

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

Luis O. Portillo Lemus, Maryline Harang, Michel Bozec, Jacques Haury, Solenn Stoeckel, Dominique Barloy

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Submitted by Solenn Stoeckel 16 Jul 2021 09:53

Abstract

Mating system influences local population genetic structure, effective size, offspring fitness and functional variation. Determining the respective importance of self- and cross-fertilization in hermaphroditic flowering plants is thus important to understand their ecology and evolution. The worldwide invasive species, *Ludwigia grandiflora* subsp. *hexapetala* (Lgh) presents two floral morphs: one self-compatible short-styled morph (S-morph) and one self-incompatible long-styled morph (L-morph). Most invasive populations worldwide are only composed of self-incompatible L-morphs, which questions the importance of sexual reproduction during the invasion. In this study, we identified the mating systems of western European experimental and natural populations of Lgh by comparing structural characteristics of pollen and style, by studying self- and cross-pollen tube elongations and the viability of the resulting seeds and seedlings in both morphs. Our results showed no differences in pollen shape and stigma surfaces among and between the two floral morphs. In the self-incompatible L-morph flowers, self-pollen tubes were stopped tardily, in the ovarian area, and were unable to fertilize the ovules. This first formal identification of a late-acting, prezygotic self-incompatible system in *Ludwigia* genus questions on the distribution of this mating system in the Myrtales order. In the self-compatible S-morph flowers, rarer in worldwide invasive populations, self-pollen always succeeded to self-fertilize the ovules that nearly all developed into viable seedlings. However, cross-pollen tubes always elongated faster than self-pollen tubes. S-morph individuals may thus advantage preferential allogamy over selfing when cross-pollen is available despite its self-compatibility. As expected in late-acting self-incompatible systems, L-morph flowers authorised 0.2% of selfed seeds during the uppermost flowering season, that increased to 1% at the end of the flowering season. Such delayed selfing resulting in a significant quantity of viable floating seeds may contribute to the local regeneration, seed bank and propagation of the L-morph, which may explain its invasion success worldwide. Management plans of Lgh would gain to consider the mixed mating system we identified.

Keywords: Delayed selfing, Mating system, Onagraceae, Pollen tube elongation, Reproductive assurance, Self-fertilization, Water primrose

Round #1

Author's Reply:

by Antoine Vernay, 20 Sep 2021 19:14

Manuscript: <https://doi.org/10.1101/2021.07.15.452457>

Decision about your manuscript

Dear Dominique and colleagues,

First of all, I would like to apologize for this late decision, summer vacations slowed the reviewing process. Two referees were assigned to your manuscript, and in my opinion, read and commented on your paper very carefully.

Unfortunately, I have decided to not consider your manuscript for recommendation by PCI Ecology for two main reasons:

- As mentioned by referees, I agree that if reproduction is a key aspect of the invasion process, your experiment was not designed to fully address this question and did not bring clear evidence of the role of *Ludwigia heterostyly* in its invasive abilities. Your paper may more fit in a biology or physiology journal.

- the *Ludwigia heterostyly* is not consensual in the botanist community. The Onagraceae family does not even have any heterostyly species for some authors. I agree with the referees that this fundamental aspect of your study is not clearly demonstrated. Referees also highlighted several (maybe too many) inaccuracies in your introduction in the different comparisons with others studies you made among the manuscript.

Referees wrote several other concerns, suggestions, and advice to improve the paper but I think the manuscript needs too many modifications and reconsideration to be recommended even with major revisions.

I attach to this email the reviews of the two referees and I hope it can help you to improve your paper. Thanks for submitting your manuscript to PCI Ecology and I hope that, despite this rejection, you will consider PCI Ecology again for future submissions.

Best regards,

Dear PCI Editorial board, dear Recommender and dear Reviewers,

First of all, we would like to thank the editor and the two reviewers for their time and works on our manuscript. We agree that this one can be improved, and that's precisely one of the two main objectives of the reviewing process: to benefit from outside looks on the presentation of results obtained along nearly 3 years of work, even if already submitted to multiple external evaluation (included a phd defense) and specialists; especially, if some terms are not adequate, if some definitions of the employed terms need to be more formally written, or if some parts of our work need to be better explain or discussed. It is what we expect from the interesting dialogue of a reviewing process, with specific, constructive and argued comments rather than unspecified definitive rejection.

We were surprised by the conclusion of this review, considering on one hand the reviewers' comments and conclusions, and on the other hand, the recommendation process in PCI. First point, the two reviewers acknowledged the quality and the interest of this manuscript: We cite Reviewer1:

“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.”

And Reviewer2

“I consider the topic of this work adequate and interesting to be published”

So, we wonder what major point or irreparable analysis error was found against this manuscript to definitely reject it from PCI Ecology or Evolutionary Biology?

Second point: Reviewer#1, from our point of view, either reviewed a previous, already-peer-reviewed and accepted paper (Portillo-lemus et al 2021, Plant-Environment Interactions), or missed the specific topic and the results presented in this manuscript. Yet, in his citation (last paragraph), he seemed to clearly identify the thematic of this manuscript: we report here *“histological work about SI and its site of recognition”* and assess the mating systems of individuals and populations, by measuring the level of permeability of the self-incompatible system in populations in which all individuals are self-incompatible of the same type (which coincide with a floral morph, but we don't mind here, because we are studying the self-incompatible system that not necessarily segregate perfectly with the floral morph). Nowhere in this manuscript, we attempted to demonstrate or establish botanical conclusions like heterostyly and the definitive identification of a heteromorphic self-incompatible system. We even do the inverse: our results rather identify a Late-acting, ovarian, self-incompatible system with at least one self-compatible type. If Reviewer#1 think we should use “type” rather than “morph”, we will follow. We exchanged with specialists, either by mails (including Spencer Barrett and Noni Franklin-Tong) or in oral discussion before submitting, and these ones recommended (some rather urge us) to use the standard nomenclature: “long-styled” and “short-styled”, and “floral morph”. That's why we used these terms, but we are clearly not interested in these expert semantic discussions as we are not botanist but rather ecologists and evolutionists. We need to define precisely the mating system of these populations (preferential outcrossing with possible delayed selfing and reproductive insurance) because as sexual reproduction (and its massive recombination events) is newly occurring in these invasive populations, it will have deep consequences on the fate of this invasion, for their ecology and evolution, and the impacted ecosystems (as explain in our introduction and in discussion on a dedicated part).

That's why we contest this final decision and ask the recommender to get back on the rude rejection of our manuscript in PCI. If decision maintained, we ask recommender and reviewers to point out the major error in our manuscript that would justify its definitive rejection. On our side, instead of this hurried rejection, we would like to continue a constructive discussion with the reviewers if they agree, especially considering that reviewer#2 raises interesting points to improve our manuscript and our outlook on our results, and that reviewer#1, when not prosecuting our previous paper or focusing on the terms we used (that can be changed), would definitively help us to better structure and communicate

our results. We also agree to contact a third reviewer or change the recommender if they don't want to recommend our manuscript, and, we can in this case, provide some more names of researchers in the thematic.

[Download reviewer's annotations](#)

Reviews

Reviewed by Juan Arroyo, 20 Sep 2021 10:51

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

Luis O. PORTILLO LEMUS, Marilyne HARANG, Michel BOZEC, Jacques HAURY, Solenn STOECKEL, Dominique BARLOY

Submitted to Peer Community in Ecology as <https://doi.org/10.1101/2021.07.15.452457>

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.

Answer: We believe there's a founding confusion here. Our manuscript aims at identifying the mating systems (or breeding system as distinguish reviewer#1) of self-incompatible and self-compatible invasive plants of *Ludwigia grandiflora* subsp. *hexapetala* (here after Lgh).

Considering the Reviewer#1 remarks, in this paper, we don't interest in heteromorphy and floral biometry. We rather study sexual reproduction (outcrossing, permeability and selfing during summer then fall) and the location of the self-incompatible reaction in the pistil of the SI plants in the invasive populations of Lgh and the respective production of viable seeds in populations, while these populations were previously supposed to be "fruitless" and "seedless" because out of their thermal comfort zone (see literature review in the introduction of Portillo et al. 2021, Plant-Environment Interactions). However, we use our previous result, showing a binary distributed coincidence of a heterostylous floral morphology with self-incompatible and self-compatible plants in 37 populations in France (Portillo et al. 2021, Plant-Environment Interactions) to put priors on the self-incompatible types of sampled plants in populations and grew in our common garden: this prior again using crosses for studying pollen tube elongations

remained valid (no exception), even if we showed a limited rate of permeability of the SI system resulting in one or two seeds in a typical fruit (small, yellow), versus big green fruits full of 60 seeds always obtained when cross-pollinated.

In the French invasive populations, we previously demonstrated the existence of self-incompatible and self-compatible individuals that co-occurs with two floral morphs characterized by, respectively, pistils above and beyond the second stamen whorls. Self-incompatibility and self-compatibility were demonstrated using 840 hand-pollinated flowers sampled from 7 populations in a common garden. We performed 105 self-pollinated flowers, 105 intra-population cross-pollinated flowers, and 630 inter-population cross-pollinated flowers, corresponding to 15 flowers per population per pollination condition and on the monitoring over two years of 37 populations, separated with a maximum distance of 600km. On a random subset of 60 total flowers, we measured 10 floral morphological traits. Measured traits were the length and width of the sepal and petal, length of the stamen and anther for the first and the second whorls, length of the pistil and width of the floral receptacle, and production of nectar (Table S2). We measured these floral traits with a digital caliper (0.01 mm accuracy). We also measured (at first 60 pistils, then to confirm our own surprise, we added more pistils) 150 styles, using 75 flowers (one per individual stem) from fruitful self-compatible populations and 75 flowers from fruitless self-incompatible populations, randomly samples in the populations. Overall experts, reviewers and listeners of our presentations, no one expressed doubts on our data, especially citing Raven 1979 that questioned himself on this point by writing in his paper “*Although it is frequent in the related family Lythraceae, heterostyly is unknown in Onagraceae*”. *Ludwigia* group seemed to have been considered as intermediate at some points between Lythraceae and Onagraceae, a point that we discuss in this manuscript, with other related clades that already include known late-acting ovarian self-incompatible plants.

Whatever, whether or not this plant is heteromorphic is not the purpose of this current manuscript and definitely doesn't matter to study and understand 1/ if self-incompatible and self-compatible plants present different shapes of pollen grain and stigma surfaces like observed in some other early-acting (homomorphic, monomorphic or heteromorphic) self-incompatible systems, 2/ where the self-incompatible reaction occurs within the pistil of the self-incompatible plants (surface rejection of self-pollen, or within the pistil or late reaction, in the ovarian area) and to quantify their level of permeability during the flowering season then, in the fall to understand if Loire populations previously described as completely sterile may produce a small quantity of selfed (thus recombined) seeds that may move to seed banks or propagate Lgh, 3/ differences in pollen tube elongation between allo- and auto-pollen, even in the pistils of the self-compatible plants, to hypothesize what will happen when self-compatible and self-incompatible populations will merge in a near future: will we observe preferential outcrossing or a spread of the self-compatible type over the self-incompatible one.

If the term “floral morph” or “L-morph” and “S-morph” is controversial, we would agree to change these terms by the ones reviewers precisely propose considering our results. We are not floral biologists and are not inclined to debate floral biology semantic and terminology at a level of precision not need to tackle our topic. Our objectives as ecologists and evolutionists are clearly to understand the ecological and evolutionary processes driving the worldwide expansion of this species out of its primary distribution area. We used “L-morph” and “S-morph” after 1/ email exchanges with Spencer Barrett and 2/ the feedbacks of associated editors from the journal “Plant Reproduction” that recommended to use these commonly used terms. Likewise, we are not phylogeneticists or plant taxonomists. We only study the morphological, the reproductive systems, the genetic diversity and the ecology of one species, Lgh; And we

still think that we have found and documented a Kuhnian anomaly that should be addressed (a late-acting SI in *Ludwigia*, and a possible dimorphic flowers in *Ludwigia*), if needed, in future studies, by botanists and taxonomists; Even if we won't study ourselves these two points (as it is out of our skills), we however think important to mention these points in our discussion for these communities.

Demonstrating that self-incompatibility co-occurs (and in fact, would segregate perfectly, this is the main reason to reject our manuscript if we understand correctly?) with some floral morphs is not needed to understand the mating system of the self-incompatible individuals and of the self-compatible individuals, deduced from monitoring pollen tube elongations and seed viabilities after hand-controlled cross or self-pollinations, within these invasive populations. To our point of view, knowing if we should call it or not "heteromorphic", "herkogamous", etc. is a debate between plant biology specialists, and it won't change what types of seeds are able to be produced in these invasive populations. We whatever identified a *Kuhnian anomaly* (Thomas Kuhn, 1962) from our point of view in the plant taxonomy, that should be published and discussed further by specialists.

If specialists can demonstrate this is not heteromorphy or something else, we would be interested to read the demonstration and explanation, out of an argument of authority about a reference that reviewed (with unspecified methods and associated references) the self-incompatible characteristics of hundreds of species, including this one, without even mentioning each species but referring to them in mass, by group or genera. The corresponding sentence in Raven paper: « Although it is frequent in the related family Lythraceae, heterostyly is unknown in Onagraceae ». For information, the family Onagraceae comprises 17 genera and about 674 species. Without questioning the work previously published by Raven in 1979, it is likely that the populations observed in the 674 species studied (number of populations not cited) are not exhaustive and that heterostyly was not observed in the populations sampled. Again, we study this species for years, on the field and by growing hundreds of samples from France, USA, Spain, etc. in our greenhouse. We only discovered only recently, by chance, this feature (we are looking at couples of millimeters of differences). Again, we will like to see contradicting results and measures, and would infirm our current results if some methodological or floral biology study demonstrate the inverse: this is the way sciences work. But for the moment, the limited returns after our first paper don't deny our results (and even confirm them), included colleagues from USA.

Finally, in this manuscript, we got attached to the facts. If you think not, we would be pleased to read the lists of points on which our results don't support our conclusions so we can correct them in our next version, preferentially reviewed by the same reviewers in *PCI Ecology* or *Evolutionary Biology*. What we do not tackle is heterostyly. We agree reviewer#1 can have an opinion on an already peer-reviewed and published paper, but the expressed opinion is in any case on the topic of this manuscript. Heterostyly doesn't interfere with the pollen shape, stigma shape, the place where the self-incompatible reaction occurs in the pistil, the speed of pollen tube elongations in self and cross-pollination, and seeds viability when coming from self- or cross-fecundation, them that are clearly studied here.

Cited literature

Thomas Kuhn (1962). *The Structure of Scientific Revolutions*, Chicago: University of Chicago Press (1970, 2nd edition, with postscript).

Reviewer comments

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.

Answer: nowhere we claim that we are tackling hypotheses about invasion. Invasion biology is the context and justification to study and characterize the mating system of this species in Europe, that was previously reported for years binarily as either fertile or unfertile, depending on the climate impacting the location rather than due to some self-incompatible system. Identifying the self-compatibility of the invasive populations is essential to understand its invasion and fertility on the invaded area (see Baker's conjecture, Barrett 2015). Quantifying the production of self-sired seeds and outcrossed seeds in populations (in the last part of our results) enable understanding the importance of our initial question, this invasion and its recent adaptation to new habitats occurred in one of the few sexually reproducing populations (i.e., dried lands, Genitoni et al. 2020; Adaptation due to recombination?). We believe that determining the reproductive modes of individuals colonizing new areas far beyond their initial ranges and measuring the viability of the resulting seeds are ecological and evolutionary questions, suitable for PCI Ecology or Evolutionary Biology. Whatever our respective opinion, this point should be addressed by Recommender and Editorial board.

Barrett, S.C.H. (2015), Foundations of invasion genetics: the Baker and Stebbins legacy. *Mol Ecol*, 24: 1927-1941. <https://doi.org/10.1111/mec.13014>

Genitoni, J., Vassaux, D., Delaunay, A., Citerne, S., Portillo Lemus, L., Etienne, M.-P., Renault, D., Stoeckel, S., Barloy, D. and Maury, S. (2020), Hypomethylation of the aquatic invasive plant, *Ludwigia grandiflora* subsp. *hexapetala* mimics the adaptive transition into the terrestrial morphotype. *Physiol Plantarum*, 170: 280-298. <https://doi.org/10.1111/pp1.13162>

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.

Answer: We discuss on the ecological consequences of our results in a dedicated section over one page in the Discussion part named “**Consequences of LSI for invasive populations of Lgh**”. Reviewer1 seemed to miss the point that sampled plants come from populations supposed, by literature and managers, to not produce seeds (and sexual reproduction was completely ignored in these populations till our study). We would ask reviewer1 to argue and give concrete examples of “*highly speculative*” discussion. We will agree to better target our discussion on the points that would suit reviewer1, with constructive comments and indications, as we are convinced that this is the main objective of the reviewing process.

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.

Answer: No, we don't explain “a pattern of invasion”, but study the factors explaining a pattern of seed production in invasive populations, that completely depends on sexual compatibility and permeability of self-incompatible plants when no compatible mates are available in the surroundings.

I must say that the data provided do not clearly demonstrate the existence of such morphs.

Answer: We are not tackling the existence of two morphs here, that was a side objective of our previous paper, demonstrated on 150 measures coming from 7 populations (not enough?). So, here, reviewer#1 is questioning our analyses in an already-reviewed and published paper, with consequent data. Okay, why not. We would be interested to see other data and analyses that would demonstrate the opposite result. Can we ask reviewer1 which additional data to Portillo-Lemus et al. 2021 (plant environment interactions) would “clearly demonstrate” the existence of two floral morphs coinciding with self-compatible and self-incompatible types?

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.

Answer: The mentioned sentence (lines 108-110) writes: “*Interestingly, floral morphs are mostly found in allopatric monomorphic populations (i.e., exclusively S-morph or exclusively L-morph populations) in Western Europe and other invasive worldwide populations (Hieda et al. 2020; Portillo-Lemus et al. 2021)*”. We don't write that Hieda et al. 2020 mention, style morph, but they only have in Japan the L-morph out of the two floral morphs we find in Europe. We write before that only Portillo-Lemus et al. 2021 identified two floral morphs (line 99-102). Hieda et al. 2020 even discuss that species identification by floral morph in *Ludwigia* is often confused.

Action: But we agree that the sentence will be clearer if written as “*Interestingly, floral morphs are mostly found in allopatric monomorphic populations (i.e., exclusively S-morph or*

exclusively L-morph populations) in Western Europe (Portillo-Lemus et al. 2021) and in other invasive worldwide populations (see for example pistil and stamen measures corresponding exclusively to the L-morph in Japan, Hieda et al. 2020)."

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.

Answer: We can remove these data, as they were already published in Portillo-Lemus et al. 2021 in its supplementary file. In this manuscript, these data bring confusion on our topic.

Moreover, in Portillo-Lemus et al. 2021, PEI, floral morphs were studied in 37 populations over a nearly 600km transect. We sampled 15 individuals per population from 7 populations to be grown for this precise experiment (floral measures) in a common garden (same environment, to avoid some putative environmental effects on floral morphs). Within these 7 populations, we randomly (explicitly: we draw random GPS coordinates in the populations, and sample one stem at these points among the multiple stems that covered the ground) sampled 15 individuals, in populations of thousands of plants. This point was clear to the reviewers eyes of this paper, and they even acknowledge our work. So we still don't understand Reviewer#1 troubles.

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 (particularlry 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 heterostyly in Onagraceae for the first time, as recognized by authors, would be a very interesting novelty, but should be properly supported by the data.

Answer: We agree that new cases of heterostyly (and other results) should be properly documented. We also understand that, for Reviewer#1, our results in Portillo-Lemus et al. 2021 don't properly document heterostyly in Lgh, which is not our question neither in our previous paper nor in this manuscript. We are not botanists. We also note that beyond the

statement, Reviewer#1 doesn't propose constructive comments on how he think we would demonstrate or not heterostyly. We are yet open to collaborate to make this demonstration on a dedicated manuscript in the future, including Reviewer#1 in the author, because this is not our current research topic, and still not the topic of this manuscript: we can delete the term "heterostyly" all along the manuscript, it won't change its results and interpretations. But it would sound strange that we don't mention it, as requested by specialists and reviewers of our previous paper, especially considering that the nearest taxonomic group (Lythracea) present multiple heterostylous species, as even stated as a surprising point by Raven (1979), we cite again: "*Although it is frequent in the related family Lythraceae, heterostyly is unknown in Onagraceae.*". Indeed, our populations were demonstrated with a sufficient, statistically-sounding dataset the coincidence of floral morphs, self-(in)compatibility and fertility/sterility. So, not mentioning heterostyly or the possibility of a heteromorphic self-incompatible system in the introduction, would be an error. Moreover, as we don't tackle this question, stating this hypothesis as rejected or void, or not mentioning it while not yet studied sounds a strange insistent request.

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 where both morphs should obviously exist.

Answer: Again, this paper is about self-incompatible permeability and pollen tube elongations in populations producing lot of seeds or few; Not about heterostyly. All observations in situ populations corresponded to monomorphic populations, L-morph or S-morph in these invasive populations. .

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.

Answer: We ask the reviewer#1: will understanding the phylogeny/coalescence of individuals in populations change their self-compatible or self-incompatible status, the permeability of the self-incompatible individuals to selfing, the potential preferential allogamy of the self-compatible one, the viability of outcrossed and selfed seeds, points that we study here? These are quite interesting questions on the origin of individuals in this invasive front. But not the topic of the current manuscript.

Again, we have early answer to these questions but they are not the objective of this manuscript. So, individuals are of the same species: they give 100% viable descendants when crossed, but some of them no seeds when self-pollinated. Will plants of subspecies follow this pattern? Moreover, (i) In France, only 2 species of *Ludwigia* sp. have been introduced. They are characterized by different levels of ploidy, diploid ($2n = 16$ chromosomes) and decaploid ($2n = 80$ chromosomes) and we know perfectly how to distinguish them (morphological difference and chromosomal counting) and resulting viability of descendants. So, we affirm that we analysed populations of only *L. grandiflora* subsp. *hexapetala*.

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.

Answer: No, not *a priori* sampled. Flowers were sampled randomly (written in the material and methods) from “fertile” and “unfertile” populations, not using some morphological prior.

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

We agree and changed in accordance.

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.

Answer: we agree that we need to clearly define this semantic point. Mating system here means as in the title preferential allogamy or preferential selfing, as in most ecological and evolutionary literature. If the fact that we are tackling mating system at the scale of individuals sampled in these populations (thus at an individual’s scale rather than population one) requires to use “breeding system” rather “mating system” (its extrapolation to

populations, because we are not exhaustive by sampling 15 individuals by populations...;but which study is??)

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.

Answer: We only wrote that differences in pollen shape and stigma structure are one of the clues of possible heteromorphic self-incompatible system, not a direct proof, as clearly stated and understood by Reviewer#2. Again, we will appreciate any constructive proposal that would match reviewer#1 nuance requirements.

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.

Answer: Again, this paper is not about heterostyly but self-incompatibility. Floral morphology was studied from 60 flowers, sampling one flower on each of the 6 randomly sampled individuals per population, sampled in 5 fruitless populations (at hundred kilometers each other's) and one flower from 15 randomly sampled individuals from 2 populations (again away from one hundred kilometers) see Portillo-Lemus et al. 2021 (published in Plant-Environment Interactions, previously reviewed in Ecology and Evolution and Journal of ecology, reviewed as sounding but not a sufficient audience, we can provide the reviews as Recommender#1 seems to question the reviewing process of these journals and his colleagues that accepted our paper) and we even added more flowers (up to 150 to measure their pistils and stamens) as we also were surprised by this results.

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

Answer: why it is critical knowing of randomly-sampled individuals in populations of thousands of plants are clones (ramets) or genets to understand their self-(in)compatibility and if their selfed and outcrossed seeds are viable?

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.

Answer: the sentence: “For reciprocal *inter-morph* pollination, to simulate free random crosses, we selected five pollen-donor flowers from short-style *or* long-style flowers to create a pollen mix.”

We didn't mix S-morph and L-morph pollen together. This sentence uses a “or” not a “and”: inter-morph pollination required that we provided pollen from unfertile plant to fertile plant and reciprocally. Thus, we mixed pollen grains from multiple S-morph plants to pollinate L-morph pistils. And, we mixed pollen grains from multiple L-morph plants to pollinate S-morph pistils. We can change this sentence to be clearer, even if it is obvious that it makes no sense to mix pollen from S-morph and L-morph plants to make intermorph pollination S-morph and L-morph pistils.

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.

Answer: no, this is not the topic of this manuscript. We will remove the data corresponding to floral morphology as they were already tackled in Portillo-Lemus et al. 2021 PEI for that, already-reviewed and published. This is consequently not the topic of this manuscript. If reviewer#1 reviewed data that are not part of the results, we understand his rejection recommendation.

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.

Answer: The discussed sentence: “*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*”. We showed that fertility is due to self-incompatibility in Portillo-Lemus et al. 2021. Even self-compatible plants seem to favor (with significant faster tube elongations of cross-pollen versus self-pollen) outcrossing, when the other type of compatibility is available. Thus, if the monotypic (for the moment) populations by extending meet a t some points of migrate into other self-(in)compatible type of populations, this breeding system will create outcrossed descendants rather than selfed, with higher invasive potential if some local adaptations occurred in some individuals. But do we really need to detail this sentence?

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.

Answer: thank for these precisions, we will change the sentence in agreement.

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.

Action: we change for monomorphic on this species.

Line 360: You should also mention that Raven (1979) explicitly mentioned that no Onagraceae is reported to have heterostyly.

Action: Yes, we can even cite his sentence (cited above).

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.

Answer: This result is important to hypothesize what will happen when the self-compatible and self-incompatible populations will merge in a near future: will we observe preferential outcrossing or a spread of the self-compatible type over the self-incompatible one.

Line 400. The concept of “in situ populations” is odd: I would suggest “wild populations”

Action: changed.

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?

Answer: Because just one brushstroke of cross-pollen in hand-controlled pollination experiment in the common garden (thus protected by cellophane bag) always resulted into fully seeded fruits with around ~60 seeds in green big fruits (see fruit morphology and colors in figure 6), while the rare fruits observed in the monomorphic self-incompatible populations looked like the fruits (small and yellow) always obtained by permeable self-pollinations of self-incompatible individuals in hand-controlled pollinations experiments in the common garden.

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.

Answer: We agree and changed these sentences. We however think its is important to write hypotheses from our experiment.

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).

Answer: In fertile populations, seeds are viable and we saw growing seedlings. We will change in accordance as we didn't explore explicit breeding systems in wild populations.

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

Changed.

Figure 1. Why are stamen measurements not provided? they are critical for definition of heterostyly as reciprocal herkogamy

Answer: we will remove all data about the floral morphology as it was already tackled in Portillo-Lemus et al. 2021, as mentioned above.

Figure 6. This kind of information is better provided as a table or bar diagram figure

Answer: we disagree as this figure with the photos illustrate that one cannot confuse the different types of mating, even in the wild, which also answer some of the previous questions made by reviewer#1.

Figure S1. If each point in the curve along time is derived from sampling, as I suppose, you should report error bars.

Answer: yes. They correspond to those of figure 3. We changed.

Figure S2. I do not see its value for understanding the manuscript.

Answer: It shows the fruits of self-compatible and incompatible plants. It is important to understand the type of experimentation we achieved and help to make them again for other studies. It is in supplementary materials, so not essential out of an element of reproducibility (that matters for us, included to be contradicted).

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.

Reviewed by anonymous reviewer, 27 Aug 2021 13:23

I consider the topic of this work adequate and interesting to be published, 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.

Action: We agree but also noticed that it is still the case of most of these species with some exception in few genera. So, we added the following sentence: “*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, 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”.

Action: Changed

· 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.

Action: yes, changed as recommended.

· 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.

Answer: Yes, in the long-styled morph, the stigma protrudes the two levels of anthers as shown in the figure 6 (see L-morph photo).

· 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 reproduction in invasive populations.

Answer: interesting question on which we have no answer for the moment but we hope to tackle in the next years. L-morph (and S-morph) were found indifferently in native area (Argentina) and in invaded countries as in Europe and in North America (see supplementary file in Portillo-Lemus et al. 2021 PEI paper).

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.

Answer: this is a negative result, answering some of the questions we had when presenting our results that sounds to be important to measure and thus to report. In the introduction, we wrote “Heterostyly [...] sometimes associated with additional features such as differences in pollen sizes and shapes, and different lengths of stigmatic papillae (Barrett and Shore 2008)”. We changed the sentence in adequation.

· 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.

Action: yes, added.

· 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?

Answer: No, we chose this timeline using early observations and the number of time points to be sufficiently reasonable to be achieved by our small team. We had previously observed that pollen germinated on stigmas around 2 hours after pollination. We also stopped our observation 24 hours after pollination because we previously observed that fertilization all occurred before 24 hours.

· 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.

Action: yes, we changed for “We also assessed the rate of self-fertilisation in self-incompatible L-morph and in self-compatible S-morph in field populations at the beginning of October”

· 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?

Action: Right, we did not use cellophane bag to protect each flower in field populations because it was not possible considering the population configuration and geography. We changed this sentence by “To evaluate the appearance of fruits, we collected fruits from free pollination between flowers of S-morph in monomorphic self-compatible populations or between flowers of L-morph in monomorphic self-incompatible populations.”

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

Action: right, we agree. We will perform again the analysis using generalized estimating equation and generalized method of moments using gee and gmm in R or statmodels in python. It should not change the results but will be more correct.

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.

Action: we decided to remove the data already presented Portillo-Lemus et al. 2021, a description in material and methods in not necessary.

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.

Answer: we agree, and had a lot of discussion on this specific point when presenting our results in conferences. We would love to find a dedicated model to make prediction. We explored yet Charlesworth literature for example. To our knowledge, after a recent discussion with Xavier Vekemans, we know that one of this model exists, and we will try to make some prediction about the possible evolution of Lgh SI system. Can we ask reviewer#2 if he/she can provide a reference? We are struggling currently using a diallel to identify the heritability of the self-incompatibility, but Lgh is a polyploid species which doesn't ease our task. Action: we will try to better review literatures and propose at least some sentences of discussion about this specific point.

· 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. triandrus* and *N. albimarginatus*. therefore, these species should be cited as stylar-dimorphic species or stylar-polymorphic species (if the authors include *N. triandrus*).

Action: changed.

· 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.

Answer: We agree but did not mean that. The sentence: "Delayed selfing is thought to have evolved in preferentially allogamous species because it would provide some reproductive assurance when populations suffer from the lack of compatible pollen (Goodwillie and Weber 2018; Ruane et al. 2020; Xu 2021).". We rather pointed out here that selfed seeds may contribute to maintain and even propagate (seeds in Lgh are floating seeds, versus clonal cuttings that present small roots and settle down quickly) these monomorphic L-morph populations. And despite the self-incompatible system of this plants at first sight, may contribute to its invasiveness (fitting Baker's conjecture on the advantage of monoparental modes of reproduction). **Action:** We will rephrase these sentences to clarify.

· 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.

Answer: The fastest speed of cross-pollen tube elongations is not specific to S-morph. We didn't explore the consequences of this mechanisms on the fate of both morphs but we also wonder how it would impact the SI evolution in such species.

· 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.

Answer: we agree on the interest of this question but we don't have the data to address this question and it constitutes a nice of perspective on this study.

· 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.

Answer: for us this interesting topic is yet addressed by the subpart entitled "Consequences of LSI for invasive populations of Lgh". If some specific discussion is still missing on some points, we would be inclined to complete.