Reviewer 1:

1- Intro: When presenting species of section Jussiaea, previous findings regarding phylogenetic relationships between diploid and polyploid species (e.g. Liu et al. 2017), previous hypotheses regarding auto or allopolyploid origin are missing; this information is provided later in the discussion, but should be presented in the introduction to more clearly highlight the unresolved questions and the need for additional investigations.

Answer: We agree with this remark and clarified information about previous phylogenetic data from Liu et al (2017) and clarified hypothesis and unresolved questions.

Action: we added this information in introduction (see lines: 92 -108 and lines 133-136)

2- Intro: Similarly, the way the species are circumscribed and the need for useful diagnostic morphological traits to distinguish the species should be mentioned to better justify the morphological analyses that have been undertaken. As it stand the link between the morphological study and the goals of the paper, is not clear

Answer: As suggested by the reviewer 1, the aim of observing morphological characteristics was more to ensure that we had the 'right' species than to describe the morphology of these species, which had already been well studied elsewhere. The added value of our analysis is the fact of observing these species at the same time in a common garden. This is why we have focused on easily identifiable traits that do not require morphological measurements.

Action: To take account of this change,

(i) We complied information about morphological traits in a new table (see table S1) with a focus on traits observed in this study, indicated lines 164-166.

(ii) We changed the objectives of the study (lines 136-138)

3- Material and Methods: Plant material: The sample size (number of individuals) indicated per species is not consistent with the number of individuals examined across the different analyses (morphology, flow cytometry, GISH): please indicate to which analyses the numbers indicated in the plant material refer.

Answer: We initially collected 5 to 15 plants in function of species studied. Then, each plant through clonal propagation gave a lot of news plants which were used to carry out different analysis described in this study. Number of individuals indicated in different analyses came from clonally propagated plants.

Action: To clarify this point, we added in plant material part following information (lines 157 to 159): “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.”

4- Material and Methods: Morphological traits: Please justify the choice of the analyzed traits and the goals of this analysis.

Action: As explain above in connection with the change in the aim of the morphological trait analysis, we modified the corresponding part in material and methods

5- Genome size estimation by flow cytometry:

5.1- L. 175: Please provide the genome size of the species used as internal standard (Trifolium and Zea)

Answer: we added information (see lines 182-183: *Trifolium rupens* (2C DNA = 2.23 pg) *or Zea mays* (2C DNA = 5.55 pg) (Zonneved et al, 2019)

Action: done

5.2- Also in these polyploid species it would be better to use “1C” as “gametic” genome size (n), rather than “haploid”, which may be confused with “monoploid” (x). In the results (table 1) 2C values (instead of C-values) are provided

Action: done (see table 1)

6- Results: Morphology: lack of statistical analysis (any data regarding variability within species?)

Answer: Morphological traits analysis concerned only visual observations and none quantitative data were collected.

7- Discussion: Please start with an introductive sentence recalling questions and approaches

Answer: Thank you for this suggestion

Action: we added the following sentence “To better understand the evolutionary history of genus Luwigia, we have evaluated the genomic relationships between diploid and polyploid species using the morphological observations, molecular cytogenetic, and crossing investigations” (lines 346-348).

8- Ludwigia species in section Jussiaeae: I would have presented the diagnostic morphological traits (li. 338-345) in either the introduction or the method section to justify the choice of the studied traits.

Answer: we added information about morphological traits in introduction (see answer point 2) and in material and methods.

Action: done (lines 178-180)

9- The sentence l. 347 stating that the morphometric approach is “comprehensive” should be moderated as it is here based on two traits only without statistical analysis across populations over the range of the species (see also comments of Reviewer 1)

Answer: agree

Action: “comprehensive” was removed

10- Origin of polyploids: rely with their native and introduced range that are not indicated

Action: we added information about their native area in introduction (lines 87-90) and gave detailed in a supplementary table (table S1) then added one point of discussion about that (lines 393-395).

11- Line 392: “phylogenetic study”: rather use “genomic relationships and origins of polyploids”

Answer: Thank you for this suggestion

Action: expression has been changed throughout the text (line).

12- Line 393: we propose the “first phylogenetic history”: this is not appropriate: you could indicate instead “first hypotheses regarding diploid-polyploid relationships”…

Answer: Thank you for this suggestion

Action: expression has been changed (see line 389).

13- When referring to previously published phylogenetic work using nuclear and chloroplast DNA markers: can you discuss the maternal inheritance of cp DNA information compared to biparental ITS-Waxy information and the present findings using GISH (any comments on the maternal progenitor?).

Answer: Thank you very much for this very interesting remark.

Action: we compared ours results with those obtained by Liu et al (2017) and added different comments in discussion part (see lines 441 - 445).

14- The section on “combination of different data to identify phylogenetic relationships” is not convincing and not warranted as it stands … The interest of combining different lines of data and the critical contribution of molecular cytogenetics (which cannot be presented as an alternative to other approaches) could be briefly mentioned in the concluding paragraph.

Answer: we reduced this part and include sentences in conclusion.

Action: see lines 417-490

Reviewer 2:

1- Firstly, the authors should explain, or remind the reader, that with GISH methods, the inference is based on the blocking DNA rather than the probe.

Answer: Thank you very much for this suggestion.

Action: We added this sentence (line) “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 as probe 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. “

2- Secondly, the role of morphological observations is unclear as the authors report many features but without any morphometric analysis (measurement or coding of discrete variables and multivariate analyses). Without these morphometric analyses, it is difficult to assess the effects and even the purpose of the morphological observations. Perhaps the authors could use it as a simple verification step to prove that the identification is correct, this would allow them to reduce the size of their text.

Answer: The authors thank the reviewer 2 for this very relevant suggestion which correspond to the real objective of these observations.

Action: see answer of reviewer 1

3- Thirdly, the inference by GISH of the origin of the two 4X is clear but it is less clear for the 6X and 10X, the authors need to give more explanation on the relevance of intensity variation (page 13).

Answer: Thank you very much for this suggestion.

Action: We added this sentence in discussion (lines 413-416) “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.”

4- Finally, we are left wanting more from the experiment cross, perhaps a figure summarizing which are the crosses that always led to a dead-end could be informative to make hypotheses on what may have happened. Currently, a simple, fast reading may lead to the inference that all the crosses failed, but that’s not true.

Answer: Thank you very much for this suggestion.

Action: We have changed the initial table (see Figure 6), indicating whether or not the crosses were successful, and indicated the original table as supplementary table (Table S2).

5- Minor

page 2, l. 44: so what is diploid if all plants have experienced at least one polyploidy event?

Answer: It is a very interesting question about genome evolution and recombination between homoeologous or homologous chromosomes and exchange between cytoplasmic organelles during diploid and polyploid events. Recent genomic analyses 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). Question that would be need to explore by scientific community.

page 5 l. 117: the word phylogeny should be kept for inference based on phylogenetic methods (e.g. cladistic). Genomic relationships or genealogy is better here.

Answer: similar remark that reviewer 1

Action: Change (line 140)

page 8 l. 191: how many genomic DNA ?

Action: added line 200 (500 ng of total genomic DNA)

page 9 l. 222: does it mean that the experimental crosses were done after GISH and based on GISH inferences?

Answer: We carried out controlled crosses according to availability of flower/pollen and before obtaining of GISH results. Unfortunately, it was only possible to make crosses with species sharing the same AA genome.

page 10 l. 227: did you used a hood on flower to prevent non-controlled pollination?

Answer: Yes. We used cellophane bags before and after pollinations to prevent non-controlled pollination. We missed to specify this in material and methods.

Action: we added in materiel and methods these information (see lines 224-226).

Figure 1: improve quality of the pictures, it is pixelized.

Action: several pictures have been changed (see Figure 1)

Table 1: estimator of variance are needed (Sd or 95% CI)

Answer: In table 1, we have indicated the genome sizes of Ludwigia sp. estimated from different measures showed in Figure S2 without given this information in the corresponding legend.

Action: we completed the legend of Figure 1 by indicating the p-values.

GISH results: The explanation of the diploid GISH is confusing. I do not understand why and how Lpm and Lh could be “genetically close” but could “correspond to different genomes”. Perhaps I miss it but the overall strategy leading to table 2 should be explained. For example why not blocking the 4X La genome by the 2X Lpm genome?

Answer: Reciprocal hybridization between Lpm and Lh gave two different results. When blocking DNA of Lh was hybridized to Lpm chromosomes, all 16 chromosomes of Lpm were stained in red, meaning that no Lh genome is shared with Lpm. In the case of reciprocal GISH experiment, the results are not so clear: ten chromosomes of Lh were stained on grey (indicating 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. This is why we considered that Lpm and Lh correspond to different genomes even if homology exist. We did not made hybridization between La and Lpm but similar GISH results were observed on Lgg or Lgh chromosomes using Lh and/or La blocking DNA or using Lpm and Ls blocking DNA. These cross-referenced results point to genomic differences between Lpm and Lh and genome sharing between Lh and La and between Lpm and Ls.

Action: We have deleted the corresponding sentence, which could have led to confusion, and modified the following sentences: “Thus, the *Lh* blocking DNA did not block any sequence present in the *Lpm* probe, meaning that no *Lh* genome was shared with *Lpm* “ (see line 276-277) and ‘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. (see lines 279-281).

Page 14, l. 329: “no plants survived at 90 days after seedling”: is it due to their genomic composition or to another (external) factor ? do you compare the survival of seedlings among inter and intra specific crosses ? as control…

Answer: We carried out intraspecific crosses (self-pollination) for Lgh, Lpm and Ls (lines 229-230) as described in material and methods to control efficiency of pollination in greenhouse and added results lines 341-343. The results of intraspecific crosses were included in table S2. At the same time in greenhouse, we carried out another experiment of intraspecific crosses in Lgh. Germinated rate was 96,6% and all seedling gave all viable plants. Results of these crosses were published in Portillo Lemus, L. O., Harang, M., Bozec, M., Haury, J., Stoeckel, S., & Barloy, D. (2022). Late-acting self-incompatible system, preferential allogamy and delayed selfing in the heteromorphic invasive populations of Ludwigia grandiflora subsp. hexapetala. Peer Community Journal, 2.

Action: done

Page 15 l. 349 and after: it’s true that morphometrics done on plants grown in common garden are very precious however morphometrics require measurement or at least standardized observation of discrete variables and analyses (e.g discriminant analysis). For example, the differences between Lh and Lpm need to be confirmed by the data.

Answer: Since we have changed the objective of observing morphological traits, as you suggested, this remark is no longer relevant.

Page 20 l. 475-478: Although I agree on the fact that cytogenetics is crucial, it does not replace molecular phylogenetics or phylogenomics. Both are complementary and both are expensive if the salary costs are considered.

Answer: Agree

Action: we changed this part (as also request by reviewer 1), see lines 417-490.

Reviewer 3:

1- The authors should adopt a single taxonomic concept that should be referred to throughout the paper. The current version, where the same taxon is in one paragraph referred to as L. grandiflora subsp. grandiflora and in the other one as L. grandiflora (or in one as L. grandiflora subsp. hexapetala and in the other one as L. hexapetala) is very confusing.

Answer: we agree

Action: we specify that we use the nomenclature proposed by Nesom and Kartesz in our study (lines 99-101). Reference: Nesom, G. L., and J. T Kartesz. 2000. Observations on the Ludwigia uruguayensis Complex (Onagraceae) in the United States. *Castanea* 65: 123–125.

2- When counting species and subspecies one should not call all of them species (the term “taxa” instead of “species” would be more appropriate). E.g., “one hexaploid species (2n=6x=48) (Ludwigia grandiflora subsp. grandiflora); and one decaploid species (2n=10x=80) (Ludwigia grandiflora subsp. hexapetala)” [lines 85-87] – these are not two separate species, but two subspecies of the same species, i.e., two taxa. The same problem is here: ”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)”

[lines 87-89] – in the concept adopted in the previous sentence, these are two subspecies of the same species.

“Taxon name” should be used instead of “Species name” should be used also in the Table 1.

Answer: the naming of these two 'species' has been the subject of numerous publications with two different conclusions. Some authors consider them to be two separate species (*Ludwigia grandiflora* and *Ludwigia hexapetala*), while others treat them as two subspecies of Ludwigia (*Ludwigia grandiflora* subsp. *grandiflora* and *Ludwigia grandiflora* subsp. *hexapetala*). This has led to a great deal of confusion, with *Ludwigia grandiflora* even appearing on the list of invasive European species, whereas species 10x is present in Europe (i.e. *L. hexapetala* or *Lgh*).

Action: We have explained these two differences in naming and specify that we have retained the name proposed by Nesom et Kartesz (see lines 95-101).

3- Line 117: “phylogenic origin” – what is the exact meaning of this term? Would just “origin” be sufficient?

Answer: similar remark that reviewers 1 and 2

Action: Change (line 139)

4- The description:

4.1 The description of methods of Chromosome counting, Genome size estimation by flow cytometry, and Genomic in situ hybridization is too long, it can be shortened, providing the reference to some other papers.

Answer: We agree

Action: We have reduced these sections by indicating bibliographic references where the initial information is given: For Chromosome counting (see lines 175-176); for genome size estimation (see line 186); for GISH (see line 208).

4.2 On the other hand, the description of morphological measurements is not sufficient. The number and origin (locality) of measured plants for each taxon and morphological character should be provided (to document the representativeness of the measurements).

Answer: The numbers and the origins of plant (species) were indicated in plant material part with GPS coordinates. For morphological data, we indicated number of plants observed in text and added a table with morphological trait observed and theirs color/ form.

Action: see table S1 and lines for GPS coordinates (lines149 – 157); number of plants (lines 168-169).

5- line 142: “Morphological observations for each species were randomly made” – this is unclear (meaning of “randomly”??).

Answer: agree

action: suppression of randomly

6- Locality details (at least geographical coordinates of the locality of origin) and number of analyzed plants should be provided for each chromosome number count and genome size measurement for each taxon.

Answer: Geographical coordinates (GPS data) of material used were indicated in material and methods (lines149 – 157) excepted for which was bought in an aquarium shop (Ruinemans Aquarium B.V. IJsselveld 9, 3417 XH Montfoort, Netherland). For chromosome counting, we have indicated number in text. For genome size, information was given in the initial version

Action: For chromosome number, we have notified that “at least 40 root tips from 5 to 15 plants were taken, depending of species studied (see line 172) and for each plant chromosome counts were estimated on a total of 20 cells at the mitotic metaphase

stage (see line 177). For genome size fresh roots from five plants were collected" (see line 182).

7- Lines 225 ("produced flowers in continuous on a shoot"), 228 ("for each of other species") – unclear meaning (language).

Action: We removed both as it was finally not necessary.

8- Lines 404, 421: "hexaploid" instead of "hexaploidy".

Answer: agree

Action: change.