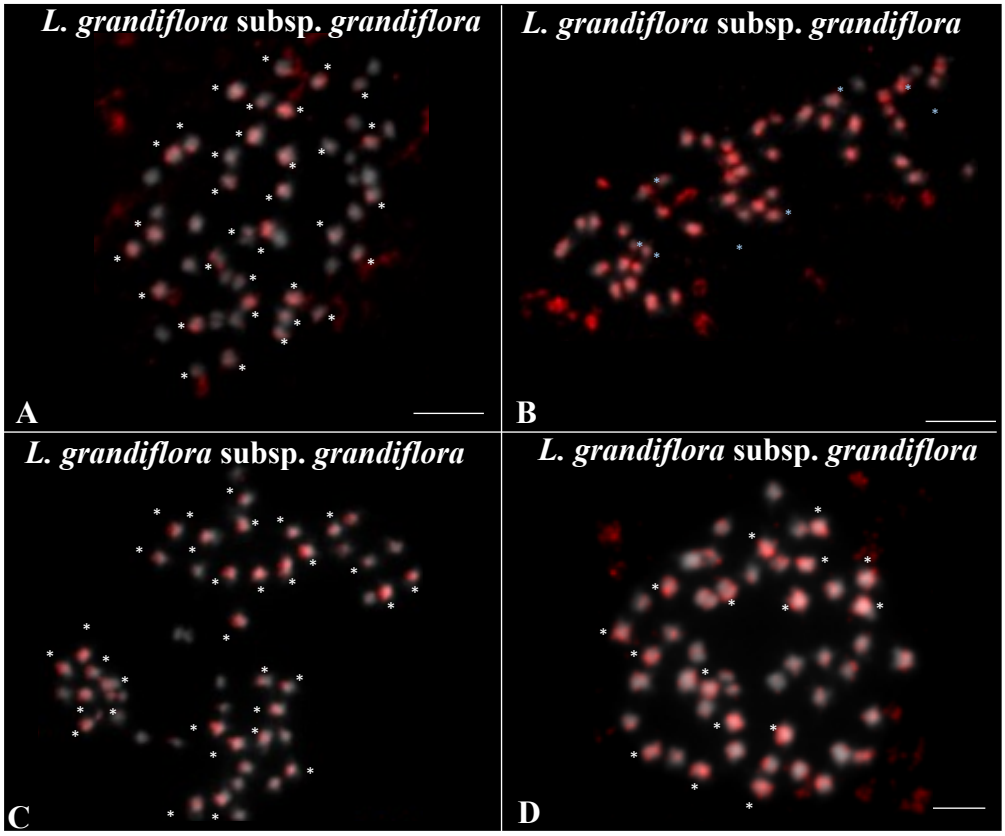

Figure 4: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwigia grandiflora subsp. grandiflora* (2n=6x=48) using *L. grandiflora subsp. grandiflora* probe (6x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (**A**), *Ludwigia helminthorriza* (2x) (10µg) as block (**B**), *Ludwigia stolonifera* (4x) (10µg) as block (10µg) as block (**C**), *Ludwigia adscendens* (4x) (10µg) as block (**D**), *Ludwigia grandiflora* subsp. *hexapetala* (10x) as block (**E**).

Thus, GISH reveals specifically 32 red signals (white star) and 16 *L. peploides* chromosomes (grey) (**A**), 48 red signals with 8 present less intensity (white star) (**B**), 32 red signals (white star) and 16 *L. stolonifera* chromosomes (grey) (**C**) and 48 red signals with 16 present more intensity (white star) (**D**). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

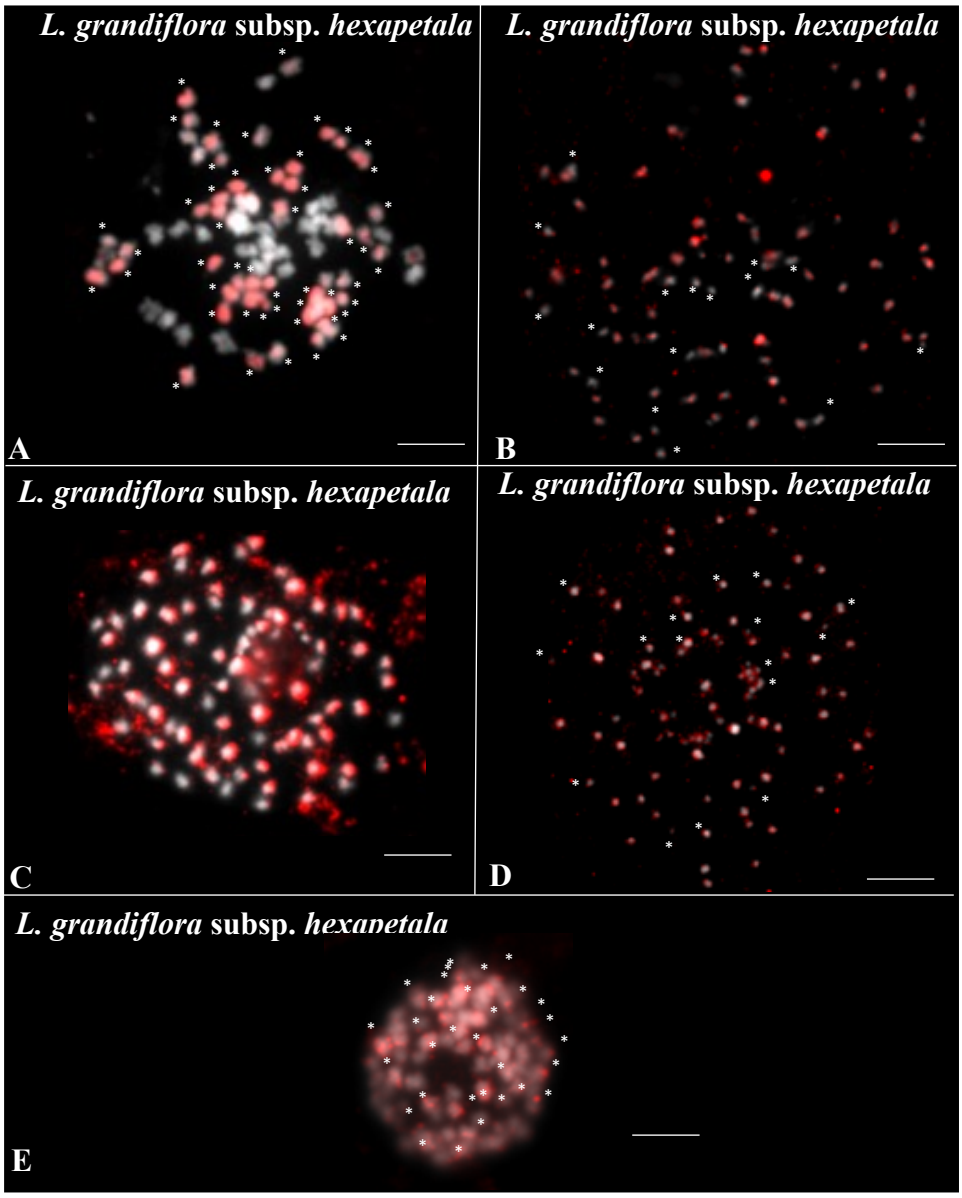

Figure 5: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwigia grandiflora* subsp. *hexapetala* (2n=10X=80) using *L. grandiflora* subsp. *hexapetala* probe (10x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (10µg) as block (**A**), *Ludwigia helminthorrhiza* (2x) as block (**B**), *Ludwigia stolonifera* (4x) (10µg) as block (**C**), *L. adscendens* (4x) as block (**D**) and *Ludwigia grandiflora* subsp. *grandiflora* (6x) as block (**E**). Thus, GISH reveals specifically 48 red signals and 32 *L. peploides* chromosomes (grey) (**A**), 80 red signals and 16 present less intensity (white stars) (**B**), 48 red signals and 32 *L. stolonifera* chromosomes (grey) (**C**), 80 red signals and 16 present less intensity (white stars) (**D**) and 32

870 red signals and 48 *L. grandiflora* subsp. *grandiflora* (grey) (E). Chromosomes were

871 counterstained with DAPI (grey). Bar represents 5 µm.

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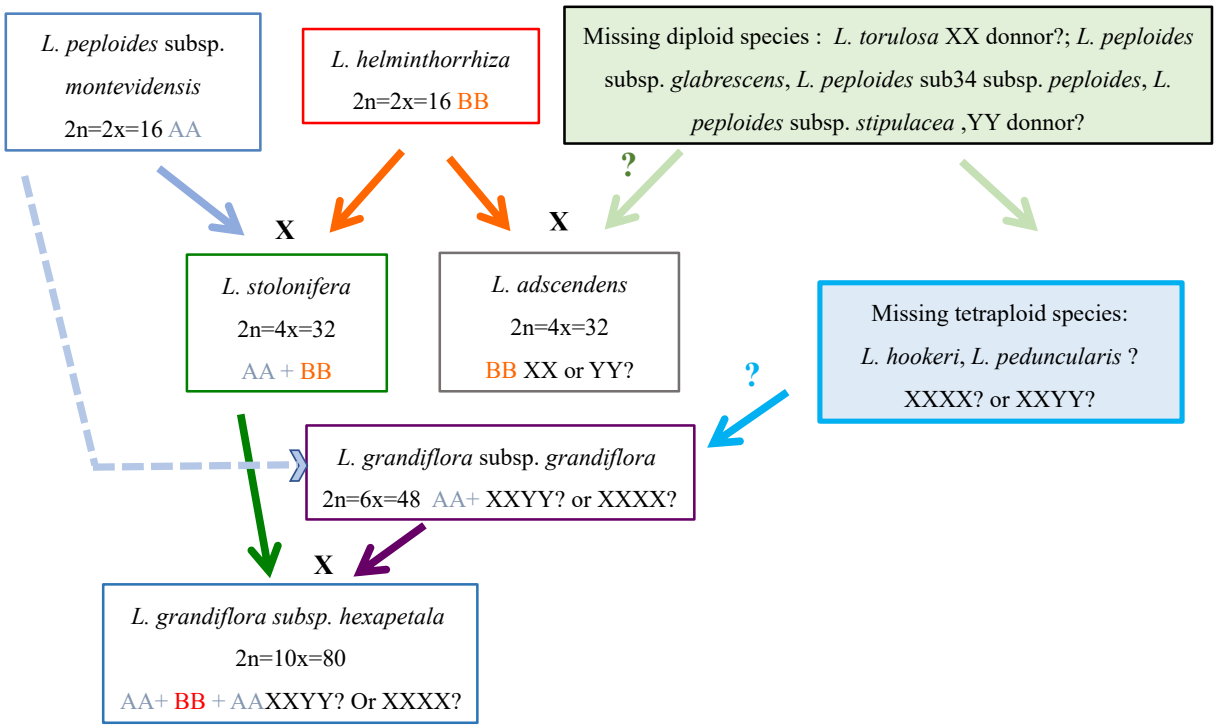

Figure 6: Hypothetical phylogenetic history of ludwigia species of section Jussieae