Reviewer 1

Review of the manuscript

Late-acting self-incompatible system, preferential allogamy and delayed selfing in the heterostylous invasive populations of Ludwigia grandiflora subsp. hexapetala

by

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This is an original study which deals with ascertaining biological traits (morphological, reproductive, genetic) of a species and their putative relationship with invasion ecology of the species. In short, the authors use their data on flower morphological variation in discrete style morphs, incompatibility systems, and reproductive output to infer processes on invasion ecology of the species invasion ecology. Thus, the whole study relies on an adequate description of morphs. I have several kinds of general comments on the suitability of this manuscript before the decision of PCI Ecology about its possible recommendation. Finally, I will provide some specific comments in the hope they will help in improving the manuscript.

About the topic

Whereas the topic is of interest to the public with a concern about biological invasions, I think that the information provided is not strictly and directly related with the process of invasion of the species. The biological traits reported are of course on potential implication on the invasion capacity of the species, but the authors are not strictly testing hypotheses about invasion, nor at local nor at global scales. Instead, the information provided is valuable in the context of botanical and plant biology literature. This information is about morphological, reproductive, embryological, and physiological aspects but by no means ecological ones, thus it is not possible to answer ecological hypotheses in a straightforward manner. In consequence, most of the Discussion is highly speculative.

About the data

Here there is my strongest concern about the manuscript. I mean that most of the study and the manuscript uses the concept of reciprocal style morphs and heteromorphic incompatibility just to support its significance for explaining a pattern of invasion. I must say that the data provided do not clearly demonstrate the existence of such morphs. This tenet is based in lines 98-102 and 108-112 of the Introduction, which is based in former studies by the authors and others (line 110: Hieda et al. 2020; Portillo-Lemus et al. 2021). I have checked carefully the information provided by these references and I do not think it demonstrates unequivocally the existence of heteromorphic incompatibility or even the existence of morphs. The reviewed manuscript neither does that. The study by Hieda et al. 2020 does not mention style morphs at

all; may be the authors of the current manuscript infer the information from the morphological information provided by those authors, but it is unclear. Most importantly, the reviewed manuscript (and the former one by Portillo-Lemus et al 2021 Plant-Environment Interactions DOI: 10.1002/pei3.10042) lacks critical points in the design:

1. You need to take random samples of individuals (genets) where to pick flowers to take proper floral measurements. Whereas floral measurements for style length and stigma height are apparently well performed, I do not see the equivalent measurements for stamens. Critically, we do not know if separate genets were properly sampled in a species and populations where vegetative reproduction is so important.
2. In this context it is very surprising that former literature on systematics, reproductive biology and morphology on Onagraceae and Ludwigia, did not report heterostyly and heteromorphic incompatibility in the family and genus, as it was not reported in the several available surveys on heterostyly presence across angiosperms. It should be taken into account that some of the most remarkable papers were written by well-known scholars on the topic (particulalry PH Raven and RH Eyde, some of them cited by the authors). Of course, it is always possible to find new cases of heterostyly, but they should be properly documented. Just as a noteworthy mention by Peter H. Raven (1979) A survey of reproductive biology in Onagraceae, New Zealand Journal of Botany, 17:4, 575-593, DOI: 10.1080/0028825X.1979.10432572 with abundant information on Ludwigia which made clear that heterostyly was specifically searched:

"No species is known to be modally pollinated by bats, by beetles, or by wind, and none has apomixis involving seeds, nor heterostyly "

"Although it is frequent in the related family Lythraceae, heterostyly is unknown in Onagraceae."

Obviously discovering of hetersotyly in Onagraceae for the first time, as recognized by authors, would be a very interesting novelty, but should be properly supported by the data.
3. Heterostyly, and related style polymorphisms is, by definition, a population trait, because it works by disassortative mating, a negative frequency dependence mechanism. Of course, there are many reports of populations of heterostylous species that are otherwise monomorphic, just due to loss of one of the morphs or to colonization of only morph, which should have an alternative mode of reproduction (either vegetative, selfing, or intramorph compatibility). However, in all these cases, the species show some dimorphic source populations. These have not been reported by the authors and either in the former paper for the species under study by Portillo-Lemus et al 2021 Plant-Environment Interactions DOI: 10.1002/pei3.10042. Even that it is mentioned that 75% of the populations are composed by only L-morph plants, and that others are composed by the two morphs, the data provided do not accomplish requisites for proper morph sampling, nor there are data from native populations were both morphs should obviously exist.
4. Therefore, the pattern shown of a self-compatible (SC) morph and a self-incompatible (SI) morph in separate populations, if they really exist, could be explained by alternative hypotheses, for instance, the two “morphs” could be two lineages or species related but distinctly different given the difficult taxonomy of the species and the different ploidy levels in the group having different reproductive modes. The shift from SI to SC has been frequently reported and it is a very interesting research avenue according to cited Baker’s law, but it would need detailed phylogeographical and phylogenetic studies. Independently, it is very possible that the authors have sampled very few genotypes, or even only one, per population if vegetative reproduction is very strong. Again, this is a very relevant issue for invasion ecology which would need appropriate use of genetic markers to determine the genets/ramets being sampled. I recommend the authors explore these possibilities, and others, in depth with proper data and, if they are convinced that the species is truly heterostylous, gather the

appropriate morphometric data for that.

5. The figures provided by the authors about flower measurements seem to show two different style length morphs (but see issues about sampling design: the flowers selected where different "morphs" a priori), but stamen length of morphs is not shown, which is needed for assessment of heterostyly. Besides, other factors such as style developmental stage across flower life span should also be considered.

Specific comments

Line 70. The species in Barranco et al. 2019 is not heterostylous, but stigma height dimorphic, although it is related with heterostyly, it has not reciprocal herkogamy as claimed by the authors.

Lines 85-87. Many of the Narcissus species included in Barrett et al. 2004 are not heterostylous, but stigma height dimorphic species, as it is the species studied by Simon-Porcar et al 2015. The species studied by Medrano et al 2012 is monomorphic.

Line 91. Ludwigia should be in italics

Line 92. were should be was

Line 121 and thereafter. What the authors report is information on breeding system or incompatibility system, nor mating system. This is a population trait which depicts what plant mates with what other plant. See Neal & Anderson Plant Syst. Evol. 250: 173–185 (2005) DOI 10.1007/s00606-004-0229-9 for clarification about these terms.

Line 125. Pollen and stigma dimorphism have never been reported as characteristic of homomorphic SI; even more, these differences cannot be taken as distinct features to separate homomorphic and heteromorphic SI systems as there are heteromorphic SI lacking the pollen-stigma dimorphism.

Lines 135-137. This comparison is meaningless as there is not phylogenetic information used here. Instead, it would be more valuable to explore the distribution of existence of different breeding systems in different morphs of the same species (if demonstrated, see above).

Lines 142-145. Why dimorphic populations were not explored? if they do not exist, that is intriguing: it is by chance that only one morph is forming each population? any selective values of morphs? I doubt it, but it should be explored. Probably they are different lineages, yet reproductively non-isolated, forming largely clonal populations, which offer a more parsimonious explanation. Additionally, 10 stems (genets, ramets? this is critical issue) is a very small sample to characterize a population in heterostylous species.

Lines 169-179. The numbers of samples in this paragraph (which are large indeed) does not refers to how many individual plants (genets) and this is critical.

Lines 189-191. I do not see what the rationale is to mix pollen from S- and L-plants to do experimental intermorph pollinations. I assume that results are clearer with appropriate pollen donors of only the opposite morph. Why simulating random crosses? in fact you did not simulate that.

Lines 228 and thereafter ("Statistical analyses"). This section should include also details on analyses of floral morphology and any other data, in addition to those details on pollen tubes already included.

Lines 341-343. What is the meaning of this advantage if populations are composed a of a single morph? This is why is so important to study also dimorphic populations, if they exist.

Lines 353-354. Narcissus tazetta and N. papyraeus are not heterostylous, but style dimorphic, and their SI is not heteromorphic, but similar to gametophytic homomorphic SI.

Line 357-358. Ipomopsis aggregata is no homomorphic, but monomorphic. The difference is

critical in heterostyly literature: monomorphism refers to one single morph in the population/species, whatever the herkogamy is; homomorphism refers to a monomorphic condition with no herkogamy.
Line 360: You should also mention that Raven (1979) explicitly mentioned that no Onagraceae is reported to have heterostyly.
Line 388 and thereafter. What is the significance of the advantage of cross-pollen tubes over self-pollen tubes if this type of cross does not occur in wild populations? I agree that this mechanism has been previously and frequently reported, but in monomorphic species.
Line 400. The concept of "in situ populations" is odd: I would suggest "wild populations"
Line 404-405. How do you discard that the small number of seeds produced by L-morph wild populations are not due to the pollen-transfer from nearby, unsampled S-morph populations as a result of natural legitimate cross-pollination between morphs?
Line 408 and thereafter. The number of seeds per se does not tell almost anything about sexual regeneration of populations, you need demographic data (number of seedlings, juveniles, adults) to address this important question of invasion ecology. Also, the balance between sexual and vegetative reproduction is crucial here.
Lines 419-426. What you mention, two breeding systems in the same species, is not mixed mating (see my comment above about appropriate terms). You need appropriate markers to demonstrate mixed mating (i.e., the relative number of seed sired by outcrossing and selfing in wild conditions).
Reference Gibbs PE 2014a and 2014b are the same
Reference Gibbs PE, Byran GW 1986 is unrelated with the topic. The first author is not the same as in the former ref.
Reference Takayama S, Isogai A 2005. Please change title to lower-case
Figure 1. Why are stamen measurements not provided? they are critical for definition of heterostyly as reciprocal herkogamy
Figure 6. This king of information is better provided as a table or bar diagram figure
Figure S1. If each point in the curve along time is derived from sampling, as I suppose, you should report error bars.
Figure S2. I do not see its value for understanding the manuscript.

On the more positive side, I must acknowledge the value of the study for plant biologists and botanists as the histological work about SI and its site of recognition is superb. If the work is properly addressed in the future, within the heterostyly field if it is finally demonstrated, or in a different context of different lineages with different histories, it will be a significant contribution.

Reviewer 2

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I consider the topic of this work adequate and interesting to be publised, however I think that some ideas must be reviewed:

A) Introduction:

· L72-L74: The authors expose: "Commonly, species with style polymorphism have a sporophytic heteromorphic (i.e. di- or tri-allelic) incompatibility system that prevents self-

fertilization and crosses between individuals of the same floral morph (Barrett 2019)." However, there are several genera, as Lithodora, Glandora, Narcissus, in which stylar polymorphism (or heterostyly is not associated with the presence of an heteromorphic incompatibility system.

· L81-82: "It concerns both homomorphic and heterostylous species (Gibbs 2014a; Simon-Porcar et al. 2015)." The study of Simon-Porcar et al 2015 is focused on a stylar dimorphic species (Narcissus papyraceus); therefore, the term heterostylous in L81-82 should be replaced to "stylar polymorphic".

· L85-87: "In the ovarian LSI heterostylous Narcissus spp. from the Amarilidacea, from 4 to 30% of their seed-sets result from self-fertilization (Barrett et al. 2004; Medrano et al. 2012; Simon-86 Porcar et al. 2015)" In the genus Narcissus there are 2 species which presents heterostyly and 12 or 13 that exhibit stylar-dimorphism. The data and the references that give us the author are from a stylar-dimorphic species (Simón-Porcar et al 2015) and a monomorphic species (Medrano et al 2012). Therefore, the term "heterostylous" in line 85-87 should be changed to stylar-polymorphism and the reference Medrano et al 2012, should be eliminated because that work is focused in Narcissus longispathus, which is a monomorphic species.

· L103-L108: The authors expose the floral architecture of both morph of this species, however they do not reveal the position of the stigma in bot morph; at this point I suspect that the short-styled morph exhibit the stigma below the two levels of anthers but I'm not sure about the long-styled morph, the stigma protrudes the two levels or anther or is it between the two levels or anthers?. Furthermore, it is not the only heterostylous species that exhibit to levels of anthers.

· L111: the authors exposed that 75% of the invasive populations worldwide are composed of L-plants. Could be this fact a consequence of vegetative reproduction? They do not say nothing about the frequency (or relevance) of vegetative vs sexual reproducition in invasive populations.

Materials and methods:

· L173-174: A brief description of the pollen morphology of this species should be given to understand why the authors decided to measure the diameter of the pollen grains.

· L174-L177: It is necessary to include a brief description of the principal differences between the stigmatic papillae in heterostylous species; and the authors should include how they are going to measure these differences.

· L197: I would like to know Why the authors fixed the flowers 2, 3, 7, 16 and 24 hours after the cross- or self-pollination. Are they following a specific protocol?

· L216: "We also assessed the rate of self-fertilisation in self-incompatible L-morph from in situ populations at the beginning of October." L-morph should be replaced by L- and S-morph; Because They estimated the rate of self-fertilisation in both morphs.

· L216-L225: They collect seed from natural populations to estimate the rate of self-fertilization, however they do not specify if these flowers were caged or not and if they were not caged, how it is possible to be sure that crosses between different plant of the same morph were avoided?

· L232: It is not crucial but, in a GEE or GMM model, the authors could include random variables as the plant or the population.

Results:

· L250-254: The authors found differences in style length and width between both morphs, however in the Materials and Methods section they do not specify how they analyzed this observation and what is the goal.

Discussion:

· L341-L43: The authors exposed: "Yet, in the two Lgh floral morphs, inter-morph pollen tubes always elongated faster than self-pollen tubes, which may give advantage to intermorph crosses when inter-morph pollen is available". This fact may have an effect on the stability of both morphs, specially for the L-morph in natural populations, and I think that I must be discussed.

· L353- L354, Narcissus tazetta and N. papyraceus are not heterostylous species. They exhibit stigma height dimorphism. In the genus Narcissus there are two heterostylous species: N. triandrius and N. albimarginatus. therefore, these species should be cited as stylar-dimorphic species or stylar-polymorphic species (if the authors include N. triandrus).

· L376-L385: I´m not totally agree with this observation and I´m not sure that this study prove that the residual number of seed that produces the L-morph after self-pollination can be a mechanism to maintain this morph in a natural population.

· L387-L309: The authors might give an explanation about why this fact is only observed in the S-morph and what is the role of this characteristic in the maintenance of both morphs in natural populations.

· As I told before, in the introduction section, it is important to include also in the discussion section a paragraph that compares rates vegetative and sexual reproduction in the invaders and native areas of this specie to understand the relevance of the mechanism described in this work.

· Finally, it could be interesting add a final paragraph in the discussion section to talk about the relevance of this work to control the invasion of this species.