Running Head: Reproductive isolation between parapatric Senecio

Immigrant inviability produces a strong barrier to gene flow between parapatric ecotypes of Senecio lautus

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/evo.12936.

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Abstract

Speciation proceeds when gene exchange is prevented between populations. Determining the different barriers preventing gene flow can therefore give insights into the factors driving and maintaining species boundaries. These reproductive barriers may result from intrinsic genetic incompatibilities between populations, from extrinsic environmental differences between populations, or a combination of both mechanisms. We investigated the potential barriers to gene exchange between three adjacent ecotypes of an Australian wildflower to determine the strength of individual barriers and the degree of overall isolation between populations. We found almost complete isolation between the three populations mainly due to premating extrinsic barriers. Intrinsic genetic barriers were weak and variable amongst populations. There were asymmetries in some intrinsic barriers due to the origin of cytoplasm in hybrids, which combined with weak selection against hybrids has the potential to facilitate gene flow in the system, specifically from Dune and Headland ecotypes into the
Island ecotype. Overall, these results suggest that reproductive isolation between these three populations is almost complete despite the absence of geographic barriers, and that the main drivers of this isolation are ecologically based, consistent with the mechanisms underlying ecological speciation.

Introduction

Speciation is the process whereby groups of individuals become reproductively isolated from other similar groups (Coyne & Orr 2004). Therefore, understanding the factors that prevent gene flow and the way they contribute to reproductive isolation (RI) can provide novel insights into the evolutionary processes driving the formation of new species (Sobel et al. 2010). While the potential barriers preventing gene flow in plants have been known for some time (Stebbins 1950), only recently have the cumulative effects of multiple barriers been investigated using a common framework (Ramsey et al. 2003; Lowry et al. 2008b; Baack et al. 2015).

Reproductive isolation can arise in a number of ways, and can either prevent hybridization (prezygotic barriers) or prevent hybrids from breeding (postzygotic barriers). These barriers may be independent of the environment in which they are measured (intrinsic) and originate from the evolution of incompatible genetic differences between populations (Orr and Turelli 2001), or act only in the local habitats of the diverging populations (extrinsic), and arise as a result of adaptation to environmental differences between populations. There is growing evidence that adaptation to local environmental conditions has a significant influence on patterns of reproductive isolation (eg. Funk et al. 2006). Although events such as genome duplications can lead to complete isolation from a single reproductive barrier, complete isolation is more commonly caused by the cumulative effect of
multiple incomplete barriers (Lowry et al. 2008a; Matsubayashi and Katakura 2009; Baack et al. 2015).

Prezygotic barriers prevent hybridization by preventing the formation of a zygote. These barriers arise from differences between populations that can prevent mate recognition, such as flower color and shape (Ramsey et al. 2003; Kay 2006) or differences in reproductive timing (Runquist et al. 2014). Environmental, or extrinsic, factors can also prevent gene flow by reducing the viability of migrants adapted to alternative environmental conditions (Nosil et al. 2005b), or can arise due to eco-geographic isolation (Sobel and Streisfeld 2015). Prezygotic barriers act earlier in life history and are often stronger than postzygotic barriers (Ramsey et al. 2003; Coyne and Orr 2004; Nosil et al. 2005a; Kay 2006), which arise after mating has occurred. Postzygotic barriers prevent backcrossing and movement of genes from one population into another. They are frequently asymmetrical, in that they may prevent gene flow in one direction but not the other (Tiffin et al. 2001; Moyle et al. 2004; Turelli and Moyle 2007; Gosden et al. 2015), and also increase in strength as populations diverge (Coyne and Orr 1989).

These different forms of isolation are not mutually exclusive, and many incomplete barriers may contribute to the overall strength of RI between populations (Martin and Willis 2007; Sobel and Chen 2014). Barriers also occur sequentially so that barriers occurring early in life history may have a larger relative impact on gene flow than late acting barriers, independent on the individual strength of a particular barrier (Coyne and Orr 2004). Subsequent barriers can only reduce gene flow that has not already been eliminated earlier in time. The sequential nature of barriers means that prezygotic barriers make a greater overall contribution to total isolation, even when postzygotic barriers are strong (Jiggins et al. 2001; Kirkpatrick and Ravigne 2002; Price and Bouvier 2002; Ramsey et al. 2003; Lowry et al. 2008b; Sobel et al. 2010). It is therefore important to include as many barriers as possible to obtain unbiased estimates of the relative importance of individual barriers (Kay 2006).
Attempting to isolate the mechanisms initially preventing gene flow is further complicated by the fact that reproductive barriers continue to evolve after complete isolation is achieved (Orr and Turelli 2001; Coyne and Orr 2004; Rieseberg and Blackman 2010). This means that contemporary studies may not indicate the causes of RI at the time of speciation, and whether they evolved in the face of gene flow. Intrinsic postzygotic barriers are important as they can complete the speciation process, but when speciation is driven by divergent selection these reproductive barriers may evolve after RI is complete (Martin and Willis 2007; Sobel et al. 2010; see Seehausen et al. 2014, Box 2). The continuous nature of divergence means that determining the barriers present between closely related populations gives the most accurate insight into the mechanisms of speciation (Sobel et al. 2010; Gosden et al. 2015).

*Senecio lautus* (Variable groundsel) is an outcrossing, short-lived perennial herbaceous plant with multiple morphological variants correlated with specific environmental conditions (Ornduff 1964; Radford et al. 2004). There is a growing body of evidence that suggests populations have repeatedly diverged in parapatry along environmental gradients (Roda et al. 2013a; Roda et al. 2013b) and that these populations have diverged recently (Roda et al. 2013a). This work concentrates on three parapatric ecotypes that form a monophyletic group at Coffs Harbour, Australia. This provides an ideal framework to identify the degree to which these adjacent populations are reproductively isolated by quantifying the effects of intrinsic genetic differences and divergent environmental conditions on gene exchange. We estimate the strength of nine possible barriers to gene flow, and calculate which of these contributes to total reproductive isolation between these three populations. Further, because our populations have contrasting but adjacent habitats and gene flow is expected to hinder local adaptation, we also investigated the degree of local adaptation between these populations of *Senecio lautus*. 
Materials and Methods

**Plant material**

All seeds for these experiments were derived from seeds collected from three wild populations at Coffs Harbour in November 2010. These three populations represent ecotypes within the *S. lautus* species complex growing in sand dunes at Boambee Beach (S 30° 18' 45.28", E 153° 8' 21.43", Dune type), rocky headland environment with clay based soil at Corambirra Point (S 30° 18' 44.09", E 153° 8' 41.51", Headland type), and a near-shore island seabird colony on Mutton Bird Island (S 30° 18' 19.67", E 153° 8' 57.27", Island type). We created Parental families through one round of controlled crossing within each wild population, and generated F$_1$ families through crosses between parental populations. All crosses were performed under glasshouse conditions at The University of Queensland.

**Prezygotic barriers affecting co-occurrence**

**Phenology**

To assess the overlap in flowering time between ecotypes, we established transects across the full distribution of each population. Transects consisted of twelve 4 x 20m plots in which all growing plants were counted and categorized as flowering or not. Plots were separated by 20-30 metres in the island and headland populations, and by 500m in the Dune, as the Dune population is distributed over a greater area. Measurements were taken monthly between February 2013 and April 2014, with the exception of March and November 2013, and February 2014.

Estimates of reproductive isolation were calculated using equation RI4S2 from the supplementary material of Sobel and Chen (2014), where the probability of conspecific vs
heterospecific matings is calculated using the proportion of individuals from each ecotype that flower in a given month while controlling for the relative abundance of each ecotype.

**Immigrant inviability**

To determine the effects of local adaptation on immigrant inviability we conducted reciprocal transplant experiments between the three populations (see plant material). In each environment, we planted Parental and F\textsubscript{1} cross types derived from the three populations. We included four individuals from 15 families per cross type in each environment leading to 60 individuals per genotype per environment. Seeds were glued to toothpicks using Selleys Quick Fix supa glue gel and planted into four blocks per environment. Each block consisted of one 400 x 600mm grid, and seeds were planted out in a completely randomized block design. To maximize germination, all blocks were covered with 50\% shade cloth, and watered daily for the first month of the experiment, after which the shade cloth was replaced with bird netting. The field transplant was conducted between April and November 2014, at which point all surviving plants were harvested, and we recorded wet mass (g) and the number of flowers on each plant. We used wet mass, instead of the more common measure of dry mass, to reduce the effect of measurement error when weighing near-zero dry mass of very small plants.

Here we calculate the strength of both pre and postzygotic immigrant inviability based upon relative growth and flower number between local and foreign crosstypes in each environment using equation RI4a from Sobel and Chen (2014), where H is the sum of biomass for all surviving plants from each immigrant genotype and C the sum of biomass for all surviving individuals from the local parent. Similarly for flower count, H is the total number of flowers attributed to the immigrant genotype, and C being the total number of flowers attributed to the local parent genotype. By creating a composite measure of immigrant viability, we can account not only for growth or flower production,
but incorporate information about the number of survivors as well as growth in each local or immigrant population.

Prezygotic immigrant inviability was calculated within transplant environment between the parental genotype of the local population and each immigrant parental population. Postzygotic immigrant inviability was calculated within transplant environment between the local parental genotype and each F₁ hybrid genotype. We condensed reciprocal crosses into a single F₁ hybrid genotype depending on the original parental populations (e.g., F₁DH, F₁DI, F₁IH). We also calculated RI from the local parental population to all F₁ hybrids to determine the strength of barriers to outcrossing.

Postzygotic barriers

F₁ viability

Intrinsic germination rate was calculated using data from two experiments conducted under controlled temperature conditions at The University of Queensland. In both experiments, seeds were scarified and placed on moist filter paper in 40mm glass petri dishes under dark conditions for three days, then exposed to a 12 hr light–dark cycle. Experiment 1 included six seeds each from 20 parental families from Dune, Headland and Island ecotypes, with an overall total of 430 seeds. Experiment 2 was smaller and included 1 seed per family for reciprocal hybrid crosses as well as parentals, with a total of 74 seeds (D=12, DH=6, HD=5, H=11, HI=8, IH=6, I=6, ID=10, DI=10). Germination was scored as the emergence of cotyledons from the seed case. Numbers from both experiments were pooled and germination rate was calculated as the proportion of total seeds that germinated per genotype. We used equation RI4a from Sobel and Chen (2014) where H equals the hybrid germination rate and C equals the parental germination rate. Reproductive isolation was calculated between parents and each reciprocal hybrid cross to determine differences between cytoplasmic origin, and parents and the mean hybrid germination rate across both reciprocal crosses.
**F₁ fecundity**

Plants were grown to maturity in greenhouses at the University of Queensland and the number of flowers produced during the flowering stage recorded. Intrinsic fecundity was measured by counting the average number of flowers per plant for each parent and both reciprocal hybrid crosses. We used equation RI4a from Sobel and Chen (2014), where H is the average hybrid flower number and C is the average parental flower number. We calculated RI between parents and each reciprocal cross, and parents to mean hybrid flower number. We also calculated RI between each parent and the mean of all other genotypes to gain an estimate of the barriers to outcrossing within all possible genotypes of the system.

**Relative contribution of barriers to overall isolation**

To calculate the contribution of each individual barrier to total RI, we aligned each estimate of barrier strength in chronological order, the substituted the values into the spreadsheet calculator provide in the supplementary material of Sobel and Chen (2014). We included flowering time overlap and growth and flower production of parental immigrants as prezygotic barriers affecting co-occurrence. Intrinsic germination rate and flower production, and growth and flower production of hybrids in the field transplant were included as postzygotic barriers. In some cases, barrier strength was one, which isolates populations completely. In these cases, we removed that barrier and repeated the analysis with all non-complete barriers. Output from both tests is presented in Table S1.

**Local adaptation**

We calculated the extent of local adaptation across the three populations by calculating the difference in viability of local versus foreign individuals extracted from a reciprocal transplant experiment. We focus our comparison between local and foreign genotypes within a single environment, rather than comparing a single genotype in home and away environments (Kawecki and
The local versus foreign approach is better aligned conceptually with our calculation of reproductive isolating barriers, such as immigrant inviability, where RI is derived from a comparison of local and immigrant fitness within a single environment. We determined relative fitness differences based on pairwise comparison of the mean days lived by each local and foreign population within each environment, and substituted these values into Equation (1) from Hereford (2009), where local adaptation is the relative fitness (W) of the local population (P1) minus the relative fitness of the foreign population (P2), standardized by the mean fitness of both populations at that site (S1):

\[
LA = \frac{W_{\text{Population 1}} - W_{\text{Population 2}}}{W_{\text{Site 1}}}
\]  

We calculated local adaptation using wild collected and glasshouse generated seeds from each ecotype to determine whether maternal environmental effects (field site versus common garden) influenced relative fitness. Local adaptation can be inferred from positive values, while negative values describe increased relative fitness of immigrants.

Results

**Prezygotic barriers affecting co-occurrence**

**Phenology**

Flowering time was largely concordant across the three populations. The number of flowering plants was consistently larger in the Dune than in the Headland or Island populations (Figure 1A), and the main flowering season occurred during the autumn and winter seasons between February and September. This also corresponded with the number of plants alive, with all populations reducing in size over the summer months.

**Immigrant inviability of parents**
There was a strong pattern of immigrant inviability between the three populations. In each environment, immigrant genotypes contributed a much smaller proportion of the overall biomass produced by surviving plants (Figure 1B), showing that immigrants had lower survival, and immigrant individuals had lower growth if they did survive. Immigrant genotypes also flowered less than local genotypes (Figure 2). In the Headland environment, local parents were the only plants to produce flowers, while in the island environment, Island locals flowered five times more than Headland immigrants, and Dune plants failed to flower. In the Dune environment, plants grew less and no flowers were produced by any genotype, most likely due to a particularly harsh season where rainfall was less than a third of the monthly average over the duration of the experiment. Strong selection against all genotypes as a result of drought conditions may reduce the effects of divergent selection and therefore reduce the contrasts between genotypes as all are suffering, although the local Dune genotype still accounts for the highest proportion of biomass production in survivors from this transplant experiment (Figure 1B).

**Postzygotic barriers**

**Intrinsic germination rate**

Intrinsic germination rate was generally lower in hybrids than in parental types, although hybrids between Headland and Dune ecotypes with Headland cytoplasm showed heterosis, and complete germination (Figure 1C). Island seeds had the lowest germination success of the parental genotypes (67%) while Headland (82%) was higher than Dune (78%). Dune-Island hybrids with Island cytoplasm had increased germination (60%) over hybrids with Dune cytoplasm (40%).

**Intrinsic flower number**

Genotype had a significant effect on number of flowers produced ($F_{(8,99)} = 6.54, P = 7.88 \times 10^{-7}$), a result largely driven by the low production of plants with an Island cytoplasm origin (Fig 1D).
Headland–Dune hybrids with headland cytoplasm again showed a pattern consistent with heterosis, with the highest flower production. Flower production was variable between genotypes, although mean flower count was 16.57 (SD = 13.69) flowers per plant.

**Immigrant inviability of F₁ hybrids**

Hybrid genotypes accumulated less biomass than local genotypes in all three transplant environments (Figure 1B). This pattern was less severe in the dune environment, where Dune parents accumulated 44%, followed by hybrids between Dune and Headland (F₁HD= 26.4%), Island-Headland (F₁IH = 16.8%) and Dune-Island (F₁ID = 7.5%). However total biomass accumulated by all genotypes in the Dune was much less than the other two environments. In the headland environment, hybrid genotypes collectively accounted for only 14.1% of biomass compared with 84.8% in parentals, while in the Island hybrids between Dune and Island populations produced 31.6% compared with the Island parents at 59.4% (Figure 1B).

**Strength and relative contributions: Pre- and postzygotnic barriers**

Due to the differential population sizes, the strength of flowering time as a reproductive barrier was asymmetrical. Flowering time provided a moderate barrier to gene flow, isolating Headland from incoming Dune pollen, while facilitating gene flow from the Headland into the Dune (Figure 2A). Similarly, there was a pattern of asymmetric isolation between Dune and Island populations, where the greater number of Dune plants flowering increases the probability of heterospecific matings occurring due to pollen flow from Dune to Island rather than Island to Dune (Figure 2B).

Although there were differences between hybrid and parental germination rates, these differences were small and therefore did not produce a large deviation from random mating expectations. Thus, it seems that intrinsic germination rates of hybrids between Dune and Headland...
populations facilitated gene flow in both directions (Figure 2A). Similarly, intrinsic germination rate provided a weak barrier to gene flow between Dune–Island (Figure 2B), and Headland–Island populations (Figure 2C). In both comparisons, there was an asymmetry in the strength of isolation based on the direction of hybridization, with a slight increase in the barrier strength when parentals were compared to hybrids with cytoplasm from the alternative population. However, the calculation of F₁ germination and fecundity in controlled conditions may be different to patterns in the wild, and therefore may have limitations when interpreting their effects on reproductive isolation in the wild.

The implications of each barrier are slightly different when we consider the strength of individual barriers and the contribution of many barriers to total RI. Strength is the ‘absolute value’ of barriers and can be contrasted to determine which categories of reproductive isolation are strongest. The cumulative effects account for the contribution of each barrier to total isolation in sequence. As such, postzygotic barriers that are strong may contribute little to overall isolation, as earlier barriers may already effectively isolate populations. This is the case between Dune and Headland populations, where postzygotic barriers associated with hybrid immigrant inviability are very strong, but contribute little to total isolation due to the effect of strong immigrant inviability between parents occurring before.

When we consider the relative effect sizes of extrinsic and intrinsic barriers to gene flow, extrinsic barriers are strong overall and prevent gene flow, while intrinsic barriers are either weak or facilitate gene flow (Figure 3A). Extrinsic prezygotic and postzygotic barriers have a similar strength (Figure 3A), while prezygotic barriers have a larger relative contribution to total isolation as they occur earlier in the life history sequence (Figure 3B). This pattern is largely driven by immigrant inviability of both parents (prezygotic) and hybrids (postzygotic).

Local adaptation

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All populations showed a pattern of local adaptation based on reduced immigrant viability. This is evident from positive values of the local adaptation index in all populations suggesting plants from local populations lived longer. The pattern was strongest in the headland environment, where Headland locals displayed a 91% fitness increase over Dune immigrants and 57% increase over Island immigrants. In the Island, the fitness of locals was 50% greater than Dune immigrants and 67% greater than Headland immigrants. Local adaptation was lowest in the Dune environment, although local Dune plants still had 12% higher fitness than Headland and 23% higher fitness than Island immigrants. This relatively low measure may be due to the overall harshness of the Dune environment producing strong selection against all genotypes that obscured the distinctions between locals and immigrants.

Discussion

The main aim of this study was to determine the strength and relative importance of intrinsic and extrinsic barriers to gene flow, and estimate levels of RI between closely related parapatric ecotypes of Senecio lautus. We quantified the strength and cumulative effect of three intrinsic and four extrinsic barriers to gene flow and found strong effect of immigrant inviability as a mechanism preventing gene flow between these three populations. Consistent with strong immigrant inviability, we also observe strong patterns of local adaptation, suggesting that adaptation to the contrasting environmental conditions between populations may be the main driver of RI.

Strength and contribution of barriers

The impact of a single barrier on gene flow depends on both the effect size of the barrier and its timing relative to other barriers. This is because subsequent barriers can only reduce gene flow that remains after preceding barriers have occurred. Consistent with previous studies, we find prezygotic barriers contribute most to overall RI (summarized in Lowry et al. 2008b; Baack et al. 2015), however the relative contributions of each barrier were different between ecotypes.
While phenology contributes weakly to total isolation in the Island population, it does not deviate from random mating in the Headland and facilitates gene flow in the Dune. The other prezygotic barriers, which contribute to immigrant inviability, largely prevent gene flow amongst the populations. In the Headland environment, the low growth of immigrants leads to near complete isolation. Therefore, although subsequent extrinsic barriers also have an equally large effect size, prezygotic barriers contribute more to total isolation (Figure 3B). A similar situation arises in the Dune environment where gene flow is facilitated by overlapping phenology, but very strong immigrant inviability leads to almost complete reproductive isolation.

An important consideration when assessing the relative importance of isolating barriers is that individual barriers are not necessarily independent (Martin and Willis 2007; Sobel et al. 2010). Lowry et al. (2008b) solve this to some extent by collapsing individual barriers into categories based on the timing (pre- or postzygotic) of intrinsic and extrinsic mechanisms, a method we also employ in this study. We find both pre- and postzygotic extrinsic mechanisms act to prevent gene flow between the three populations of S. laetus from Coffs Harbour while intrinsic mechanisms weakly facilitate gene flow. Consistent with recent reviews of the comparative strength of reproductive barriers (Lowry et al. 2008b; Baack et al. 2015), prezygotic barriers have a larger contribution to overall isolation than postzygotic barriers, and measures of immigrant inviability were the strongest individual barriers. Similarly, we found the strength extrinsic postzygotic barriers to be variable, producing strong isolation of the Headland population, but weaker in measures of inviability in the Dune and asymmetric between Dune-Island hybrids.

As the bulk of reproductive isolation is produced by extrinsic rather than intrinsic mechanisms, our results also implicate ecologically based divergent natural selection as an important factor driving speciation (Nosil et al. 2005a). This pattern also suggests that these Senecio populations may be in the early stages of divergence as they have a genetic basis for divergent traits that may
confer local adaptation (Schluter 2001, Table1), but have not yet accumulated sufficient genetic differences to lead to intrinsic hybrid breakdown (Seehausen et al. 2014).

Asymmetry due to cytoplasm

Asymmetry in the strength of barriers between populations may be common although it is generally greater in postzygotic barriers (Lowry et al. 2008a). We find asymmetry in both pre and postzygotic barriers, although the explanations for each stage are probably different. Asymmetry in flowering time is present between the Dune and the other two populations but not between the Headland and Island populations. This result is largely driven by the difference in relative abundance of each population rather than the genetic mechanisms that asymmetrically reduce gene exchange, such as unidirectional inheritance (Tiffin et al. 2001; Turelli and Moyle 2007) implicated in postzygotic asymmetry.

There is also asymmetry in the intrinsic postzygotic barriers associated with hybrid flower production in crosses involving the Island ecotype. Between Headland and Island populations, asymmetries could imply gene flow is more likely from the Headland population toward the Island rather than the opposite direction. The result is more complex between Dune and Island hybrids as the origin of the hybrid cytoplasm also affects the asymmetry. For instance, in the Island environment, hybrids formed between immigrant pollen and local ovules (i.e., Dune pollen, Island cytoplasm) may have higher flower production than hybrids derived from immigrant seed (Island pollen, Dune cytoplasm). This result is consistent with the prediction that gene flow is more likely from a single migration event, pollen migration from site 1 to site 2, is than multiple events, pollen migration from site 1 to 2 then return of hybrid seed from site 2 -1. Asymmetries in specific barriers may allow us to predict the probabilities of different modes of gene flow occurring, although it is important to note that intrinsic flowering measures were observed under glasshouse conditions, which may be quite different to flower production under field conditions.
Estimates of the barrier strength of intrinsic flower production suggest that the low flower production by Island plants in the common garden has the potential to facilitate introgression when hybrids have local Island cytoplasm. This then leads to the hypothesis that pollen mediated migration may occur more readily than seed dispersal due to the barrier strength against pollen being weaker than seed. This scenario is intuitively more likely as the flow of pollen into the island population from the other populations may occur more frequently than the flow of pollen out of the island, fertilization and production of hybrid seeds in Dune or Headland populations, then the return of hybrid seeds to the Island environment.

Local adaptation

Many factors can contribute to local adaptation, however when local adaptation itself reduces gene flow between divergent populations it implies a direct link between ecologically divergent natural selection and the process of speciation. As populations colonize or adapt to different environments, selection against migrants can lead to reproductive isolation (Hendry 2004). This link is the basis of immigrant inviability (Nosil et al. 2005b) and is fundamental to the predictions of ecological speciation. In all populations, immigrant inviability based on growth provides a strong barrier, with a mean strength of 0.84 (SD=0.26) between parents, and 0.73 (SD = 0.27) in hybrids. We also see high values of local adaptation, calculated by the difference in lifespan of immigrants and locals. Greater barriers toward parents rather than hybrids have been reported in a range of plant species (Lowry et al. 2008c; Baack et al. 2015) which suggests a tradeoff between fitness and adaptation to alternative habitats in immigrants has a greater effect than the extrinsic postzygotic fitness effects which manifest in hybrids (Baack et al. 2015, Table 2).

Collectively, the strong patterns of immigrant inviability and local adaptation provide strong evidence for the role of divergent natural selection in maintaining discontinuities between these three populations (Schluter 2001). While we are unable to determine the initial causes of divergence by
looking at current barriers acting in populations, the presence of extrinsic barriers and general lack of barriers based on intrinsic genetic incompatibilities suggests that these populations are at an early stage along the speciation continuum (Seehausen et al. 2014, Box 2), and that divergence is following a pattern of ecological speciation rather than the alternatives of mutation order or chromosomal duplications (Schluter and Conte 2009).

**Considerations and conclusions**

Throughout all calculations of reproductive barriers we make some assumptions. We assume that ecotypes occur in sympathy, which effectively scales eco-geographic isolation to zero. Ecotypes do show strong spatial organization, so eco-geographic isolation probably influences gene flow between populations to some degree, however the close proximity between populations (a few meters between the Dune and Headland, 1.2km between Dune and Island and 850m between Dune and Headland) means that populations are well within the foraging range of generalist pollinators such as honey bees (*Apis mellifera*) and painted lady butterflies (*Vanessa (Cynthia) kershawi*), both of which have been observed in all populations. Determining the appropriate spatial scale to classify eco-geographic overlap requires detailed knowledge of seed and pollen dispersal (Baack et al. 2015), which we lack in this study. Therefore, to maintain conservative estimates of isolation we assume sympathy, as the strict definition of shared and unshared area (Sobel and Chen 2014) would scale eco-geographic isolation to 1, which may inflate estimates of reproductive isolation within the system, and obscure the signal of any subsequent barriers.

Similarly, we assume pollinator guilds are consistent between populations based upon the presence of generalist pollinators in all three environments (Richards pers. obs.) and consistency of pollinators in other studies of *Senecio laetus* (White 2008). While there is some evidence to suggest there may be strong effects of pollinator heterogeneity within environments (Janovsky et al. 2013), we assume pollinator constancy here to maintain conservative estimates of reproductive barriers.
The measures of intrinsic $F_1$ fitness in this study were derived from experiments under controlled glasshouse conditions. Although intrinsic barriers do not have a large effect on total RI between the populations, it is important to acknowledge that hybrid fitness may be considerably different between glasshouse and wild conditions. The main purpose of including these measures is to illustrate that there is little fitness reduction in hybrids as a result of genetic incompatibilities between parental populations, however there may be significant environmental influences on fitness, as seen in the measures of immigrant hybrid growth and flowering (Figure 2), that strongly influence patterns of reproductive isolation.

Overall, these results suggest that reproductive isolation between the ecotypes is primarily driven by ecological factors. The strong pattern of local adaptation coupled with the strong barriers associated with immigrant inviability in both parents and hybrid genotypes, and the negligible effects of intrinsic genetic barriers are all consistent with a recent divergence driven by adaptation to divergent environmental conditions. Along with other evidence from the *S. lautos* species complex (Roda et al. 2013b), this suggests a fundamental role for ecology in driving reproductive isolation in this system.

Acknowledgements

We acknowledge the custodianship of the Gumbaynggirr people, for whom Giidany Miirlarl (Mutton Bird Island) holds special significance. We thank Jeff Holmes from Coffs Harbour City Council, and Ann Walton from New South Wales Parks and Wildlife Service for permission to conduct experiments at these sites. We also thank Tom Gosden and Andrew Mather for helpful discussions. Funding was provided by Australian Research Council grants DP0986172 and DP120104559 to DO.

Author contributions
TJR performed experiments, and analyzed data. TJR and DO designed research and wrote manuscript.

Authors declare no conflict of interest.

Bibliography


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Figure Legends

Figure 1. Isolating barriers between ecotypes of S. laetus from Coffs Harbour, NSW, and their hybrids

(A) Flowering time overlap between three ecotypes of S. laetus. Bars represent mean and 95%CI of number of flowering plants in each environmental block during a 1 year period. (B) Proportion of surviving population depicts the proportion of total biomass accumulated during a reciprocal field transplant attributable to each cross type. This is a composite fitness measure that accounts for the number of plants surviving and their growth performance. Intrinsic germination rate (C) and flower production (D) were derived from common garden glasshouse experiments. The dotted line represents the mean flower production for all crosstypes. Population/cross abbreviations are as follows: PD4 (Dune), PH5 (Headland), PI (Island), F1HD (Headland-Dune F1), F1ID (Island-Dune F1), F1IH (Island-Headland F1)
Figure 2. Strength of extrinsic and intrinsic barriers to gene flow between ecotypes of *S. lautus* from Coffs Harbour

These panels display the influence of each reproductive barrier (y axis) on RI (x axis). RI value of zero suggests random mating between populations, -1 means outcrossing is facilitated over con-specific mating, and 1 means populations are completely isolated from one another. Isolating barriers are: (A) Flowering time overlap, (B) parental immigrant mass, (C) parental immigrant flowers, (D) intrinsic hybrid germination rate (mean), (E) intrinsic hybrid germination rate (local cytoplasm), (F) intrinsic hybrid germination rate (alternative cytoplasm), (G) intrinsic hybrid flower number (mean), (H) intrinsic hybrid flower number (local cytoplasm), intrinsic hybrid flower number (alternative cytoplasm), (J) hybrid immigrant mass, (K) hybrid immigrant flowers.
Figure 3. Mean strength and contribution of reproductive barriers across three ecotypes of *S. lautus* from Coffs Harbour.

Mean and 95%CI of Strength (A) and Contribution (B) of barriers to gene flow between Dune, Headland and Island ecotypes of *S. lautus* from Coffs Harbour, NSW. Barriers include: (A) Flowering time overlap, (B) parental immigrant mass, (C) parental immigrant flowers, (D) intrinsic hybrid germination rate (mean), (E) intrinsic hybrid flower number (mean), (F) hybrid immigrant mass, (G) hybrid immigrant flowers. Barriers occur consecutively from A-G. Total describes mean isolation between populations and is the sum of all barriers to gene flow.
Supplementary material

Table S1. Barrier strength and contribution of isolating mechanisms between *S. lautas* ecotypes. Data was compiled using the Reproductive isolation calculator provided in the supplementary material of (Sobel and Chen 2014). Analysis was repeated with and without complete barriers as barrier strength estimates of 1 remove information about any subsequent barriers.