Genomic relationships among diploid and polyploid species of the genus Ludwigia L. section *Jussiaea* using a combination of molecular 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 (Husband et al., 2013; Alix et al., 2017; Otto and Whitton, 2000). 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 (Soltis et al., 2015; Alix et al., 2017). 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*). While most species are native to the New World, particularly South America, two species are restricted to the Old World, *Ludwigia stolonifera* and *Ludwigia adscendens*, in Africa and tropical Asia, respectively (Wagner et al., 2007) (Table S1). 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). Octoploid hybrids between *L. grandiflora* subsp. *hexapetala (Lgh)* and *L. grandiflora* subsp. *grandiflora* (*Lgg*) were found in southern Brazil which for both species is their native area (Zardini et al, 1991). Studies of Liu et al (2017) confirmed close relationship between *Lgg* and *Lgh*. So, Nesom and Kartesz (2000) suggested that as *Lgg* and *Lgh* shared genomic portions and possible hybridization between them, both species were recognized as subspecies within the single species *L. grandiflora*. However, several authors, including Okada et al. (2009) and Grewell et al (2016), continue to recognize two distinct species. In this paper, species were named as described by Nesom and Kartesz (2000) and Armitage et al (2013), i.e., considered

as two subspecies of *L. grandiflora* (*Lgg* and *Lgh*). So, phylogenetic studies (Liu et al 2017) revealed that the *L. peploides* (2x) or a relative and the *L. adscendens* (4x) have probably contributed its genome to the origin of *L. stolonifera* and of the triploid hybrid for *L.* × *taiwanensis* (3x), respectively. Furthermore, based on morphological observations, Zardini et al. (1991) suggested that *Lgh* may be result of interspecific hybridization between *Lgg* and *L. hookeri*. So, in view of the diversity of ploidy levels present in the ludwigia sect. *Jussiaea*, results of morphological and molecular analysis, polyploid species could be probably the result of hybridization between diploid species or combinations of diploid and polyploid species. 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 morphological traits, molecular cytogenetic 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. However, while many authors have highlighted the possibility of interspecific hybridization between the species presents in the Jussieae section, there is a lack of data enabling the polyploid origin of these species to be identified, i.e., the auto or allopolyploid origin as well as that of the progenitor species. First, we observed some morphological traits as a simple verification step to prove that the species collected were those expected. Second, we characterized the different species by analysis of their genome size using flow cytometry and their ploidy level by cytogenetic observations. We identified the genomic relationships 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* (hereafter, *Lpm*) (2x) 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. IJsselveld 9, 3417 XH Montfoort, Netherland). Five plants of *Ludwigia adscendens* (L.) H. Hara (hereafter, *La*) (4x), and of *L. stolonifera* (hereafter, *Ls*) (4x) and ten of *L. grandiflora* subsp. *grandiflora* (hereafter, *Lgg*) (6x) 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. As all Ludwigia species growth preferentially by clonal reproduction; each plant was used as mother plant giving new plants from the development of buds present on its stem which are then used for all experiments (Okada et al., 2009; Glover et al., 2015). The plants were easily maintained in the greenhouse at Institut Agro Rennes – Angers (France) before analysis (Portillo-Lemus et al, 2021).

Morphology

To confirm that the collected Ludwigia species corresponded to the expected species, we carried out qualitative observations using visual morphological traits such as the colour of the flowers and roots and the pneumatophore form as reported in Table S1. Morphological observations for each species were 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

At least 40 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. Chromosome preparations were performed according to procedures detailed in Ksiazczyk et al. (2011). At least four different roots per species were observed and chromosome counts 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* (2C DNA = 2.23 pg) or *Zea mays* (2C DNA = 5.55 pg) (Zonneved et al, 2019) (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. Estimation of genome size for each species was obtained as described by Boutte et al, 2020. 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-value= 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. 500 ng of total genomic DNA were labelled by random priming with biotin-14-dCTP (Invitrogen by Thermo Fisher Scientific) used as probes.

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 from different species were used as competitors to block the common sequences at both species. Genomic In Situ Hybridization (GISH) was carried out as described in Coriton et al., 2019, using a 5 µg of blocking DNA (~50-fold excess). 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).

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 on a shoot until July to October, with at one time only one flower per shoot at the good stage of mature for pollination. To carry out interspecific pollinations, flowers were enclosed in cellophane bags to protect them from external pollen before and after pollination. Flowers used as 'female' were emasculated before anthesis. A mix of pollen from flowers of five 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 for interspecific crosses was estimated by the number of fruits, fruit size and weight, the number of seeds, viable plantlets, and the number of plants ultimately produced. For intraspecific crosses, the number of fruits obtained were noted.

RESULTS

Morphological traits of Ludwigia species

The qualitative traits observed in the species collected were consistent with the morphological traits described in the species selected for our study, as summarized in Table S1.

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 (Figure 1). For the tetraploid

species, *La* had pink roots, white petals with yellow base, and had few conical pneumatophores. *Ls* had white roots, petal color light yellow 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. 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, Figure S1). *Ludwigia* spp. exhibited an ~0.77-fold range of C-values. The lowest value, 0.265 pg/1C, was found in *Lpm* and the highest, 1.045 pg/1C, in *Lgh* (Table 1, Figure S2). The tetraploid species *Ls* (0.265 pg/1C) and *La* (0.53 pg/1C) have C-values that were twice that the value for the diploid *Lpm* (0.265 pg/1C) and *Lh* (0.275 pg/1C). The hexaploid species *Lgg* had C-value 0.885 pg/1C. 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

GISH is used to distinguish chromosomes from different genomes in interspecific/intergeneric hybrids or allopolyploids. Total genomic DNA of a genitor involved in the formation of a hybrid is used at the same time as an unlabeled DNA from another genitor, at a higher concentration, which serves as a blocking DNA, hybridizing with the sequences in common with both genomes. This method is based on repetitive sequences which are more often in plant species-specific. Thus, we compared the level of relatedness between the genomes of the studied species and hypothetical parental species.

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, meaning that no *Lh* genome was shared with *Lpm*. 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). This observation seems to indicate a certain genome homology with the *Lpm* genome but four chromosomes were stained in red, meaning that there are nevertheless differences in *Lpm* and *Lh* genomes. Due to the absence of chromosomes marked by *Lh* blocking DNA in *Lpm*, we can suggest that *Lpm* and *Lh* correspond to different genomes, even if homology exist, 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 different blocking DNA combinations 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* (10x) 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*.

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 with grey signals whereby 48 chromosomes showing red signals (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 (Figure 6, Table S2). 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 (Figure 6, Table S2). 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 (Figure 6, Table S2, Figure S3). Similarly, fruits were produced after *Ls* (4x) x *Lgh* (10x) crosses with a mean number of seeds per fruit of 37 (Figure 6, Table S2) 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 effectiveness of the greenhouse pollination conditions.

DISCUSSION

To better understand the evolutionary history of genus Luwigia, we have evaluated the genomic relationships between diploid and polyploid species using morphological observations, molecular cytogenetic and crossing investigations.

Validation of Ludwigia species sect. Jussieae studied and identification of new discriminating traits.

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) The long-standing taxonomic confusion surround aquatic *Ludwigia* species required a approach combining morphometric traits and cytogenetic evaluations to differentiate the species and improve taxonomic identification (Grewell et al., 2016). Furthermore, distinguishing *Ludwigia* species in field presents a real challenge.

In this study, qualitative morphological traits were observed for the six Lg ssp. grown in a common garden, which represents a real opportunity to compare these species growing under the same conditions. Our results confirmed that all the species collected corresponded to the expected species. However, our cross observations of the different species in a common garden revealed additional differences between these species. For example, 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 (Figure S4). *Lh* plants studied had these same characteristics as those described (Rocha and Melo, 2020), but the petals were more creamy-white than white and were sharply narrow at the petiole. Difference in pneumatophore form, petal and root coloration could differentiate these both species in field (Figure 1). For the tetraploid species, flowers of *La* are described as creamy white petals with yellow at the base (Wagner et al., 2007) but we observed white petals similar to *Lh* (Figure 1). As *Ls* had light yellow petals, the floral color may a good characteristic with which to distinguish these two tetraploid species in natura. For the hexaploid species *Lgg*, we only saw pink roots and more morphological investigations are required. Finally, the decaploid species *Lgh* had white roots and bright yellow petals (Figure 1).

Grewell et al., (2016) reported that distinguish in field *Lgg* and *Lgh* was complicated. 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). As regards the distinction between *Lpm* and *Lgh*, the differences in stipule shape are often cited, 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 (Figure S4). *Lgg* seems to have pink roots at a young plant stage (Figure 1). Whether this characteristic is also true at all stages of *Lgg* development, it could also be a promising way to distinguish *Lgg* and *Lgh*.

Genomic relationships and origins of polyploids in section Jussieae

We propose the first hypotheses regarding diploid-polyploid relationships of *Ludwigia* diploid to decaploid species belonging to the section *Jussiaea* (Figure 7). 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* (BB), sharing same genome with *Ls* even though the *La*, native to Asian-Pacific, and *Ls*, native to African, do not currently co-occur (Table S1). Our results are in agreement with phylogenetic analysis of Liu et al. (2017) which suggested through analysis of nuclear tree that *Lp* or a close relative contributed to the origin of *Ls* and shared a same genome (here designated as genome AA). Similarly, Liu et al. (2017) reported that *L. adscendens* (4x) is close to *L. helminthorrhiza* (2x) (genome BB). GISH analysis revealed that *Lh* and *Ls* shared at least one genome, which was not shown by Liu et al. (2017) phylogeny analysis.

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 hexaploid *Lgg* genome. The decaploid species *Lgh* seems to have emerged from interspecific hybridization and allopolyploidization events between the tetraploid species *Ls* (4x) and the hexaploid 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*.

All chromosomes of *Lgg* and *Lgh* were tagged by *Lh* blocking DNA, but had strong or light hybridization intensities for 16 chromosomes respectively. The intensity of fluorescence could be explained by the existence of numerous repetitive sequences shared by closely

related species or specific to a given species. Thus, Liu et al. (2008) could distinguish the subgenomes of Triticeae allopolyploids due to differences in element abundance and the resulting probe signal intensity. In addition, in a Silene hybrid, Markova et al. (2007) showed that the intensity of fluorescence varied quantitatively based on the relatedness of the species. 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 hexaploid 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).

Success of interspecific hybridization and contribution to hypothetical phylogenetic origin of Ludwigia species, sect. Jussieae

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). Liu et al. (2017) observed through the chloroplast (cp) tree analysis that *La* and *Ls* are grouped suggesting that both tetraploid species shared at least one maternally inherited genome, probably the BB genome from *Lh*. Unfortunately, *Lh* was not include in cp tree analysis by Liu et al. (2017). The combined data from the interspecific crosses carried out in this study and the phylogenetic analysis carried out by Liu et al. (2017) allows us to hypothesize that in Ludwigia sp. sect. Jussieae, interspecific hybrids can be obtained when the species used as a female has the lowest ploidy level.

Natural hybrids within section *Jussieae* have been reported between *La* (2n =4x= 32) and *L. peploides* subsp. *stipulacea* (2n= 2x =16), with production of a triploid sterile hybrid (2n = 3x= 24) named *L. x taiwanensis* (Peng, 1990). Between *Lgg* (2n = 6x = 48) and *Lgh* (2n = 10x = 80), an octoploid hybrid was produced (2n = 8x = 64) and between *Lgg* (2n = 6x = 48) and *L. hookeri* (2n = 2x = 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.

CONCLUSION

In this study we demonstrated the interest of a truly novel combination of data to identify genomic relationships and origins of polyploids 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). Another way 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 various approaches used here,

combining morphological traits and cytogenetic analyses, in situ hybridization and interspecific crosses, could constitute a first step towards phylogenetic studies of species belonging to poorly known species complexes for which there are few genomic resources.

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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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article

Data

Data are available in supplementary Figure S2 and Table S3

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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 and calculated from data in Figure S2 (+/- = Confidence Interval, p-value=0.5). For Ls, only one sample was analyzed.

Species name	**Ploidy and chromosome numbers**	**Genome size (1C/pg)**	**Genome size (Mb)**
Ludwigia peploides subsp. *montevidensis (Lpm)*	2n= 2x= 16	0.265	261.7 +/- 9.2
Ludwigia helminthorrhiza (Lh)	2n= 2x= 16	0.275	267.7 +/- 1.7
Ludwigia adscendens (La)	2n= 4x= 32	0.53	498.6 +/- 31.4
Ludwigia stolonifera (Ls)	2n= 4x= 32	0.535	522
Ludwigia grandiflora subsp. *grandiflora (Lgg)*	2n= 6x= 48	0.885	863.9 +/- 2.6
Ludwigia grandiflora subsp. *hexapetala (Lgh)*	2n= 10x= 80	1.045	1418.9 +/- 107.3

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**

Figures

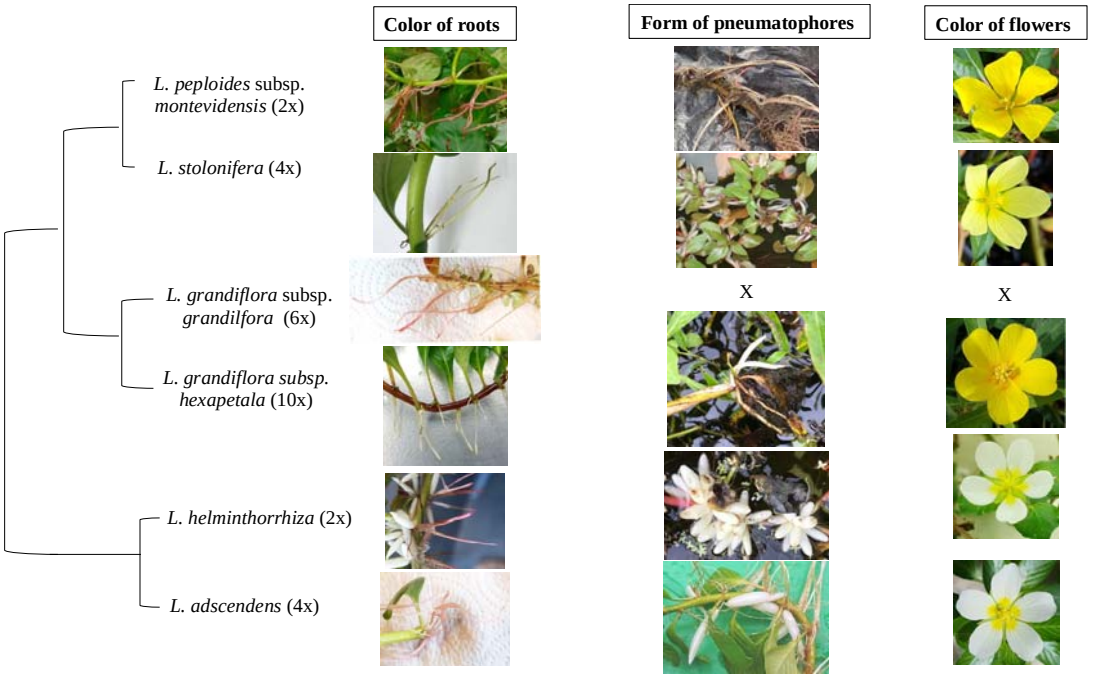


Figure 1: Morphological traits of Ludwigia L. species in section *Jussiaea*. Ludwigia L. species are classified in a phylogenic tree as proposed by Liu et al (2017). Three morphological traits were observed (color of roots, pneumatophore form, color of flower).

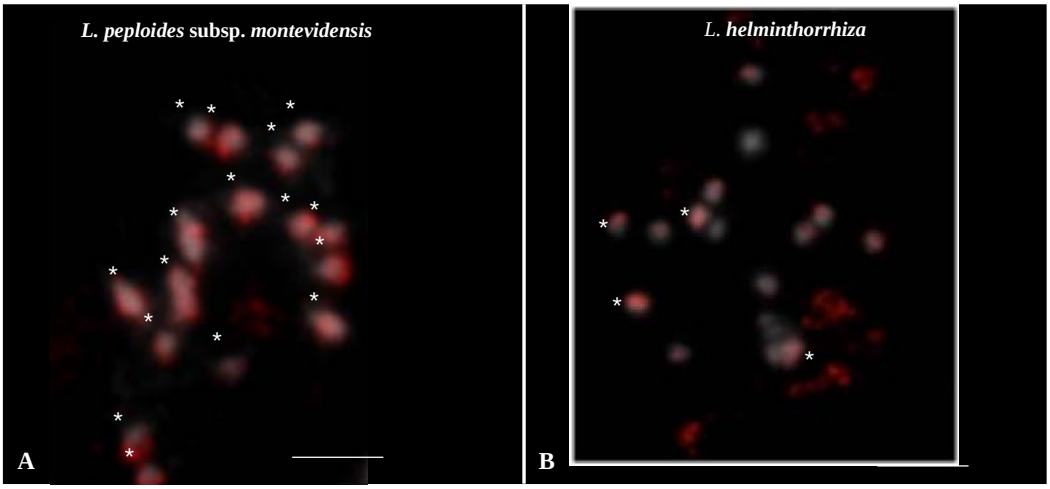


Figure 2: Genomic in situ hybridization (GISH) on mitotic metaphase chromosomes from *Ludwigia peploides* subsp. *montevidensis* (2n=2x=16) using *L. peploides* subsp. *montevidensis* probe (2x) (red) and *Ludwigia helminthorrhiza* (2x) (10µg) as blocking DNA (A) and from *L. helminthorrhiza* (2n=2x=16) 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.

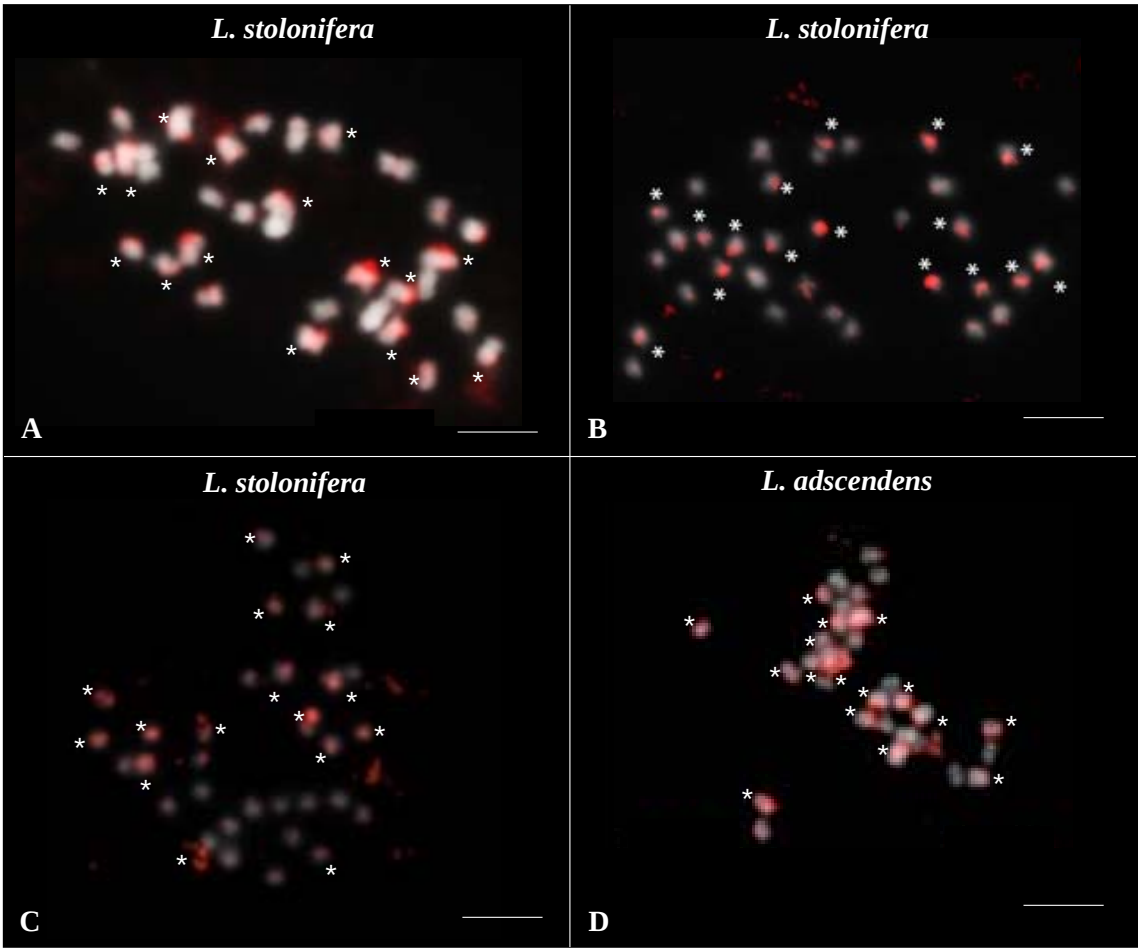


Figure 3: Genomic in situ hybridization (GISH) on mitotic metaphase chromosomes from the tetraploid species, *Ludwigia stolonifera* and *Ludwigia adscendens* (2n=4x=32).

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.

801

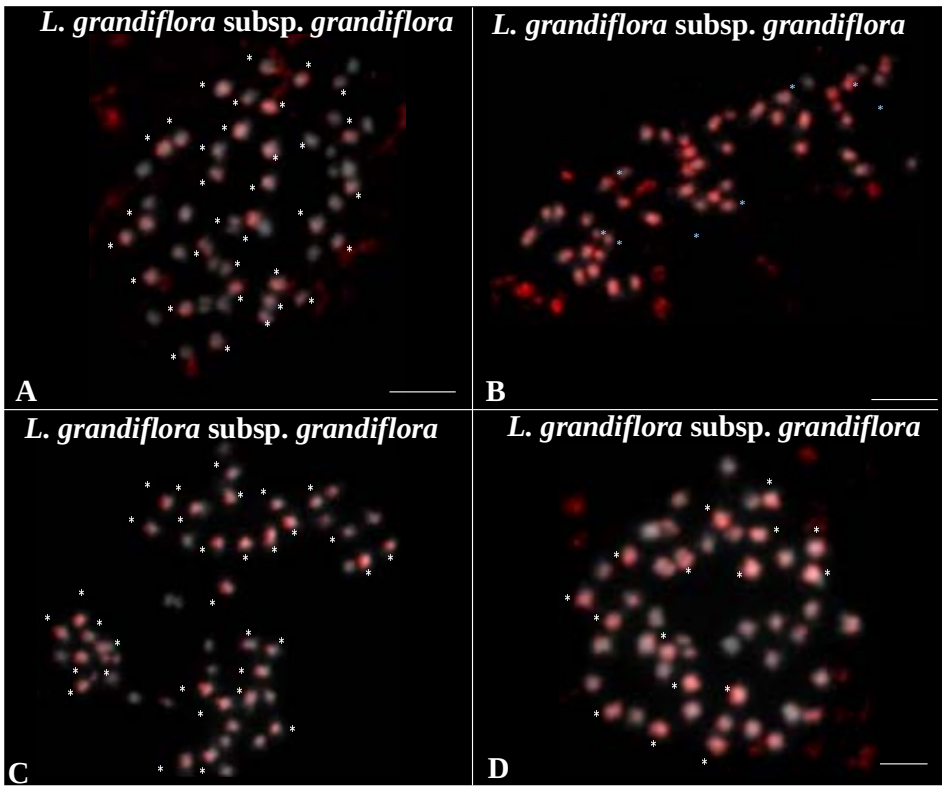


Figure 4: Genomic in situ hybridization (GISH) on mitotic metaphase chromosomes from *Ludwigia grandiflora subsp. grandiflora* (2n=6x=48) using *L. grandiflora subsp. grandiflora* probe (6x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (**A**), *Ludwigia helminthorriza* (2x) (10µg) as block (**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.

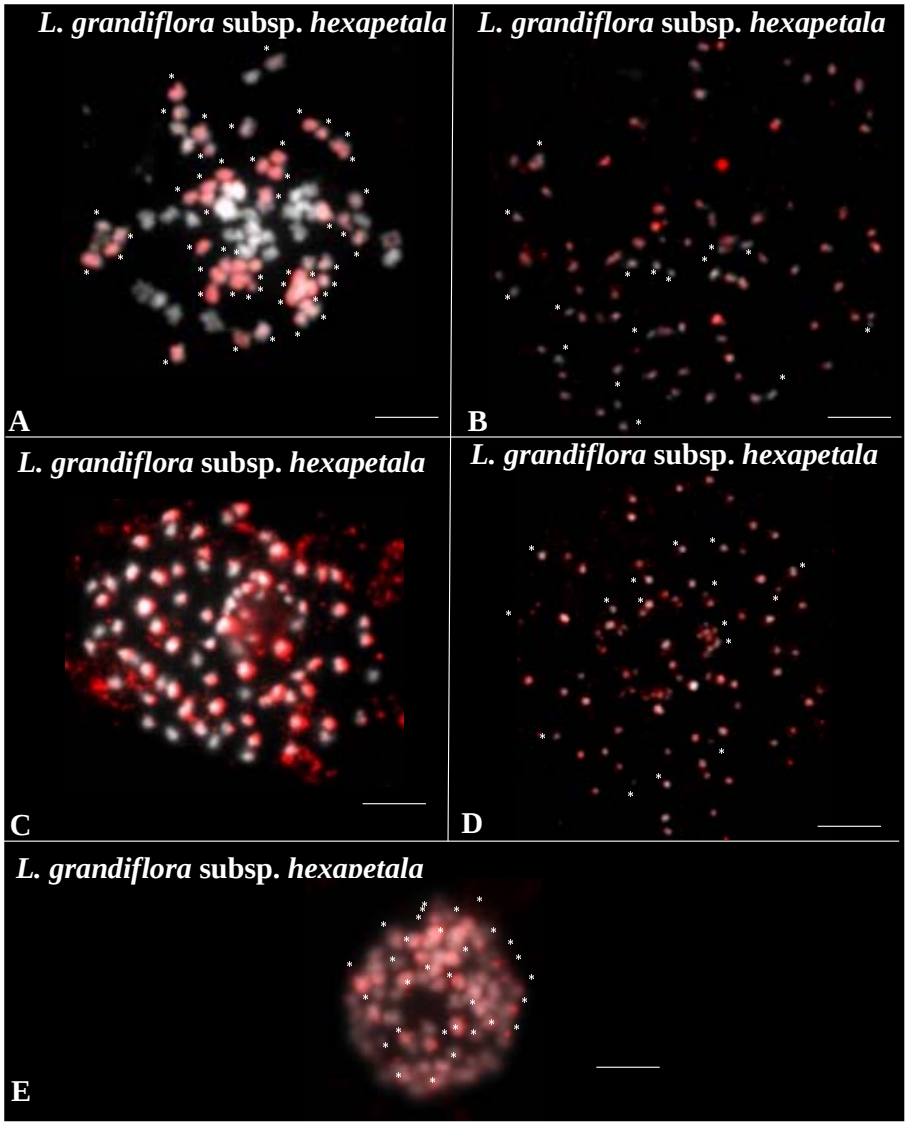


Figure 5: Genomic in situ hybridization (GISH) on mitotic metaphase chromosomes from *Ludwigia grandiflora* subsp. *hexapetala* (2n=10X=80) using *L. grandiflora* subsp. *hexapetala* probe (10x) (red) and *Ludwigia peploides* subsp. *montevidensis* (2x) (10µg) as block (**A**), *Ludwigia helminthorrhiza* (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.

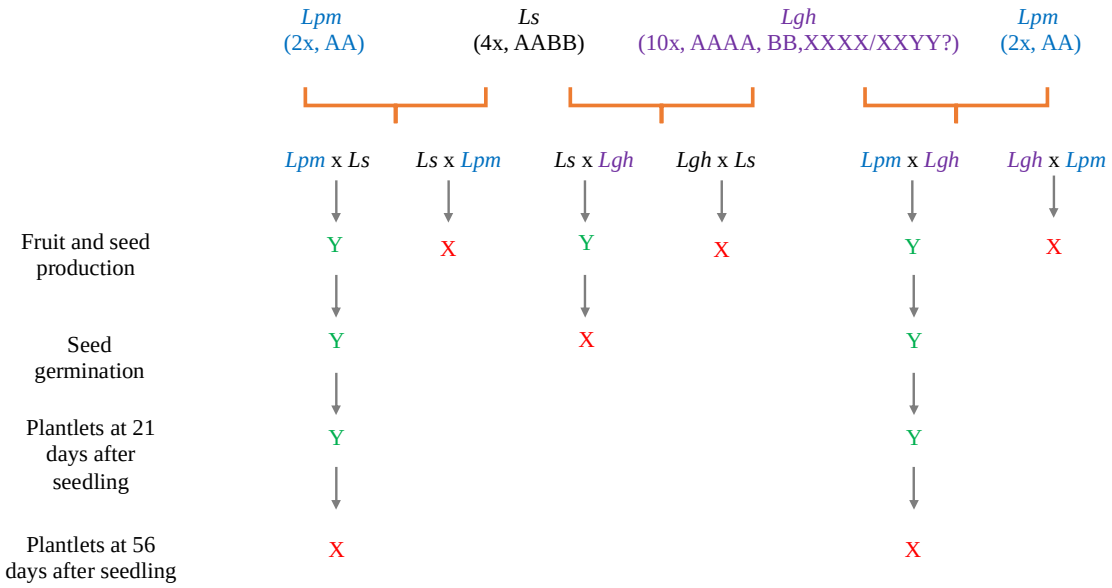


Figure 6: Reproductive success after controlled interspecific crosses between different Ludwigia sp belonging to the section Jussiaea.

Interspecific hybridizations were made between the three species, *Ludwigia peploides* subsp *montevidensis* (*Lpm*), *Ludwigia stolonifera* (*Ls*) and/or *Ludwigia grandiflora* subsp. *hexapetala* (*Lgh*) used as female or male. All species possess same genome A: *Lpm* (2x, AA); *Ls* (4x, AABB); *Lgh* (10x, AAAA BB XXXX or XXYY). Y letter (green) indicated success of step and the cross (red) failed of the corresponding step. Intraspecific crosses (*Lgh* x *Lgh*; *Lpm* x *Lpm*; *Ls* x *Ls*) were used to control pollination efficiency in greenhouse and all pollinated flowers produced fruits. For details, see Table S2.

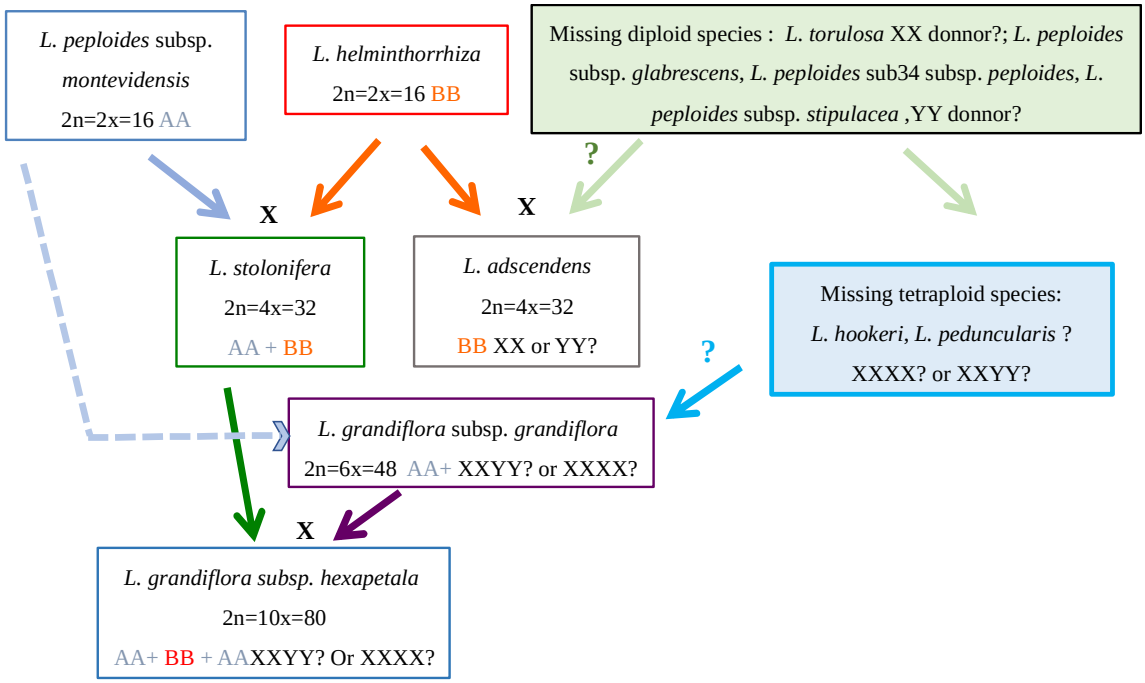


Figure 7: Hypothetical phylogenetic history of ludwigia species of section Jussieae

Supplementary files

Table S1: Native area and floral and root traits of the six ludwigia sp. studied. ND: not described

	Ludwigia peploides* subsp. *montevidensis	***Ludwigia helminthorrhiza***	***Ludwigia adscendens***	***Ludwigia stolonifera***	***Ludwiga grandiflora* subsp. *grandiflora***	***Ludwigia grandiflora* subsp. *hexapetala***
Native area *(Munz, 1942; Raven, 1963, Wagner et al, 2007, Grewell et al, 2016)*	southern South America (Argentina, Urugauay and Southern Brazil)	South America	Asian-Pacific	Africa, Madagascar	Central and southern South America (from south of theBrazil to Uruguay)	southern South America (southen Brazil, eastern Paraguay, Argentina)
Flower and root traits						
Color of flower	Yellow (Dandelot et al, 2005)	White (Raven and Tai, 1979)	Creamy white, to pale yellow, bright yellow, or dark yellow (Van Anh et al, 2021)	Lemon Yellow (Amer et al, 2016)	Yellow (Grewwell et al, 2016)	Yellow (Portillo-Lemus et al, 2021)
Color of Petal	White yellow (Dandelot et al, 2005)	White with yellow spot at the base (Rocha et al 2020)	White with bright yellow base (Van Anh et al, 2021) ; light yellow with a deeper yellow base (Oziegbe et al, 2012)	Lemon-yellow with a darker spot at the base (Amer et al, 2016)	Yellow or light orange (Grewwell et al, 2016)	Yellow with a orange spot at the base ((Portillo-Lemus et al, 2021)
Size of sepal (long x width)	5-9 x 1.5-2 mm (Zotos and Sarika, 2006)	6-8 × 1.5-2 mm (Rocha et al 2020)	/	15-20 x 8-10 mm (Amer et al, 2016) ; 5.5–12× 2.5–3.5 mm (wordfloraoneline)	8-11 mm long (Nesom and Kartesz, 2000)	11-19 mm long (Nesom and Kartesz, 2000) ; For morph-S: mean length=18.3 mm and mean width = 4.2mm - for morph-L: mean length = 14.9 mm; mean width = 3.2 mm) (Portillo-Lemus et al, 2021)
Size of petal (long x width)	10-15 × 5-8 mm (Zotos and Sarika, 2006)	13-16 × 10-11 mm (Rocha et al 2020)	8-20 x 8-10 mm (Van Anh et al, 2021)	7-15 × 4-8 mm (wordfloraoneline)	8-25 long (Nesom and Kartesz, 2000; Grewell et al, 2016)	7-15 mm long ((Nesom and Kartesz, 2000) ; for morph-S : mean length = 27.2 mm and mean width 22.4mm - for morph-L: mean length = 21.0 mm and mean width = 16.3 mm (Portillo-Lemus et al, 2021)
Colors of roots	/	/	/	Bright red or black roots, thin and elongated (wordfloraoneline)	/	/
Pneumatophores	Rarely produced (Armitage et al, 2013)	White spongy pneumatophores emerging from each branch knot (Rocha et al, 2020)	On nodes of floating stems (Barua 2010, Oziegbe et al, 2012)	At nodes of loating stem from abundant (up to 10/node) to occasional (up to 3/node) and conical, cylindrical and fusiform forms (Soliman et al, 2018)	/	Frequently produced (Armitage et al, 2013)

Table S2: Reproductive success after controlled interspecific and intra crosses between different Ludwigia sp belonging to the section Jussiaea.

Interspecific hybridization between the three species, *Ludwigia peploides* subsp *montevidensis* (*Lpm*), *Ludwigia stolonifera* (*Ls*) and/or *Ludwigia grandiflora* subsp. *hexapetala* (*Lgh*) used as female or male. All species possess same genome A: *Lpm* (2x, AA); *Ls* (4x, AABB); *Lgh* (10x, AAAABBXXXX or XXYY). Number of plantlets and plants were counted three and 8 weeks after seed germination, respectively. NA: data not available. (+/-= confidence interval, p-value=0.05).

Controlled interspecific crosses (female x male)	***Lpm* x *Ls***	***Lpm* x *Lgh***	***Ls* x *Lpm***	***Ls* x *Lgh***	***Lgh* x *Lpm***	***Lgh* x *Ls***	***Ls* x *Ls***	***Lpm* x *Lpm***	***Lgh* x *Lgh***
Number of cross pollination	8	25	10	2	10	10	50	45	75
Number of fruits	8	25	0	2	0	0	50	45	75
Mean length of fruit (mm)	15.08 (+/- 0.78)	16.64 (+/- 0.82)	/	NA	/	/	NA	NA	NA
Mean fruit weight (g)	62.04 (+/- 6.46)	64.64 (+/- 6.02)	/	NA	/	/	NA	NA	NA
Number of total seed	221	1101	/	74	/	/	NA	NA	NA
Number of germinated seeds	118	34	/	0	/	/	NA	NA	NA
Number of plantlets	118	3	/	0	/	/	NA	NA	NA
Number of plants	0	0	/	0	/	/	NA	NA	NA

Table S3: Data of fruit length and weight, number of seed and germinated seeds for the interspecific crosses of *Ludwigia peploides* subsp. *montevidensis* (*Lpm*), *Ludwigia stolonifera* (*Ls*) and *Ludwigia grandiflora* subsp. *hexapetala* (*Lgh*). Interspecific crosses = (female x male)

Interspecific crosses	Fruit length (mm)	Fruit weight (mg)	Seed number	Number of germinated seeds
Lpm x *Lgh*	17,01	72,6	52	0
	11,71	126,46	14	0
	15,85	62,7	44	0
	19,46	62,4	59	0
	18,12	62,2	45	0
	16,27	66,3	47	0
	19,87	82,4	40	0
	15,34	58,3	41	0
	16,63	50,7	34	1
	16,25	63,2	47	0
	18,32	72,9	59	1
	15,67	82,4	38	31
	16,95	46,4	49	0
	17,6	75,5	50	0
	14,07	70,2	41	0
	13,74	50,6	23	0
	15,15	52,7	49	1
	17,96	63,2	35	0
	17,04	71,7	42	0
	21,07	81,2	48	0
	15,46	44,6	45	0
	14,19	52,5	20	0
	15,55	50,4	47	0
	17,16	51,5	52	0
	17,99	52,7	42	0
	18,08	62,8	38	0
Total			**1101**	**34**
Lpm x *Ls*	15,28	72,7	44	26
	12,44	50,1	16	6
	14,63	70,9	37	18
	15,71	55,6	19	8
	15,5	52,7	25	18
	15,81	59,6	27	15
	15,01	68,4	23	19
	16,25	66,3	30	8
Total			**221**	**118**
Ls x *Lgh*	NA	NA	32	0
	NA	NA	42	0
Total			**74**	**0**

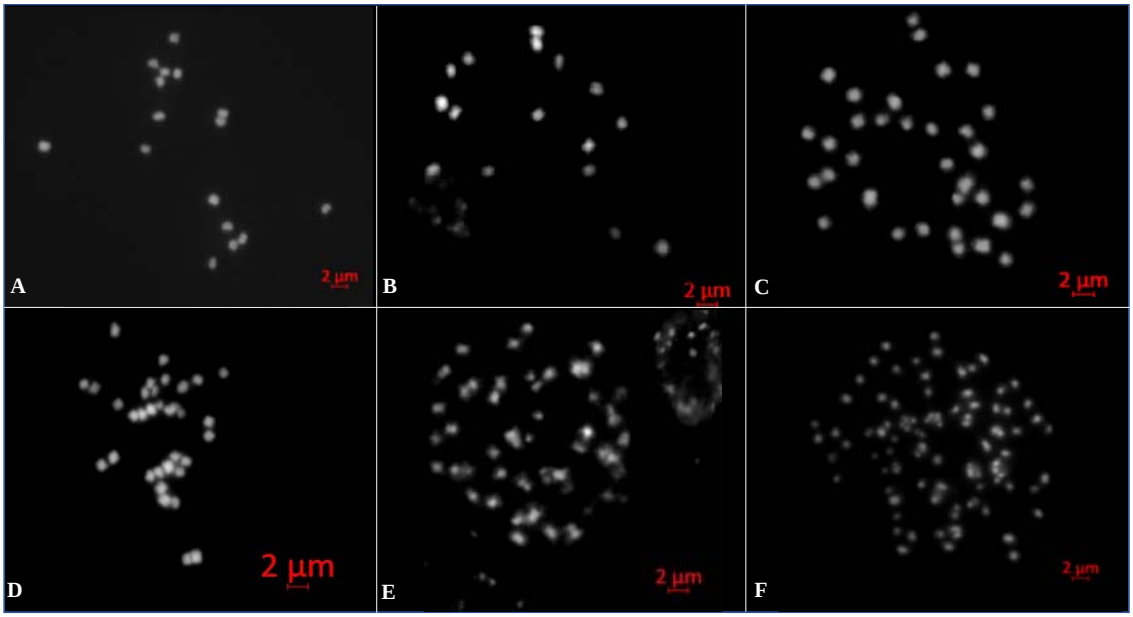
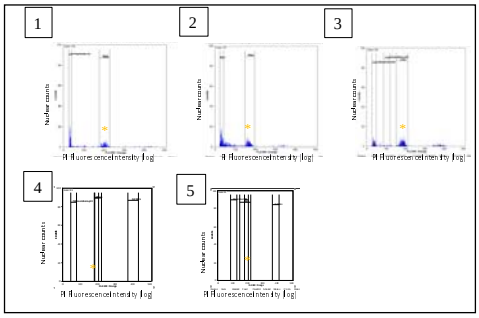


Figure S1: Polyploidy levels of different species of ludwigia sp section Jussieae. (**A**) *Ludwigia peploides* subsp. *montevidensis* chromosomes (2n=2x=16), (**B**) *Ludwigia helminthorrhiza* chromosomes (2n=2x=16); (**C**) *Ludwigia stolonifera* chromosomes (2n=4x=32); (**D**) *Ludwigia adscendens* 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)

A

Species	Mean size of genome (Mb)	Standard déviation	Confidence interval
L. peploides subsp. *montevidensis* (2X)	261,66	8,09	9,15
L. helminthorrhiza (2X)	267,66	1,19	1,65
L. stolonifera (4X)	/	/	/
L. ascendens (4X)	503,95	22,65	31,39
L. grandiflora subsp. *grandiflora* (6X)	863,88	2,33	2,63
L. grandiflora subsp. *hexapetala* (10X)	1418,86	94,80	107,28

B



C

		Cytometric peaks	pg [1]	Mb [2]
Trifolium Repens	1C	101, 32	1, 12	
L. peploides subsp. *montevidensis* (2X) Roots	1C	23, 56	0, 26	254
Trifolium Repens	1C	181, 96	1, 12	
L. peploides subsp. *montevidensis* (2X) Leaves	1C	45, 01	0, 28	270
Trifolium Repens	1C	93, 97	1, 12	
L. peploides subsp. *montevidensis* (2X) Leaves	1C	22, 55	0, 27	262
Trifolium Repens	1C	92, 68	1, 12	
L. helminthorrhiza (2X) Roots	1C	22, 82	0, 27	268
Trifolium Repens	1C	94	1, 12	
L. helminthorrhiza (2X) Roots	1C	23	0, 27	267
Zea mays	1C	199, 01	2, 7	
L. stolonifera (4X) Leaves	1C	39, 32	0, 53	522
Zea mays	1C	180, 69	2, 7	
L. ascendens (4X) Leaves	1C	35, 58	0, 53	520
Zea mays	1C	184	2, 7	
L. ascendens (4X) Leaves	1C	34	0, 50	488
Zea mays	1C	202, 55	2, 77	
L. grandiflora subsp. *grandiflora* (6X) Roots	1C	64, 39	0, 88	861
Zea mays	1C	201, 65	2, 77	
L. grandiflora subsp. *grandiflora* (6X) Roots	1C	64, 42	0, 88	865
Zea mays	1C	204, 61	2, 77	
L. grandiflora subsp. *grandiflora* (6X) Roots	1C	65, 33	0, 88	865
Zea mays	1C	189, 47	2, 77	
L. grandiflora subsp. *hexapetala* (10X) Leaves	1C	106, 88	1, 56	1528
Trifolium Repens	1C	98, 23	1, 12	
L. grandiflora subsp. *hexapetala* (10X) Roots	1C	122, 45	1, 39	1359
Trifolium Repens	1C	96, 96	1, 12	
L. grandiflora subsp. *hexapetala* (10X) Leaves	1C	121, 73	1, 40	1369

[1] : 1 pg DNA = 978 Mbp (from Doležel et al. 2003) ; [2] : Zonneveld et al, Forum geobotanicum (2019) 8: 24−78

Figure S2: Genome size results (**A**) and examples of peak profiles (logarithmic) in the flow cytometer of nuclei stained from roots with propidium iodide (PI) (**B**) and Flow Cytometry results (**C**).

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*.

(**A**) Fruit produced from the interspecific cross *Lpm* x *Lgh*

(**B**) Plantlets and plants produced from the interspecific cross *Lpm* x *Ls*

30 das

90 das

Figure S3: 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 days after seedling

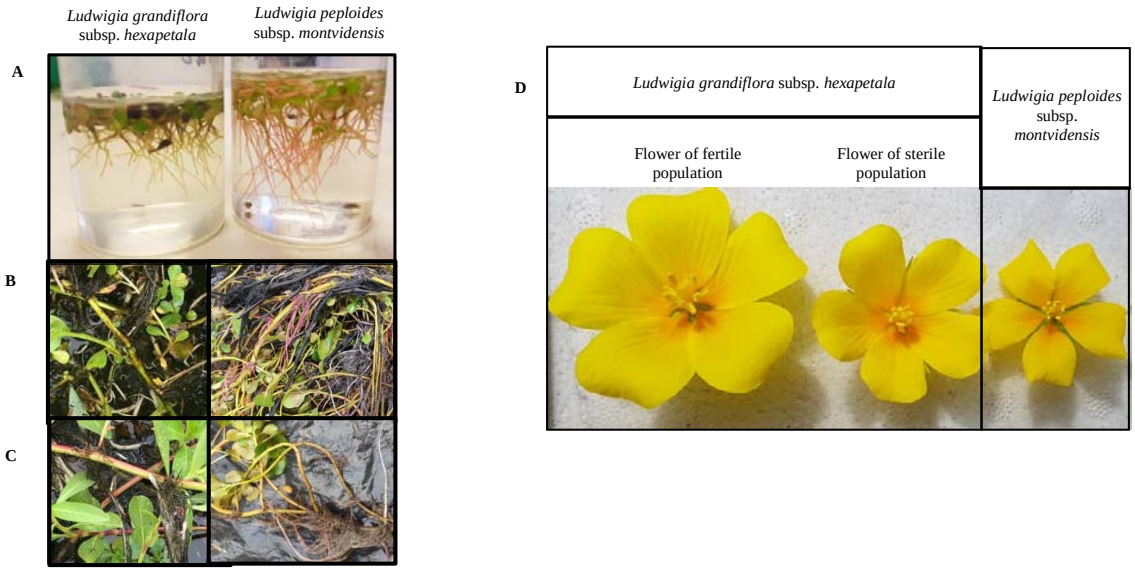


Figure S4: Morphological traits to distinguish *Ludwigia peploides* subsp. *montevidensis* and *Ludwigia grandiflora* subsp. *hexapetala*.

(**A**) roots at seedling stage; (**B**) adult roots in natura; (**C**) pneumatophores in natura; (**D**) flowers.