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

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

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

MATERIAL AND METHODS

Plant material

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

of *L. grandiflora* subsp. *hexapetala* (hereafter, *Lgh*) (10x) were collected in France at the marshes of la Musse (47°14’27.5”N, 1°47’21.3”W) and Mazerolles (47°23’16.3”N, 1°28’09.7”W), respectively. Ten plants of the diploid species *L. helminthorrhiza* (hereafter, *Lh)* was purchased in aquarium store (provider Ruinemans Aquarium B.V. Netherland). Five plants of *Ludwigia adscendens* (L.) H. HARA (4x) (hereafter, *La*), and of *L. stolonifera* (4x) (hereafter, *Ls*) and ten of *L. grandiflora* subsp. *grandiflora* (6x) (hereafter, *Lgg*) were collected in Flores island, Indonesia (Pulau Flores; 8°49'40.8"S, 120°48'39.0"E), Lebanon (Hekr al Dahri; 34°37'54.5"N, 36°01'28.9"E), and the USA (Co. Rd 73, outside Greensboro, AL; 32°61’51.41”N, 87°68’65.4”W), respectively. All Ludwigia species growth preferentially by clonal reproduction (Okada et al., 2009; Glover et al., 2015) and the plants were easily maintained in the greenhouse at Institut Agro Rennes - Angers before analysis (Portillo-Lemus et al, 2021).

Morphology

We recorded the colour of the flowers and roots and the pneumatophore form to characterize collected *Ludwigia* species, following the morphological traits considered in the *Ludwigia* sp. description (Armitage et al., 2013; Thouvenot et al., 2013). Morphological observations for each species were randomly made on at least 30 plants in the greenhouse and confirmed in natura on 15 plants in 15 and 36 populations of *Lpm* and *Lgh* in France, respectively.

Chromosome counting

Root tips of 0.5 - 1.5 cm in length were taken for each *Ludwigia* sp. as follows from 15 *Lpm*; ten *Lh*; five *La*; five *Ls*; ten *Lgg* and 15 *Lgh* different plants and were incubated in 0.04% 8-hydroxiquinoline for 2 hours at room temperature in the dark, followed by 2h at 4°C to

accumulate metaphases. Tips were then fixed in 3:1 ethanol-glacial acetic acid for 48 hours at 4°C and stored in ethanol 70 % at -20 °C. Before use, tips were washed in 0.01 M citric acid-sodium citrate buffer (pH 4.5) for 15 min and then digested in a solution of 5% Onozuka R-10 cellulase (Cat No. C1794, Sigma), 1% Y23 pectolyase (Cat No. P5936, Sigma) at 37 °C for 45 min. The digested root tips were then carefully washed with distilled water for 30 min. One root tip was transferred to a slide and macerated with a drop of 3:1 fixation solution. The slides were dried at room temperature and stored at -20°C until 4',6-diamidino-2-phenylindole (DAPI) staining. At least four roots per species were observed. The chromosome counts per species were estimated on a total of 20 cells at the mitotic metaphase stage using the visualization software Zen 2 PRO (Carl Zeiss, Germany).

Genome size estimation by flow cytometry

To explore the genome size among the different *Ludwigia* spp., we used flow cytometry. Approximately 4 mg of fresh roots or leaves from five plants of *Ludwigia* spp. and of fresh leaves from five plants of *Trifolium rupens or Zea mays* (used as an internal reference standard for *Lpm*, *Lh* and *Lgh* species and *Ls*, *La*, *Lgg* and *Lgh* species, respectively) were harvested and transferred to a Petri dish. This material was finely chopped using a sharp razor blade in 500 µl of staining buffer (from Cystain PI OxProtect, Cat No. 05-5027) and incubated at room temperature for 30 sec to 90 sec. The solution was then filtered through a 50 µm nylon mesh and 1.5 ml of solution (0.0166 mg of RNase A and 10 µl of Propidium Iodide) was added per sample. Incubation at room temperature was made for 30 min to 60 min, protected from light. Estimation of genome size for each species was obtained using a CyFlow space cytometer (Sysmex Corp., Kobe, Japan). This instrument was equipped with a 488 nm blue laser 50 mW and a band-pass filter LP590 used as an emission filter. Prior to running the samples, gain and linearity of the instrument were adjusted by using DNA control PI from Sysmex. Finally, G1

peaks in *Ludwigia* spp. and *Trifolium rupens* or *Zea mays* were collected for each sample to calculate nuclear DNA content (1C) and haploid genome size (Mbp). For the different Ludwigia spp., two or three measures of genome size were made, excepted for *Ls* (only one measure). From each species, the mean ratio of DNA content was calculated (mean + CI (Confidence Interval), P= 0.05)). Genome sizes were converted from picograms (pg) to Megabases (Mb) using 1 pg = 978 Mbp (Dolezel et al., 2003).

Genomic in situ hybridization (GISH)

DNA was extracted from 30 mg of freeze-dried buds taken from 15 *Lpm*, ten *Lh*, five *Ls*, five *La*, ten *Lgg,* and 15 *Lgh* plants, using the Macherey-Nagel extraction kit NucleoSpin® Food to which we have made following modifications to obtain a polysaccharide free DNA: (1) after lysis step with Buffer CF, we mixed freeze-dried buds with an equivalent volume of PCIA 25:24:1 (parts of phenol, chloroform, isoamyl alcohol) for 5 minutes ; (2) then we transferred the whole in a tube containing phase-look gel and centrifuged at 800rpm for 5 minutes (Quantabio, Massachusetts, USA); (3) then the DNA was precipitated using absolute ethanol at -18°C instead of QW and C5 buffers. Finally, the DNA was resuspended after an incubation of 5 min in 100 ml elution buffer with 5 mM TRIS at pH 8.5 at 65°C. Total genomic DNA were used as probes and labelled by random priming with biotin-14-dCTP (Invitrogen by Thermo Fisher Scientific).

Total genomic DNA used as a blocking DNA was autoclaved to yield fragments of 100-300 bp. The ratio DNA probe / blocking DNA was 1:50. The hybridized probes correspond to the chromosomes present on the slide (i.e., same species) and genomic DNA (blocking DNA) from different species were used as competitors in to block the common sequences at both species.

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

Controlled interspecific crosses

Controlled interspecific pollinations were carried out in the greenhouse between *Ludwigia* species which putatively shared the same parental genome. Thus, interspecific hybridizations were made between *L. peploides* subsp. *montevidensis*, *L. stolonifera* and/or *L.*

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

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

RESULTS

Morphological traits of Ludwigia species

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

Genome size and ploidy level

The chromosome numbers were as excepted: for both diploids, *Lpm* and *Lh* 2n = 16, for both tetraploids *Ls* and *La* 2n =32, for hexaploid *Lgg* 2n=48 and for decaploid, *Lgh* 2n=80 (Table 1, Appendix S2). *Ludwigia* spp. exhibited an ~0.77-fold range of C-values. The lowest value, 0.53 pg/2C, was found in *Lpm* and the highest, 2.9pg/2C, in *Lgh* (Table 1, Appendix S3). The tetraploid species *Ls* (1.07pg/2C) and *La* (1.06pg/2C) have C-values that were twice that the value for the diploid *Lpm* (0.53pg/2C) and *Lh* (0.55pg/2C). The hexaploid species *Lgg* had C-value 1.77pg/2C. Thus, the genome size by ploidy level revealed that the monoploid genome sizes (1Cx-value, 0.133-0.147 pg) of the tetraploid, hexaploid, and decaploid species are the same (0.34-0.49 pg/1Cx). The difference is accounted for by the higher ploidy levels.

Ludwigia genome sizes of diploid and tetraploid species were similar between species with the same ploidy level and varied proportionally with ploidy levels (i.e., 2x≈260 Mb, 4x≈ 500 Mb; Table 1, Appendix S3). The genome size of hexaploid and decaploid species were closer than those expected with regard to ploidy level (i.e., ratio (6x/2x) = 1.07; ratio (10x/2x) = 1.06; Table 1) with 864 Mb and 1419 Mb, respectively.

Genomic relationships using the GISH technique

For the diploid species, when we hybridized slides of *Lpm* with a *Lpm* probe (red) and *Lh* blocking DNA (grey), 16 chromosomes were tagged in red signals and zero chromosome showed a grey signal (Figure 2A). Thus, the *Lh* blocking DNA did not block any sequence present in the *Lpm* probe. But, when slides of *Lh* were hybridized with a *Lh* probe and *Lpm* blocking DNA, ten chromosomes of *Lh* showed grey signal corresponding to *Lpm* chromosomes (Figure 2B). These two diploid species seem to be genetically close to each other. Due to the absence of chromosomes marked by *Lh* blocking DNA in *Lpm*, we can suggest that *Lpm* and *Lh* correspond to different genomes, arbitrarily noted A for *Lpm* and B for *Lh*.

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

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

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

Hybridizations performed on slides of *Lgg* with *Lh* (2x) and *La* (4x) blocking DNA exhibited hybridization profiles that were more challenging to interpret with 48 red chromosomes, but with different hybridization intensities (with 16 more intense signals with *La* blocking DNA and 8 less intense signals with *Lh* blocking DNA; Table 2, Figures 4C, 4D). The 16 more intense signals could correspond to a 2x component (16 chromosomes) specific to *Lgg*. However, the concentration of the *La* blocking DNA (10 µg) was probably not sufficient to completely block the common sequences in the 6x compared to the 4x which would explain the 48 red chromosomes with 16 chromosomes more intense compared to 32 chromosomes.

For the decaploid species, *Lgh*, slides were hybridized with a *Lgh* probe and five blocking DNA of different ploidy levels, including *Lpm*, *Lh*, *Ls*, *La* and *Lgg*, respectively (Table 2). The *Lpm* DNA competitor blocked 32 chromosomes whereby 48 chromosomes showing red signals and 32 grey chromosomes (Figure 5A). An identical hybridization result was obtained with the *Ls* blocking DNA with 48 chromosomes with red signals and 32 grey chromosomes (Figure 5C). Thus, the 2x component, *Lp*, also present in *Ls* (4x), is found in a double dose (32 chromosomes) in *Lgh* (10x). The results obtained with the *Lh* and *La* DNA blocking showed 80 red chromosomes but 16 with lower intensity (Table 2, Figures 5B, 5D). After GISH hybridization of *Lgg* (6x) DNA on *Lgh* (10x) chromosomes, 32 of 80 *Lgh* chromosomes showed a red signal (Figure 5E). This result revealed that *Lgg* was probably one of progenitors of *Lgh*.

Interspecific hybridization

Interspecific hybridization between species sharing the AA genome were carried out and reproductive success was observed by fruit production when the species used as female possessed the lower ploidy level (Table 3). No fruits were obtained after crosses between *Ls* (4x) used as female and *Lp* (2x) used as male or between *Lgh* (10x) used as female and *Lpm* (2x) or *Ls* (4x) used as male. Thus, all interspecific crosses with the diploid species *Lpm* (2x)

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

DISCUSSION

Ludwigia species in section *Jussieae*

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

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

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

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

species *Lgh* had white roots, one to two pneumatophores per node, and bright yellow petals (Figure 1).

Distinguishing *Ludwigia* species in field presents a real challenge. Nesom and Kartesz (2000) suggested that few morphological distinctions between *Lgh* and *Lgg* exist and broadly overlapping: plants with larger leaves and flowers and less dense vestiture characterize *Lgh*, whereas smaller leaves and flowers and denser vestiture would describe *Lgg*. However, comparing flower morphology in sterile and fertile French *Lgh* populations, two flower sizes were observed which may call into question the criterion for distinguishing flower size between *Lgh* and *Lgg* (Appendix S5, Portillo-Lemus et al., 2021). Furthermore, Dandelot (2004) and Armitage et al. (2013) summarized the principal morphological traits that distinguish both species as emergent leaves, leaf surface, venation, stipules, sepals, and pneumatophores. The presence of pneumatophores observable on summer when populations were largely developed, appears as a late criterion to distinguish *Lpm* and *Lgh* and not adapted at early stage of growing. Likewise, the stipule is reniform for *Lpm* and oblong and acuminate for *Lgh* (Thouvenot et al., 2013), but this character is also not easily used. For all these reasons, we propose new criteria to help field managers: the color of roots. *Lpm* has red roots, whereas *Lgh* has white roots. Importantly, this character can be observed at different stages of plant development (Appendix S5). *Lgg* seems to have pink roots at a young plant stage. Whether this characteristic is also true at all stages of *Lgg* development, it could also be a promising way to distinguish *Lgg* and *Lgh*.

Phylogenetic relationships in section Jussieae

We propose the first phylogenetic history of *Ludwigia* diploid to decaploid species belonging to the section *Jussiaea* (Figure 6). The diploid species studied here were composed of two different genomes, we have called AA and BB for *Lpm* and *Lh*, respectively. Both diploid *Lpm* and *Lh* were the progenitors of *Ls,* with the latter composed of AABB (Figure 3). We also found that *Lh* was a progenitor of *La*. Our results are in agreement with phylogenetic

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

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

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

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

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

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

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

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

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

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

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Author Contributions

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

Supporting Information

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

Appendix S2: Polyploidy levels of different species of ludwigia sp section Jussieae. (**A**) *Ludwigia peploides* subsp. *montevidensis* chromosomes (2n=2x=16), (**B**) *Ludwigia helminthorrhiza* chromosomes (2n=2x=16); (**C**) *Ludwigia stolonifera* chromosomes (2n=4x=32); (**D**) *Ludwigia adscendens* chromosomes (2n=4x=32); (**E**) *Ludwigia grandiflora*

subsp. *grandiflora* (2n=6x=48); *Ludwigia grandiflora* subsp. *hexapetala* (2n=10x=80). Chromosome number correspond to ploidy level: 16 chromosomes for diploid species (**A**) and (**B**); 32 chromosomes for tetraploid species (**C**) and (**D**); 48 chromosomes for hexaploid species (**E**) and 80 chromosomes for decaploid species (F)

Appendix S3: Flow Cytometry results (**A**) and examples of peak profiles (logarithmic) in the flow cytometer of nuclei stained from roots with propidium iodide (PI) (**B**). The 'trifolium repens' peak (1C=1,12 pg) or "Zea mays" peak (1C=2,77 pg) is used as internal standard to determinate the DNA contents of the sample nuclei (*). (1) *Ludwigia peploides* subsp. *montevidensis*; (2) *L. helminthorrhiza;* (3) *L. adscendens*; (4) *L. grandiflora* subsp. *grandiflora* and (5) *L. grandiflora* sp. *hexapetala*

Appendix S4: Fruit production and seedling from interspecific hybridization between ludwigia species possessing A genome ; *Lpm* = Ludwigia peploides subsp. montevidensis (2n=16, AA) ; *Ls = Ludwigia stolonifera* (2n=32, AABB); *Lgh = Ludwigia grandiflora* subsp. *hexapetala* (2n=80, AAAABBXXXX/XXYY). (a) the seeds produced from *Lpm* x *Lgh* interspecific cross are large, which has led to the fruit bursting. (b) 30 days after seedling, green plantlets from *Lpm* x *Ls* interspecific cross were obtained. But, 60 days later, plants showed chlorotic development, stopped growing and died. Das : Number of day after seedling

Appendix S5: Morphological traits to distinguish *Ludwigia peploides* subsp. *montevidensis* and *Ludwigia grandiflora* subsp. *hexapetala,* (**a**) roots at seedling stage ; (**b**) adult roots in natura ; (**c**) pneumatophores in natura ; (**d**) flowers.

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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Tables

Table 1: Ploidy levels, chromosome numbers and genome sizes estimated by flow cytometry in Ludwigia L. spp. sect. *Jussiaea*.

Species names are mentioned according to the revised nomenclature by Hoch et al. (2015). Genome sizes were converted from picograms (pg) to Megabases (Mb) using 1 pg = 978 Mbp.

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

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

Table 3: Reproductive success after controlled interspecific crosses between different Ludwigia L. spp. belonging to the section *Jussiaea*.

Interspecific hybridization (female x male) between the three species, *Ludwigia peploides* subsp. *montevidensis* (Lpm), *Ludwigia stolonifera* (Ls) and/or *Ludwigia grandiflora* subsp. *hexapetala* (Lgh, AAAA XXXX/XXYY) used as female or male. All species possess same genome A: Lpm (2x, AA); Ls (4x, AABB); Lgh (10x, AAAA XXXX or XXYY). Number of plantlets and plants were counted three (21 days) and 8 weeks (56 days) after seed germination, respectively. NA: data not available. (+/-= confidence interval, α=0.05). For control interspecific crosses *Lgh* x *Lgh* and *Lpm* x *Lpm*, a set of randomly selected plantlets were 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

Legends of figures:

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).

Figure 2: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwiga peploides* subsp. *montevidensis* (2n=2x=16) using *Ludwigia peploides* subsp. *montevidensis* probe (2x) (red) and *Ludwigia helminthorrhiza* (2x) (10µg) as blocking DNA (A) and from *L. helminthorrhiza* (2n=2x=16) using *L. helminthorrhiza* probe (2x) and *L. peploides* subsp. *montevidensis* (2x) (10µg) as blocking DNA (B).

Thus, GISH reveals specifically 16 red signals (white stars) and 0 *L. peploides* subsp. *montevidensis* chromosomes (grey) (A) and 4 red signals (white stars) and 10 *L. helminthorrhiza* chromosomes (grey) (B). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

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 *L. grandiflora* subsp. *grandiflora* (2n=6x=48) using *Ludwigia 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), *Ludwigia adscendens* (4x) as block (D) and *L. 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 red signals and 48 *L. grandiflora* subsp. *grandiflora* (grey) (E). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

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

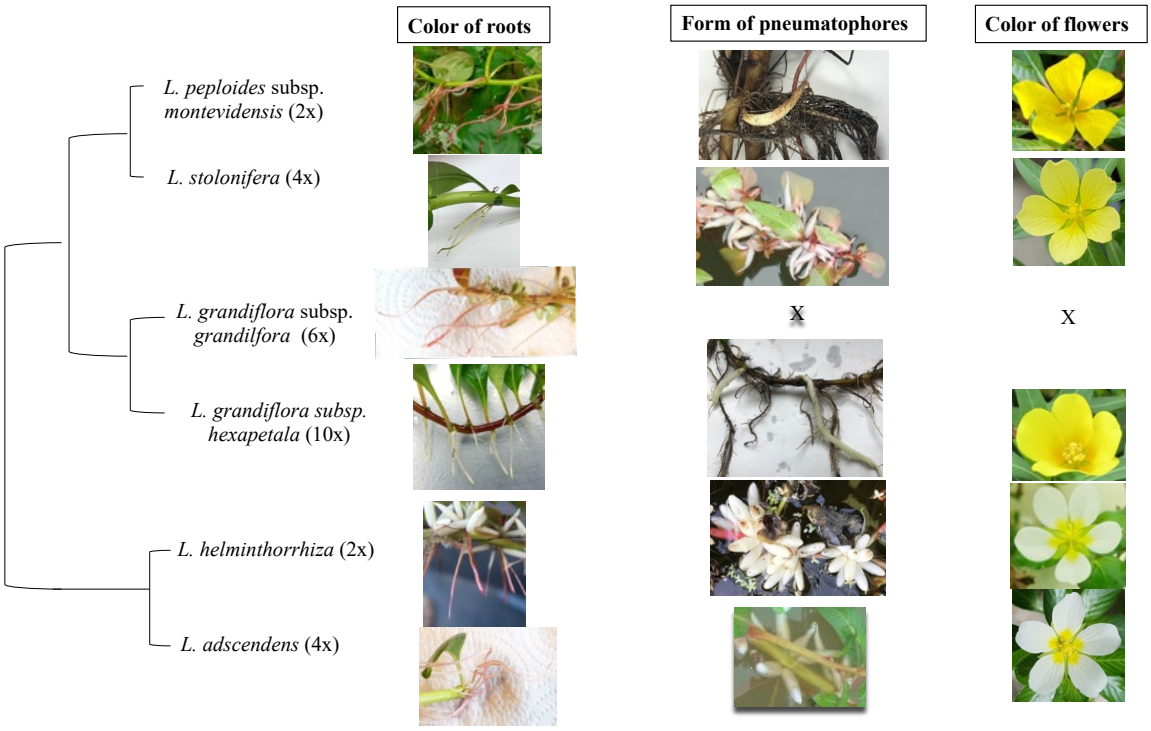

Figure 1: Morphological traits of Ludwigia L. species in section *Jussiaea*. Ludwigia L. species are classified in a phylogenic tree as proposed by Liu et al (2017). Three morphological traits were observed (color of roots, pneumatophore form, color of flower).

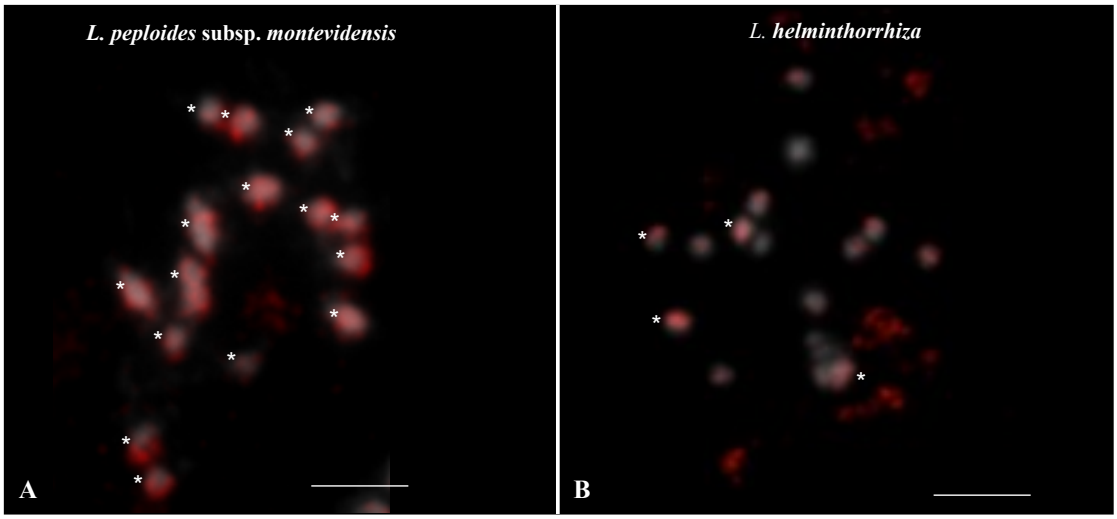

Figure 2: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwigia peploides* subsp. *montevidensis* (2n=2x=16) using *L. peploides* subsp. *montevidensis* probe (2x) (red) and *Ludwigia helminthorrhiza* (2x) (10µg) as blocking DNA (A) and from *L. helminthorrhiza* (2n=2x=16) using *L. helminthorrhiza* probe (2x) and *L. peploides* subsp. *montevidensis* (2x) (10µg) as blocking DNA (B).

Thus, GISH reveals specifically 16 red signals (white stars) and 0 *L. peploides* subsp. *montevidensis* chromosomes (grey) (A) and 4 red signals (white stars) and 10 *L. helminthorrhiza* chromosomes (grey) (B). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

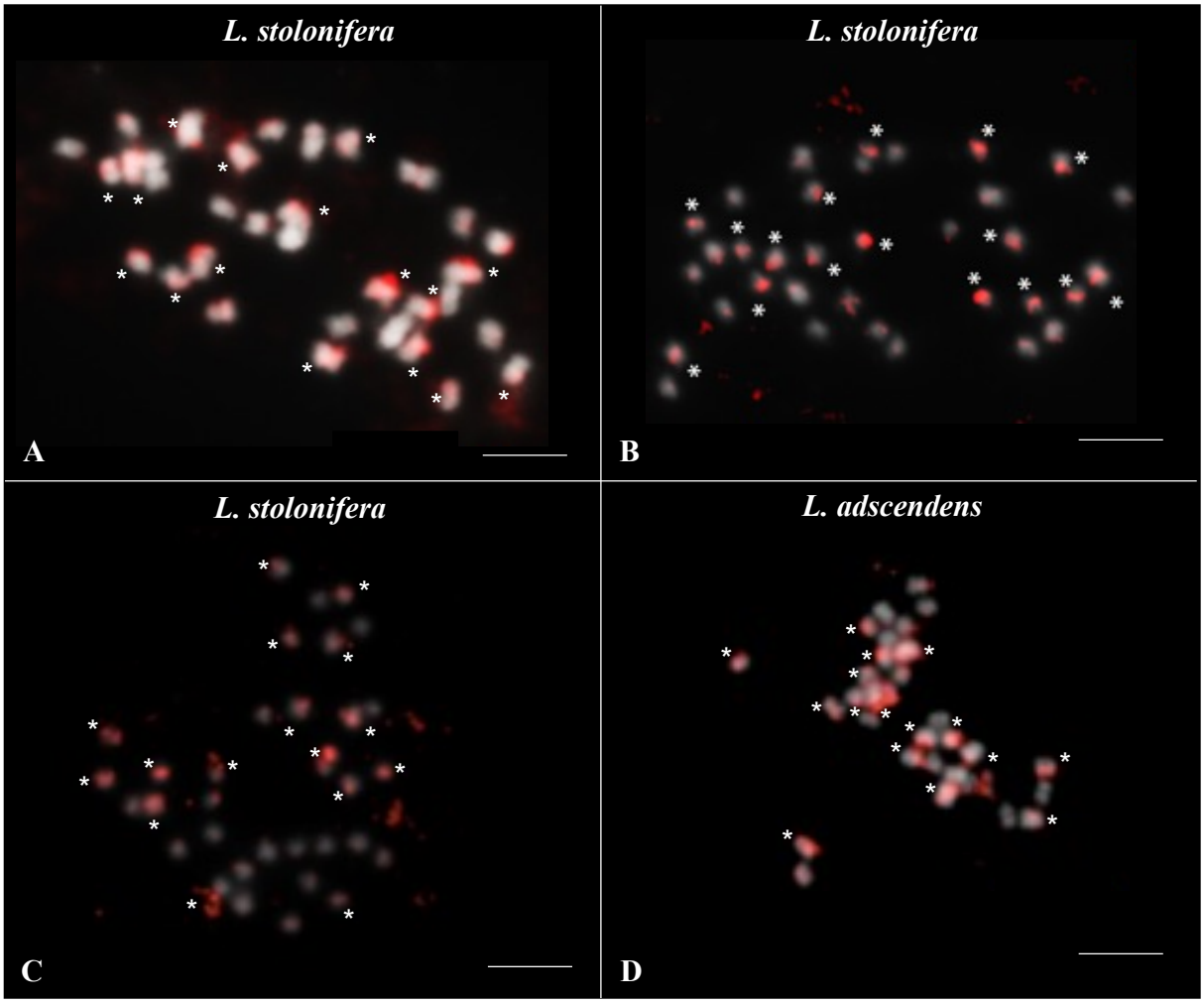

Figure 3: In situ genomic hybridation analyses of somatic metaphase chromosomes of the tetraploid species, *Ludwigia stolonifera* and *Ludwigia adscendens* (2n=4x=32).

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.

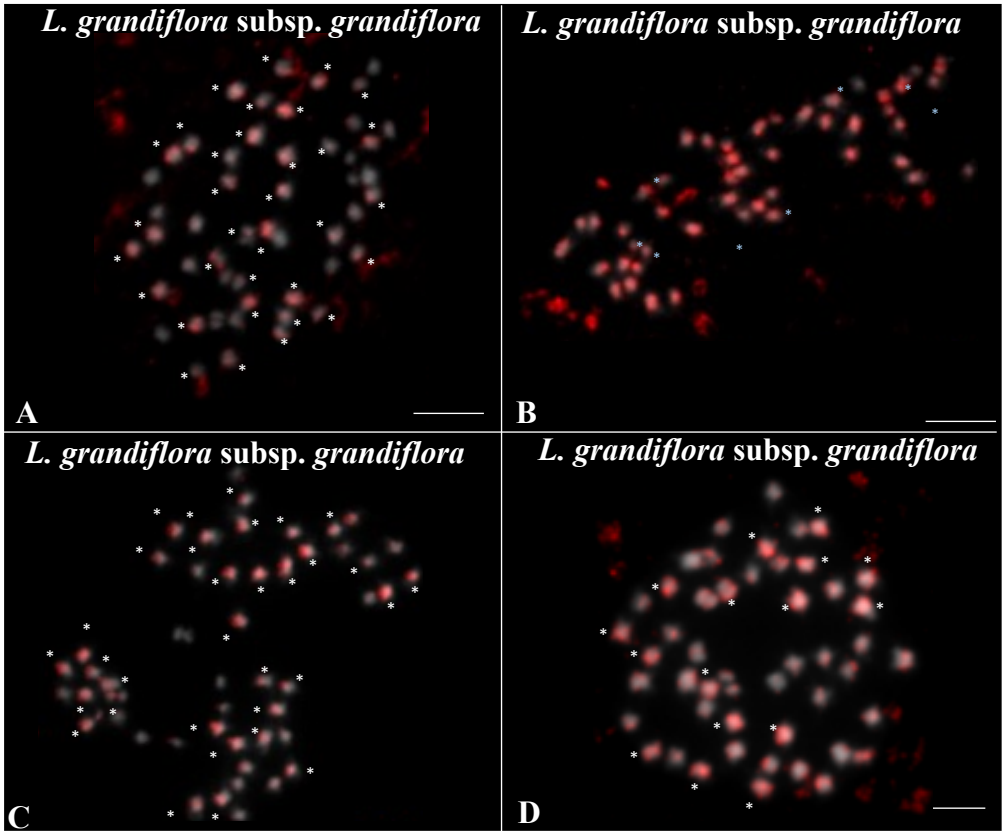

Figure 4: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwigia grandiflora subsp. grandiflora* (2n=6x=48) using *L. grandiflora subsp. grandiflora* probe (6x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (**A**), *Ludwigia helminthorriza* (2x) (10µg) as block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

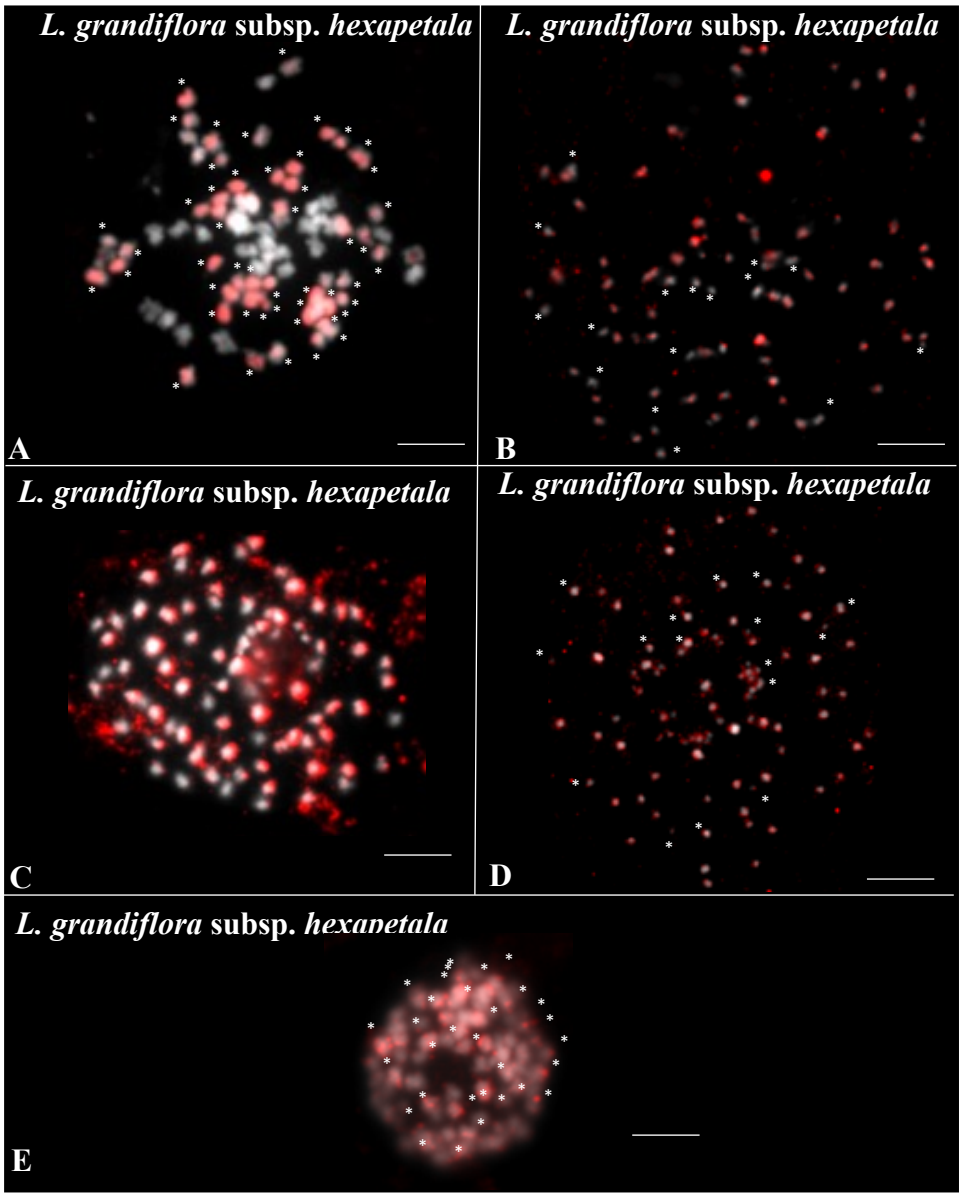

Figure 5: In situ genomic hybridation analyses of somatic metaphase chromosomes from *Ludwigia grandiflora* subsp. *hexapetala* (2n=10X=80) using *L. grandiflora* subsp. *hexapetala* probe (10x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (10µg) as block (**A**), *Ludwigia helminthorrhiza* (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

red signals and 48 *L. grandiflora* subsp. *grandiflora* (grey) (E). Chromosomes were counterstained with DAPI (grey). Bar represents 5 µm.

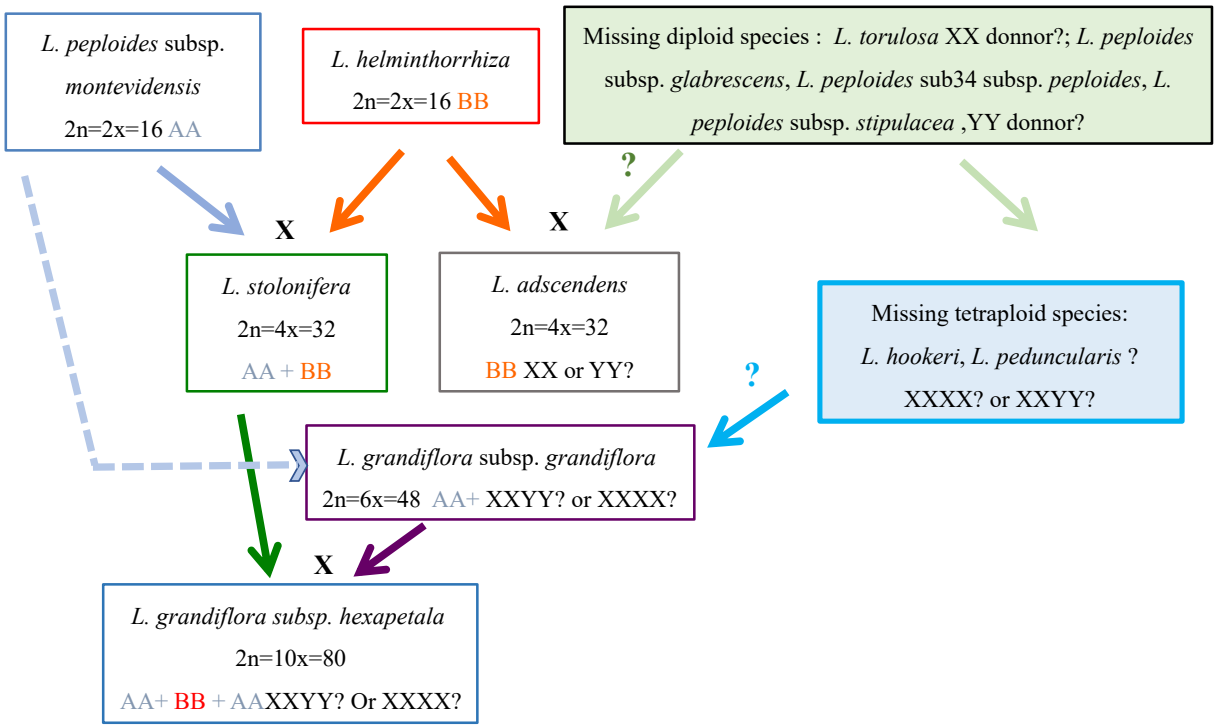

Figure 6: Hypothetical phylogenetic history of ludwigia species of section Jussieae