Evidence for mutation-order speciation between convergent ecotypes

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In a number of animal species, divergent natural selection repeatedly and independently drove the evolution of reproductive isolation between populations adapted to contrasting, but not to similar environments[1]. This process is known as parallel ecological speciation, and examples in plants are enigmatically rare[2]. Here, we perform a comprehensive test of the ecological speciation hypothesis in an Australian wildflower where parapatric populations found in coastal sand dunes (Dune ecotype) and headlands (Headland ecotype) have repeatedly and

independently diverged in growth habit. Consistent with a role for divergent natural selection driving the evolution of reproductive isolation, we found that Dune populations with erect growth habit were easy to transplant across sand dunes, were largely interfertile despite half-a-million years of divergence, and were reproductively isolated from equally divergent Headland populations with prostrate growth habit. However, we unexpectedly discovered that both extrinsic and intrinsic reproductive isolation evolved between prostrate Headland populations, suggesting that populations evolving convergent phenotypes can also become new species rapidly. Mutation-order speciation[2], where the random accumulation of adaptive alleles create genetic incompatibilities between populations inhabiting similar habitats, provides a compelling explanation for these complex patterns of reproductive isolation, and is consistent with the increasingly common observation that there are multiple biochemical avenues to create convergent phenotypes. Our results suggest that natural selection can drive speciation effectively, but environmental and genetic complexity might make parallel ecological speciation uncommon in plants despite strong morphological convergence.

Convergent and divergent phenotypes can evolve when populations adapt repeatedly to contrasting environments. For instance, stickleback fishes adapted to freshwater lakes from the northern hemisphere lack armour and are similar in size when compared to their armoured and smaller relatives from the ocean[3]. This pattern of convergence and divergence is also seen in cave fishes[4], stick insects[5], and marine snails[6], eucalypts[7], and dandelions[8,9], and is thought to be driven by the repeated and independent action of

divergent natural selection between multiple population pairs occupying contrasting environments.

Senecio lautus is a species complex from Australia and the pacific islands, generally known as the common groundsel, that has repeatedly evolved two coastal ecotypes adapted to contrasting environments[10]. The Dune (D) ecotype occupies the sheltered areas behind the sand dunes of the beach, whereas the Headland (H) ecotype lives in exposed and salty rocky outcrops and cliff tops that interrupt the beach. These ecotypes have contrasting growth habits, where Dunes grow erect and Headlands grow prostrate and form mats on top of the ground[10,11] (Figure 1A). Multiple transplant experiments have shown that Dune and Headland populations are exposed to strong divergent natural selection[12-14], which creates strong ecologically-dependent reproductive isolation between them[12,14,15]. However, it remains unknown whether intrinsic reproductive isolation has evolved as a by-product of adaptation to contrasting or similar environments. Here we test the fundamental predictions of the parallel speciation hypothesis for the evolution of both extrinsic and intrinsic reproductive isolation in *S. lautus* by using a range of glasshouse, field, and laboratory experiments.

We first confirmed if divergent natural selection drove the independent and repeated evolution of these two coastal ecotypes (Figure 1A). As we observed before[10], Dune and Headland populations that live next to each other (parapatric DH pairs) are phylogenetically more related to each other than to any other Dune or Headland population, respectively (Figure 1B, Table S1). Using 13 neutral nuclear loci common to sixteen populations of *S. lautus* we show that DH pairs are sister taxa. DH pairs sorted into two main clades (South-Eastern, and Southern), which we also detected in a

population genetics analysis of the same loci using the STRUCTURE algorithm (Supp. Figure 1). Adjacent populations showed little genetic differentiation, possibly suggesting that high levels of ancestral polymorphism are still present in these population pairs, or that gene flow after secondary contact could have homogenised them (Supp. Figure 1 and 2). To disentangle these scenarios we used coalescent models of isolation with migration[16] to estimate migration rates in each coastal pair. If gene flow after secondary contact were responsible for the phylogenetic pattern predicted by parallel speciation, we expect to detect high levels of gene flow in DH pairs. Alternatively, if DH pairs originated multiple times independently, we expect low to nil levels of gene flow in each pair, indicating that genetic similarities between populations reflect segregation of ancestral polymorphism. We failed to detect migration in each DH pair (Table S1), except in pair (D03H02), which showed asymmetric gene flow ($Nm_{H02>D03}$ = 23.01, 95% credible interval 12.30-34.98, Table S2). The phylogenetic pattern detected in coastal *Senecio* and the lack of gene flow between parapatric pairs strongly suggests that Dune and Headland morphologies have evolved independently multiple times.

Next, we asked if the independent evolution of Dune and Headland populations lead to the evolution of reproductive isolation between individuals occupying contrasting environments (DxH crosses or transplants), and between individuals found in similar environments (DxD and HxH crosses or transplants). We performed crosses in the glasshouse both within and between the Southern (S) and South-Eastern clades (SE), thus correcting for divergence time, and reanalysed previous transplant experiments in the field across multiple Dune and Headland habitats[13]. In each glasshouse cross, we measured the ability to produce hybrid seed as well as ability to germinate relative to

the average of the parents. In transplant experiments in the field, we previously measured the viability component of fitness by tracking individual mortality since germination. Consistent with parallel speciation driven by divergent natural selection, we found that populations that inhabit similar environments were on average reproductively and ecologically compatible, while populations inhabiting different environments were reproductively and ecologically isolated. We asked if reproductive and ecological compatibility was similar between Dune populations or between Headland populations. Surprisingly, we found that only Dune populations were easily crossed or transplanted into each other's habitat, whereas Headland populations were difficult to cross and transplant between headland sites (Figure 2, 3 Supplementary Table S3 and S4). Altogether, it appears that costal parapatric pairs of *S. lautus* have evolved by divergent natural selection, but Headland populations have done it uniquely every time, thus creating further reproductive isolation among them.

Contrary to expectations from the parallel speciation hypothesis, our results indicate that the evolution of the same morphology does not guarantee reproductive compatibility between populations. A straightforward explanation is adaptive polygenic divergence between populations inhabiting similar environments. Here, populations evolving similar values of a complex trait accumulate adaptive differences in random order[1,2] some of which incidentally cause intrinsic reproductive isolation. This speciation mode is referred to as mutation-order speciation, and it has been used as an alternative view to speciation driven by divergent natural selection. Our findings suggest that the two processes might have more in common than previously anticipated. Previous results in *S. lautus* have suggested that DH pairs are divergent in similar signalling pathways even if the differentiated genes are not common across populations

119 pairs[17]. One of these pathways controls auxin movement, which is known to affect
120 variation in growth habit in Arabidopsis and other model plant systems. Re-use of a
121 genetic pathway might be an easy solution for creating convergent phenotypes, yet it
122 might incidentally lead to the origins of new species. This might explain why parallel
123 ecological speciation might be rare in some organisms, such as plants.

124 **Methods**

125 **Study populations and sample preparation**

126 Leaf samples were collected from six parapatric *Senecio lautus* Dune and Headland
127 populations (*S. pinnatifiolius* var. *pinnatifolius*, *S. pinnatifolius* var. *maritimus*) as well
128 as two Inland and two Alpine populations (*S. pinnatifolius* var *serratus* and *S.*
129 *pinnatifolius* var. *alpinus*) (Table S1 and map in Figure 1, and supplementary
130 materials[10] for more details). *Senecio madagascariensis*, a closely related species to *S.*
131 *lautus* from Africa was included as an outgroup[10]. We extracted DNA with a modified
132 CTAB protocol and samples were standardised to 30ng/μL[10].

133 **Library construction and sequencing**

134 We undertook targeted re-sequencing in the Fluidigm Access Array system[18], which
135 enables the simultaneous amplification of 48 primers across 48 individuals, whilst
136 barcoding individuals for next generation sequencing. Individuals were barcoded using
137 the 4-primer PCR process with 454 barcodes[18]. Individuals were randomised and pooled
138 in equimolar quantities into two pools, and sent for sequencing at the Beijing Genomics
139 Institute. Pools were cleaned with an Agencourt AMPure XP purification kit, and then

140 sequenced using the emPCR (Lib-A) kit for bi-directional sequencing on two lanes of
141 the Roche GS FLX Titanium platform.

142 **SNP calling**

143 After trimming barcodes with TagCleaner[19], reads that had more than 40% low quality
144 bases (<Q20), had Ns higher than 2%, or were shorter than 50bp were removed.
145 PRGmatic [20] was used for read alignment. The PRGmatic pipeline aligns reads into loci
146 and constructs haplotypes for each individual using dependent programs including
147 CAP3[21], BWA[22], SAMtools[23], and VarScan[24]. We used default parameters and an
148 overlap of 100bp for read alignment, as suggested for 454 data[20]. A locus was only
149 considered for further analyses if haplotypes were assigned in six or more individuals
150 per population. BLAT[25] was used to map loci from PRGmatic to the expected
151 amplicons. 26 loci were selected and aligned in MUSCLE[26] and subsequently used in
152 phylogenetic ananylses and neutrality tests. See Tables S5-S7 for genes and primer
153 sequences.

154 **DNA sequence polymorphism analyses**

155 Deviations from neutrality[27] for each gene within each population were tested using
156 HKA[28], Tajima's D[29] and Fu and Li's tests[30] using DNAsp. Loci selected for
157 phylogenetic and population genetics analyses did not reject neutrality via the HKA test,
158 or via the other two tests if HKA was impossible to perform because a locus did not
159 amplify in the outgroup species. As a consequence, 26 loci were used in estimating gene
160 flow between different pairwise comparisons between populations, and 13 neutral loci
161 across populations were used for the phylogeny (Table S5-S7).

Phylogenetic analysis and population differentiation for neutral markers

A Bayesian phylogenetic analysis was performed using *Beast for species tree estimation[31]. Analysis was performed with a chain 300,000,000 long using a strict molecular clock. ITS was used as the reference locus, with a mutation rate for herbaceous plants of $4.13*10^{-9}$ subs/site/year [32], from which the rate of the other genes were estimated. According to Bayesian Information Criterion values found using jModeltest[33], the best substitution model for 17 out of the 26 genes was the HKY model[34]. We used a *Yules* species process for species tree estimation (this assumes that lineages split at a constant rate).

We explored population structure across the species complex by inferring the number of genetic clusters (K) using STRUCTURE v2.3.4[35]. This was undertaken at two levels: *i)* including all populations, and *ii)* by pairs (six parapatric and two allopatric). STRUCTURE was run using an admixture model and the correlated allele frequency model[36]. Parameters used for *i)* K=1-16, 20 iterations per K, burnin 100,000, MCMC 100,000; and for *ii)* K=1-9, 20 iterations per K, burnin 100,000, MCMC 100,000, as suggested by *Gilbert et al.* (2012) . To choose the most likely K value, both methods by Pritchard *et al*. (2000) and Evanno *et al*. (2005) were examined. Because both methods tend to overestimate K, and high K values did not add any additional clustering information compared to smaller Ks, the smallest K that captured the major structure in the data was chosen, ensuring that all summary statistics converged.

Gene flow

Gene flow estimates were derived from genes that did not reject neutrality tests in most populations and were non-recombining. Intra-locus recombination was assessed with

185 the four-gamete test[39] in DNAsp[40]. The longest non-recombining DNA segment per
186 locus was included in the analysis, as sequences including recombination events could
187 lead to a false signature of gene flow[41]. Estimates of gene flow were assessed using
188 IMa2[16] with pairwise comparisons between the six parapatric pairs and two allopatric
189 comparisons. Gene flow between proximate populations inhabiting the same habitat was
190 also estimated (between Dunes and between Headlands). We used a 100,000 burnin for
191 a minimum of 100,000 genealogies. Runs involved 150 to 180 independent chains,
192 effective sample size (ESS) among all parameters ranged from several hundred to
193 >10,000. Upper bounds of the prior distributions for each parameter were set based on
194 the results of a preliminary run. Mutation rates for each locus were obtained using the
195 relationship D=2*u*T, where D is the average number of substitutions separating the
196 *Senecio lautus* complex from *S. madagascariensis*, and T is the age of the *S. lautus*
197 clade[17] (Roda *et al.* 2013a). Gene flow estimates did not change if we ignored mutation
198 rates (data not shown). We ran three independent IMa2 analyses varying the seed
199 number to ensure parameter estimation was consistent.

200 **Estimates of reproductive isolation**

201 Seeds from 30 individuals from four population pairs, two from the eastern coast (D01-
202 H01 and D04-H05) and two from the southern coast (D32-H12 and D23-H21), were
203 collected and stored in dry conditions at 4°C in the Ortiz-Barrientos Laboratory at The
204 University of Queensland, Australia. Seeds were scarified (1mm trimmed at the
205 micropyle side) and germinated on moist filter paper in petri dishes. Seeds were kept in
206 dark and controlled conditions for three days to induce root elongation, and
207 subsequently placed under the light for 7 days to induce vegetative growth. Seedlings

were then transplanted into 0.25L pots filled with standard potting mix and transferred to a glasshouse with constant temperature (25°C) and 12h:12h light:dark cycle. After two months, flowering individuals were crossed by rubbing flower heads over a period of three to five days to create seed stocks for each population.

F1 hybrid seed set

15 families for each population were germinated and grown under glasshouse conditions (described above). Intra- and inter-population crosses were performed twice a day by gently rubbing flower heads (capitula): each flower head was crossed at least three times to saturate the number of fertilised florets. Due to logistic constraints, crosses were blind with respect to research assistants but not with respect to MCM who supervised the experiments. We calculated seed set by estimating the proportion of fertilised (filled seeds)[14] seeds in flower heads. We divided the number of fertilised seeds in an inter-population cross by the average number of seeds produced in parental intra-population crosses; we subtracted this fraction from one to calculate postmating prezygotic reproductive isolation in the system[17].

F1 viability at early stages of the life cycle

We tested for the viability of F1 hybrids between multiple inter-population crosses (see above) by counting the proportion of seeds that germinated in relation to the parental germination proportions. A total of 260 families equally distributed across all parental and hybrid cross types were germinated, placing five seeds of each family into moist filter paper in petri dishes (one family per petri dish). We placed petri dishes into trays (20 per tray) while randomising the position of all families. Germination conditions are the same as described above, except for tray position on the shelves, which in this case

231 was switched daily. We did not scarify seeds to investigate the intrinsic ability of
232 embryos to germinate. Data collection was blind, where cells in a tray were assigned a
233 number that was cross-referenced with the original genotype and family ID at the end of
234 the experiment. We calculated F1 inviability by estimating the proportion of germinated
235 seeds in a cross, divided by the average number of seeds that germinated of the two
236 parental populations.

237 **Strength of reproductive isolation**

238 Estimates of reproductive isolation were done for two reproductive isolating barriers in
239 the following way: *Hybrid seed set (I)* in the glasshouse or whether the proportion of
240 fertilised seeds in a flower head from an inter-population cross differed from an intra-
241 population cross as $RI_{seed\ set}$=1-(Pf_{inter}/Pf_{intra}) were Pf stands for proportion fertilised.
242 *Hybrid viability (I)*, or whether hybrid seedlings germinated equally well as their
243 parents in the glasshouse, as H_{hf}=1-($v_{F1}/v_{parents}$), where v_{F1} is the average germination of
244 F1 hybrids, and $v_{parents}$ is the average germination of the two parents.

245 **Estimates of local and non-local field performance**

246 To identify whether local adaptation was stronger among populations for the Headland
247 versus the Dune ecotype, we reanalysed field transplant experiments that used seeds
248 from three populations of each ecotype (n=150-180 seeds/population/transplant
249 environment). Methods of the transplants into the two natural environments at Lennox
250 Head (NSW), including the local population are described in detail in Walter et al.
251 2016, but briefly, we fixed each seed to a toothpick using non-drip supa glue. The
252 toothpicks were then placed in a fully randomised grid under natural conditions in both
253 the sand dunes and rocky headland at Lennox Head. We used six blocks to sample each

environment. Shadecloth (50%) was suspended 15cm above the grids and seeds watered for three weeks to replicate ideal germination conditions. We recorded seedling emergence as a binary trait, we then tracked seedling survival and recorded whether plants reached 10 leaves (as a measure of seedling establishment).

To understand whether local populations performed better than non-local populations (from the same habitat), we first implemented a survival analysis within the R package 'coxme'[43] where survival was censored at day 320, and we included population as a fixed effect, and environmental block as a random effect. Next, to test whether local populations had a higher probability of reaching seedling establishment, we implemented a generalised linear mixed effects model within the R package 'lme4'[44]. To do so, we included seedling establishment as a binary response variable, with population as a fixed effect, and environmental block as a random effect. In both analyses we wanted to test whether the two non-local populations performed more poorly than the local population. Thus, for each analysis we designated the local population as the intercept, testing each non-local population relative to the intercept.

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Figure Legends

Figure 1. A) Morphological differences between the two ecotypes: Dunes grow erect and have big leaves, while Headlands grow prostrate and have smaller succulent leaves. Modified from[10], with permission from the authors. **B)** Geographic distribution of 16 populations in the study and phylogeny based on 13 neutral markers using Bayesian inference of the populations in the study. S and SE respectively refer to the Southern and South-Eastern clades observed in the phylogeny. Numbers above nodes are estimates of divergence time obtained in IMa2, whereas numbers below nodes are credible posterior probabilities obtained in BEAST*.

Figure 2. F1 hybrid seed set and F1 hybrid inviability in crosses between coastal populations in *Senecio lautus*. Each panel represents a combination of crosses within ecotype (DxD, with Dune mother; HxH, with Headland mother) or between ecotypes (DxH, with Dune mother; HxD, with Headland mother) from the same or from a different clade. Positive values of Strength of RI imply that hybrids perform worse than parents, and negative values that hybrids perform better than parents.

Figure 3. Field performance of three replicate populations from each ecotype in their native habitat at Lennox Head (NSW) in the form of Estimated S (survival). Coloured populations represent the local population and error bars represent 95% confidence intervals. (**A**) Survival was similar for the three Dune populations, with only a significant difference between the local population (Lennox Head) and the Cabarita Beach population. The local Headland population survived much better than the two non-local populations. Tables inset describe tests of each non-local population relative

to the local population. (**B**) Probability of reaching seedling establishment was similar for all Dune populations, but was much higher for the local Headland population (asterisks denote significant difference to local population).

Figure 4. Observed pattern of extrinsic and intrinsic reproductive isolation in *Senecio lautus*. Rectangles represent sets of reproductively or ecologically compatible populations. For instance, all Dune (orange) and Headland (green) populations are reproductively compatible within clades (top right panel). However, only Dune populations are ecologically compatible within clades as they cannot be transplanted easily into rocky headland sites, and Headland populations perform best in their own rocky site (top left panel). This pattern is also found when examining intrinsic reproductive isolation between clades and ecotypes (bottom right panel).

Figure 1.

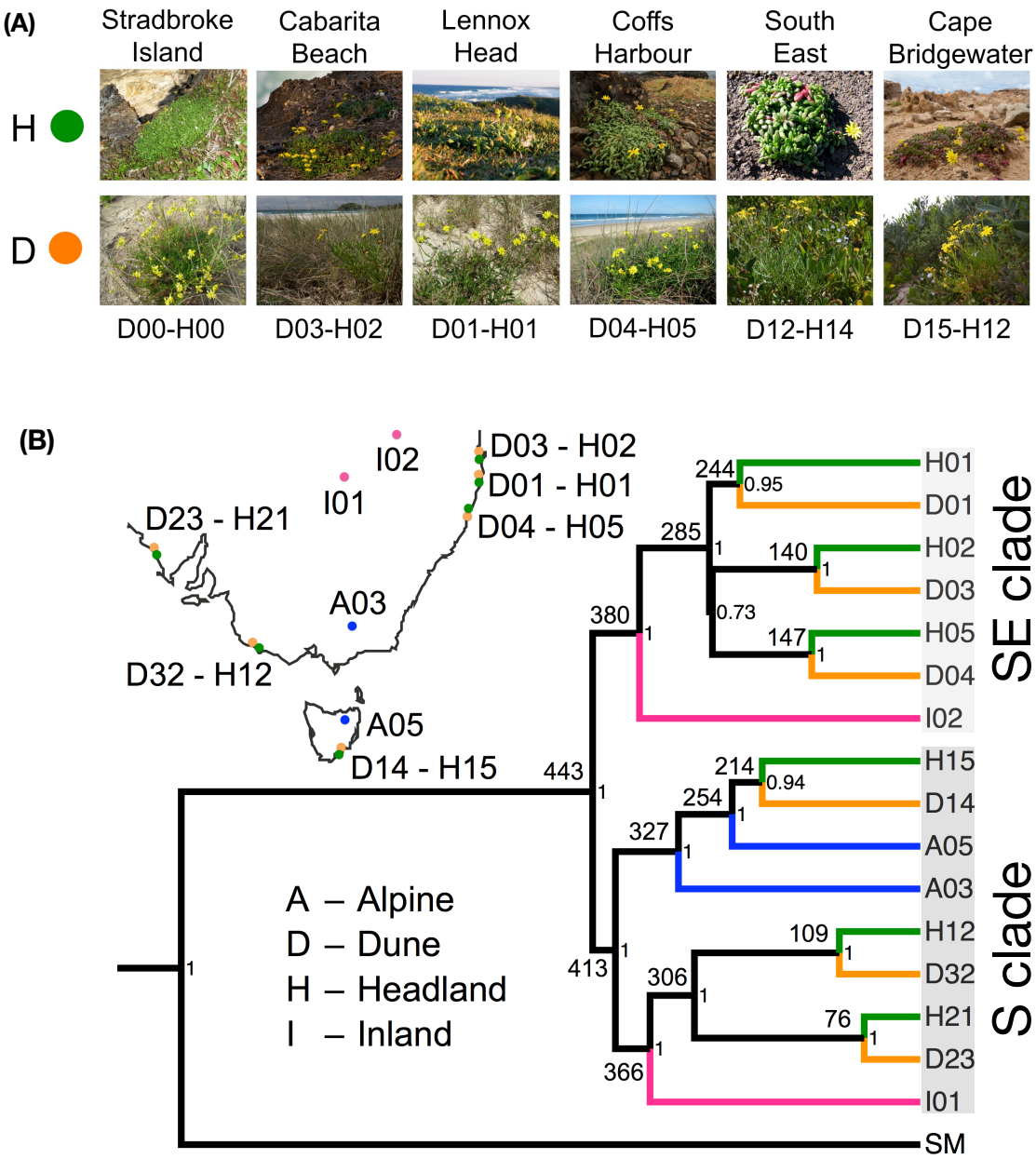

(A)
Stradbroke Island
Cabarita Beach
Lennox Head
Coffs Harbour
South East
Cape Bridgewater
H
D
D00-H00
D03-H02
D01-H01
D04-H05
D12-H14
D15-H12
(B)
I02
I01
D03 - H02
D01 - H01
D04 - H05
D23 - H21
A03
D32 - H12
A05
D14 - H15
A – Alpine
D – Dune
H – Headland
I – Inland
244
0.95
285
140
0.73
147
380
443
214
0.94
254
327
109
306
76
413
366
H01
D01
H02
D03
H05
D04
I02
H15
D14
A05
A03
H12
D32
H21
D23
I01
SM
SE clade
S clade

Figure 2

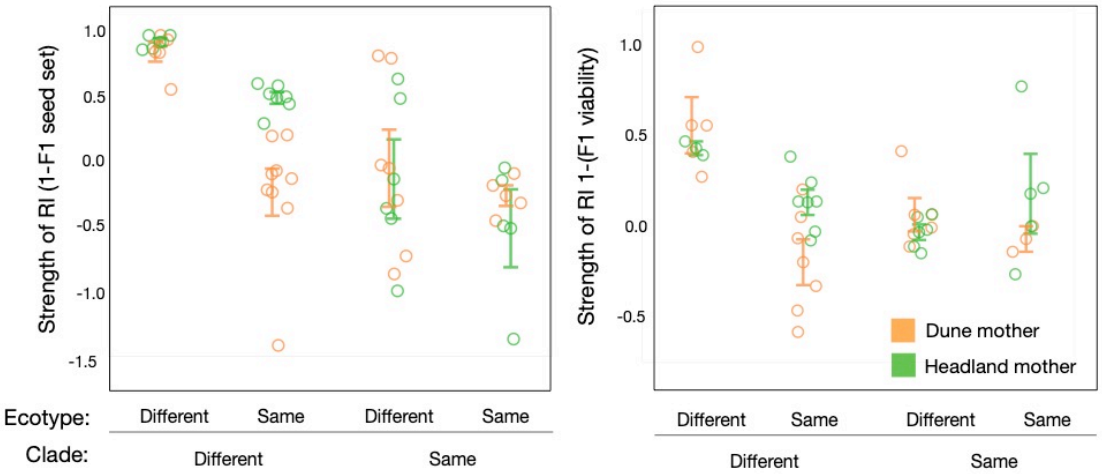
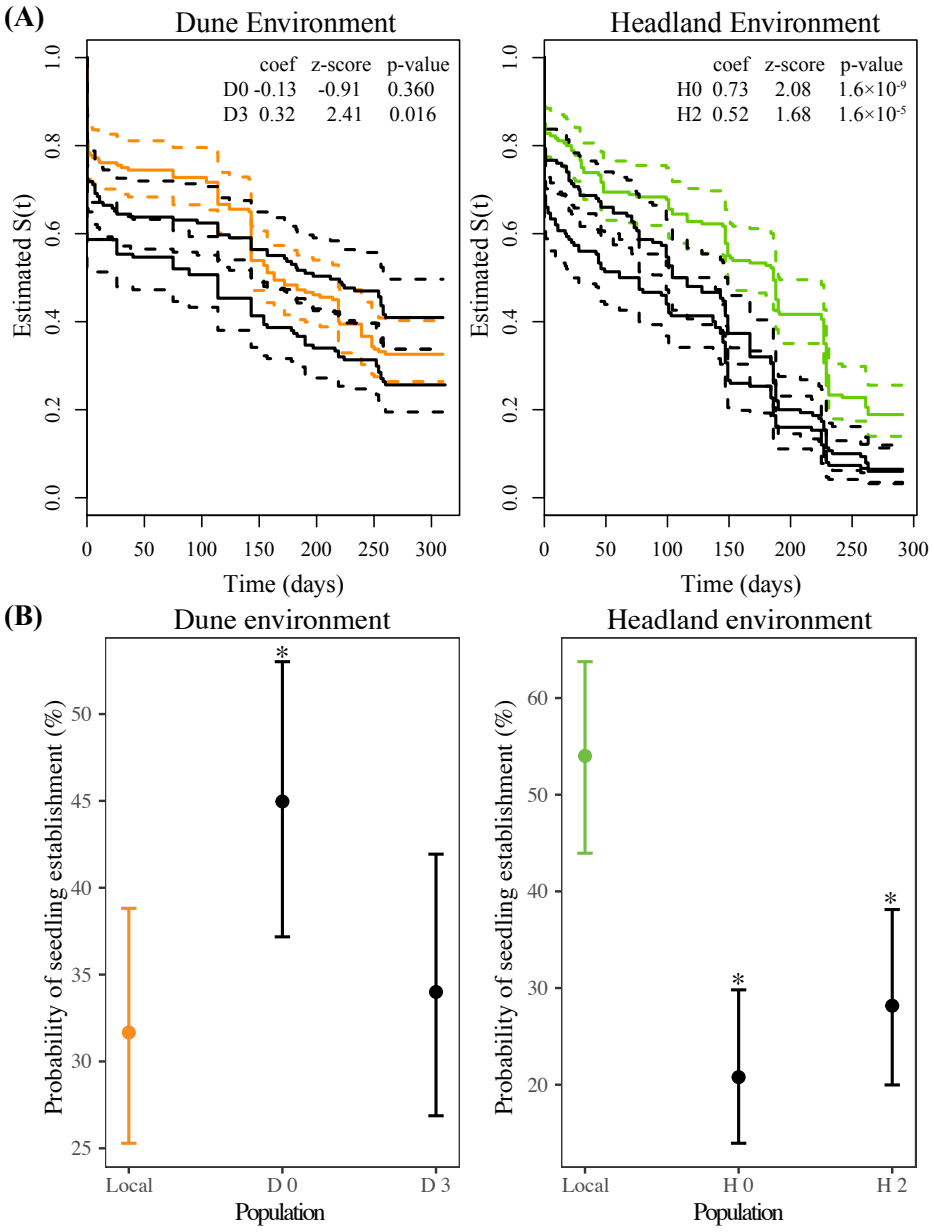

Strength of RI (1-F1 seed set)
1.0
0.5
0.0
-0.5
-1.0
-1.5
Ecotype:
Different
Same
Different
Same
Clade:
Different
Same
Strength of RI 1-(F1 viability)
1.0
0.5
0.0
-0.5
Dune mother
Headland mother
Different
Same
Different
Same
Different
Same

Figure 3


(A)
Dune Environment
coef z-score p-value
D0 -0.13 -0.91 0.360
D3 0.32 2.41 0.016
Estimated S(t)
Time (days)
Headland Environment
coef z-score p-value
H0 0.73 2.08 1.6×10-9
H2 0.52 1.68 1.6×10-5
Estimated S(t)
Time (days)
(B)
Dune environment
Probability of seedling establishment (%)
Local
D 0
D 3
Population
Headland environment
Probability of seedling establishment (%)
Local
H 0
H 2
Population

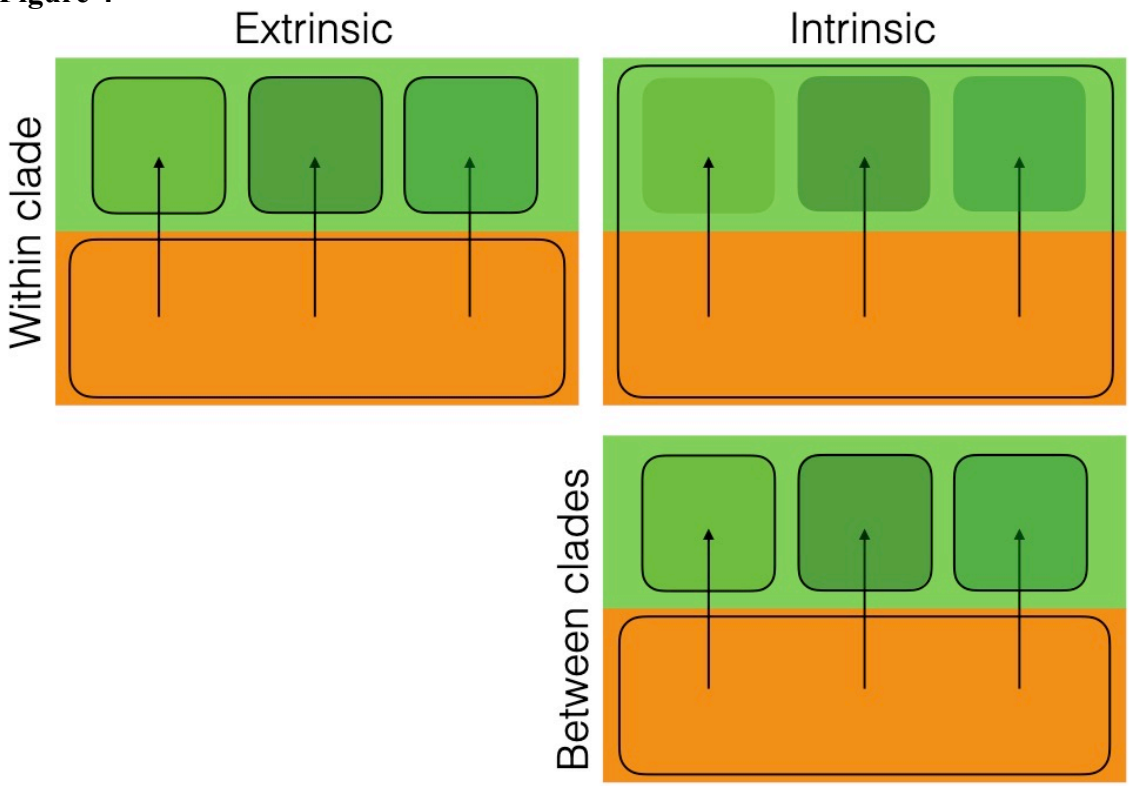



Figure 4