Minireview

Reinforcement in plants

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Summary

A major goal of evolutionary biology is to understand how diverging populations become species. The evolution of reproductive isolation (RI) halts the genomic homogenization caused by gene flow and recombination, and enables differentiation and local adaptations to become fixed between newly forming species. Selection can favor the strengthening of RI through a process termed reinforcement. Reinforcement occurs when selection favors traits that decrease mating between two incipient species in response to costly mating or the production of maladapted hybrids. Although this process has been investigated more frequently in animals, there is also evidence of reinforcement in plants. There are three strategies for the investigation of the process of reinforcement: case studies of species or diverging taxa; experimental evolution studies; and comparative studies. Here, I discuss how all three strategies find evidence consistent with reinforcement occurring in plants. I focus largely on case studies, and use research on *Phlox drummondii* to illustrate the importance of testing alternative hypotheses. Although the existing evidence suggests that reinforcement can occur, further investigations, particularly using large-scale comparative studies, are needed to determine the importance of reinforcement in plant speciation.

Introduction – what is reinforcement

Reinforcement is the process by which selection against hybrids or mating between diverging taxa causes the evolution of greater reproductive isolation (RI) between emerging species (Fig. 1) (Howard, 1993; Servedio & Noor, 2003). The hypothesis that natural selection can favor increased RI is often attributed to Alfred R. Wallace and his efforts to persuade Darwin of the role of selection in speciation (Wallace, 1889). Consequently, reinforcement has been referred to as the Wallace effect (Grant, 1966). During the modern synthesis, Dobzhansky (1940) advocated for a role of reinforcement in the formation of reproductively isolating mechanisms, by arguing that maladaptive hybridization can generate selection to decrease or prevent interbreeding between incipient species. This work helped spark decades of research aimed at understanding how RI mechanisms evolve in sympatry.

Although the role of reinforcement in speciation was debated in the second half of the 20th century (reviewed in Howard, 1993; Servedio & Noor, 2003), empirical and theoretical work has concluded that reinforcement can occur (reviewed in Ortiz-Barrientos *et al.*, 2009; Pfennig & Pfennig, 2009). The focus of research can now shift towards an understanding of how and why it occurs through investigations of the mechanisms and strength of selection, the genetic basis of trait variation, and how other sources of selection affect reinforcement.

Case studies, experimental evolution studies and comparative studies have found evidence that reinforcement can contribute to the evolution of RI in plants. Indeed, some of the earliest empirical work (Grant, 1966; Levin & Kerster, 1967; McNeilly & Antonovics, 1968; Paterniani, 1969; Whalen, 1978) and theoretical work (Dickinson & Antonovics, 1973; Caisse & Antonovics, 1978) on reinforcement involved plants. Although many aspects of reinforcement are generalizable across animals and plants, recent reviews have focused largely on animals (Ortiz-Barrientos *et al.*, 2009; Pfennig & Pfennig, 2009). Here, I discuss the empirical research performed on plants.

Fig. 1 A schematic description of the reinforcement process. Divergence between incipient species can involve morphological, cellular, developmental and genetic changes. Divergence can result in hybrids that are maladapted, nonviable or infertile, or in costly matings between the incipient species. In the diagram, the hybrid is less vigorous and does not attract pollinators. Selection favors a new adaptation that increases RI and decreases costly hybridization or mating. In the diagram, flower color change results in a switch in pollinator type. The reinforcement process is often recognized because it can result in a pattern of greater RI between individuals from sympatry than individuals from allopatry.

During reinforcement, selection can result from hybrids with low fertility or viability, or from mating costs such as wasted gametes and stigma clogging (Servedio & Noor, 2003). Selection only favors RI that occurs before the cost of hybridization, and therefore reinforcement usually involves pre-zygotic RI mechanisms. Early-acting post-zygotic RI, such as seed abortion, could potentially be favored by reinforcing selection as well. Studies of reinforcement in plants have found a range of traits that respond to reinforcing selection. The mechanisms involved in decreasing hybridization include shifts in flower color (Levin & Kerster, 1967; Hopkins & Rausher, 2012), changes in flowering time (McNeilly & Antonovics, 1968; Silvertown *et al.*, 2005), increased self-fertilization rates (Fishman & Wyatt, 1999), altered floral morphology (Whalen, 1978) and new pollen–stigma incompatibilities (Kay & Schemske, 2008). Reinforcing selection acts only in geographic regions of sympatry or close parapatry in which two diverging taxa have the opportunity to hybridize, but does not act in allopatric populations. This geographic variation in selection can create a pattern of increased pre-zygotic RI in sympatric populations relative to allopatric populations.

Processes other than reinforcement can cause patterns of reproductive trait divergence across the range of a species (Fig. 2) (Noor, 1999; Hoskin & Higgie, 2010). In particular, ecological character displacement (ECD) can result in greater divergence in sympatry relative to allopatry. ECD was proposed as a phenomenon in animals to alleviate competition between species for limited resources, such as food or habitat (Dayan & Simberloff, 2005; Pfennig & Pfennig, 2009). Trait evolution resulting from selection to alleviate competition can pleiotropically affect mate choice and decrease hybridization between sympatric species. For example, in plants, ECD has been shown to result from competition for pollinators (reviewed in Dayan & Simberloff, 2005). Sympatric species that utilize the same pollinators may suffer fitness costs from pollen limitation. Even though the source of selection is pollen competition, divergence in floral morphology or flowering time can decrease mating opportunity between sympatric taxa. In addition to ECD, local adaptation to abiotic or biotic environmental variation can also contribute to divergence between allopatry and sympatry. Therefore, much of my discussion of reinforcement focuses on understanding the evolutionary processes that could interact with, or mimic, the process of reinforcement.

Fig. 2 Multiple processes can explain divergence between allopatric and sympatric populations. Reinforcement, ecological character dis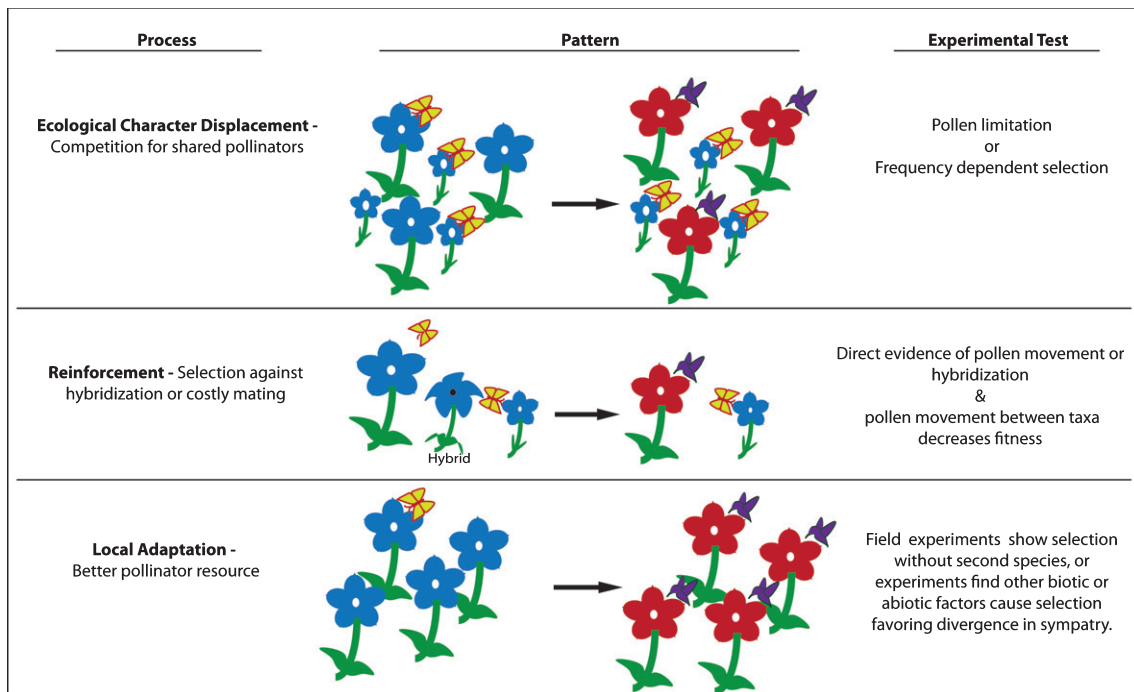placement (ECD) and local adaptation can result in a similar pattern of trait divergence. Experimental tests can be used to identify the source of selection. If competition for pollinators limits fruit set for the ancestral morphology (in this case, blue flower), a red flower mutation that attracts a new pollinator can be favored (ECD scenario, top panel). If the ancestral blue flower in sympatry enables maladapted hybridization, a red flower mutation that attracts a new pollinator will be favored because it decreases hybridization (reinforcement scenario, middle panel). If a part of the range that happens to be sympatric with another species has a better or more abundant pollinator, floral divergence to attract that pollinator would be favored (local adaptation scenario, bottom panel).

Case studies – criteria

The most common strategy for the investigation of reinforcement is through case studies on specific plants (Table 1). Case studies have shown evidence consistent with reinforcement but, in many systems, further research is required to understand whether and how reinforcement causes divergence. I present four criteria for the determination of whether reinforcement causes divergence (Table 2). Although these criteria are partially drawn from previous criteria for the testing of whether character displacement is responsible for trait differentiation between allopatric and sympatric populations (Schluter & McPhail, 1992; Howard, 1993), I focus more on understanding the forces of selection and not only on the reduction in hybridization.

As I discuss each topic, I use, as an example, research performed on flower color variation in the native Texas wildflower *Phlox drummondii* (Fig. 3). *Phlox drummondii* and its congener *Phlox cuspidata* have a light-blue flower color throughout allopatric regions of their ranges but, where the two species co-occur in sympatry, *P. drummondii* has dark-red flowers. Research has shown that divergence of flower color in sympatry is caused by reinforcement (Levin, 1985; Hopkins & Rausher, 2012).

Mating cost

Reinforcement acts to decrease costly interbreeding; therefore, it is paramount to demonstrate that hybridization or mating is costly.

Most studies have documented this cost by finding either reduced seed production as a result of hybridization (Fishman & Wyatt, 1999; Smith & Rausher, 2008) or reduced hybrid viability or fertility (Levin & Kerster, 1967; Whalen, 1978; Kay, 2006). Some studies just assume that hybrids are maladapted as a result of apparent differential local adaptation of the parents (McNeilly & Antonovics, 1968; Silvertown *et al.*, 2005; Zeng *et al.*, 2011). In the *Phlox* system, hybrids between *P. cuspidata* and *P. drummondii* are vigorous, but suffer low fertility (0–30% compared with nonhybrid individuals), indicating a high cost to hybridization (Ruane & Donohue, 2008).

Reduction in hybridization

Reinforcement acts to decrease costly hybridization, and therefore it is important to establish that divergence in sympatry decreases mating or hybridization between diverging taxa. Some studies assume a decrease in hybridization as a result of the nature of the trait variation. For example, flowering time overlap is assumed to correlate with hybridization rate (McNeilly & Antonovics, 1968; Silvertown *et al.*, 2005). Other studies measure components of hybridization rate using glasshouse crosses (Kay & Schemske, 2008) and pollinator observations (Levin & Kerster, 1967), and extrapolate the findings to estimate how divergence in sympatry probably decreases hybridization. In *Phlox*, common-garden experiments were used to measure hybridization rates in a natural environmental setting. Each possible *P. drummondii* flower color

Table 1 Case studies of reinforcement in plants

Species	Life cycle	Trait	C1: Cost of hybridization	C2: Quantify hybridization	C3: Quantify selection	HA: Test for local adaptation	HA: Test for ECD	Post-zygotic RI	Citation
Agrostis tenuis	Perennial	Flowering time	Assumed hybrids maladapted	Assumed co-flowering corresponds to hybridization rate	No	No	N/A	Unknown	McNeilly & Antonovics (1968)
Anthoxanthum odorantum	Perennial	Flowering time	Assumed hybrids maladapted	Assumed co-flowering corresponds to hybridization rate	No	No	N/A	Unknown	McNeilly & Antonovics (1968)
Anthoxanthum odorantum (Park Grass Experiment)	Perennial	Flowering time	Assumed hybrids maladapted	Assumed co-flowering corresponds to hybridization rate	No	Pattern appears inconsistent	N/A	Unknown	Silvertown *et al.* (2005)
Arenaria uniflora	Annual	Self-fertilization	25% reduction in seed set	Relative seed set with and without other species	No	Yes: selfers have inbreeding depression	Yes: pollinator competition occurs	Complete	Fishman & Wyatt (1999); Wyatt (1992)
Costus scaber & *C. pulverulentus*	Perennial	Pollen–pistil incompatibility	Low hybrid seed set, germination and fitness	Glasshouse crosses	No	No	N/A	0% or 82%	Kay & Schemske (2008); Kay (2006)
Ipomoea hederacea	Annual	Floral morphology	Reduction in seed set	Relative seed set with and without other species	Yes	Yes	No	Complete	Smith & Rausher (2008)
Phlox drummondii	Annual	Flower color	Hybrids are nearly 90% sterile	Direct common-garden measures	Yes	Yes: no other selection on flower color	Yes: no evidence of competition	70–100%	Hopkins & Rausher (2012); Levin (1985)
Phlox pilosa	Perennial	Flower color	Low hybrid seed set	Pollen movement in potted arrays	No	Yes: pollinator preference for allopatric color	No	Almost complete	Levin & Kerster (1967)
Quercus mongolica & *Q. liaotungensi*	Perennial	Unknown: molecular signature of hybridization	Assumed	Molecular signature of introgression	No	No	No	Unknown	Zeng *et al.* (2011)
Solanum grayi	Perennial	Flower size	Hybrid seed abortion	Assumed different sizes have different pollinators	No	No	No	Unknown (probably high)	Whalen (1978)
Solanum rostratum	Annual	Flower size	Hybrid seed abortion	Assumed different sizes have different pollinators	No	No	No	Unknown (probably high)	Whalen (1978)
Zea mays	Annual	Unknown	Hybrid lethality	% hybrid kernels on ears	Yes	N/A	N/A	Complete	Paterniani (1969)

ECD, ecological character displacement; C1, C2, C3, testing criteria 1, 2, 3 in Table 2; HA, alternative hypothesis; N/A, not applicable; RI, reproductive isolation.

Table 2 Criteria for the verification that reinforcement is responsible for divergence

1. Mating between diverging taxa and/or hybridization is costly.
2. The novel trait in sympatry decreases hybridization or mating between diverging taxa.
3. The decrease in mating between diverging taxa or hybridization increases net fitness of individuals with novel trait.
4. Other evolutionary processes do not explain divergence, such as:
   ecological character displacement;
   local adaptation to environmental variation.

variant was grown with *P. cuspidata* and the hybridization rate was calculated by genotyping offspring at a marker differentiating the two species (Hopkins & Rausher, 2012). This type of common-garden experiment allows for a realistic assessment of how trait variation in sympatry affects natural hybridization rates.

Net fitness

Measurement of the costs and benefits of divergence in sympatry is important because trait variation that influences hybridization could have detrimental effects on other aspects of reproduction. For example, in *Phlox pilosa*, flower color divergence decreases hybridization with *Phlox glaberrima*, but pollinators disfavor the diverged 'sympatric' flower color (Levin & Kerster, 1967). If the loss of pollinator visitation caused by preference for other colors outweighs the benefit of reduced hybridization, it is likely that flower color evolved for other reasons and not just reinforcement. The aim of this criterion is to determine whether reinforcement will result in alleles causing RI to increase in a sympatric population. A common-garden experiment is one method to measure the effects of divergence in sympatry on multiple aspects of fecundity and survival in a more natural setting. In *P. drummondii*, data from a common-garden experiment in the sympatric range indicate that net selection favors at least one of the derived flower color alleles, because it decreased hybridization with *P. cuspidata* (Hopkins & Rausher, 2012).

Alternative hypotheses

As already discussed, other evolutionary processes can cause divergence in reproductive traits between allopatric and sympatric populations. Even if reinforcement is acting to decrease hybridization, ECD and local adaptation can influence trait evolution in sympatry (criterion 4).

ECD hypothesis Without identifying the source of selection, it is difficult to determine whether divergence is caused by competition for resources (ECD) or costly hybridization (reinforcement). For example, Smith & Rausher (2008) found that selection on *Ipomoea hederacea* floral morphology was dependent on the presence of *Ipomoea purpurea*. The traits under selection could be associated with either increased self-fertilization causing reproductive assurance under pollinator limitation (ECD), or mechanical blocking of the stigma to prevent mating between taxa (reinforcement).
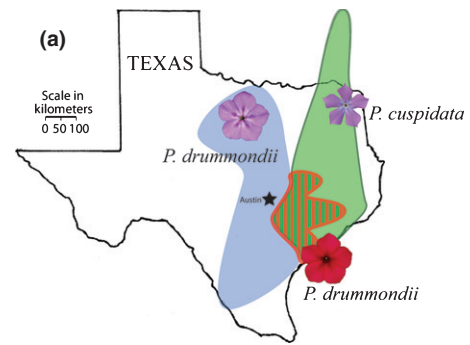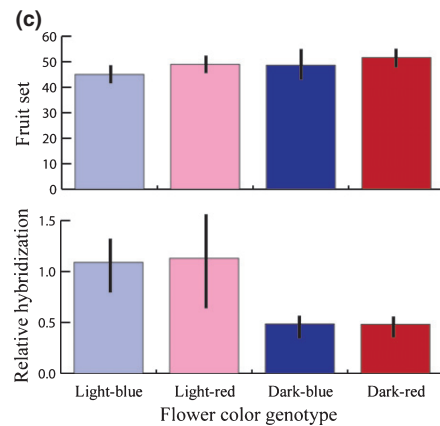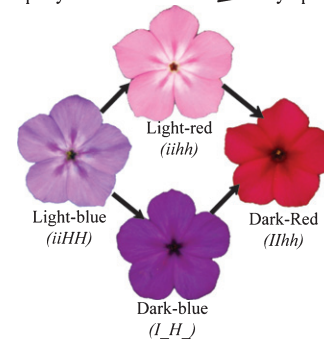
Fig. 3 Case study of *Phlox drummondii*. (a) A schematic diagram of the distribution of *P. drummondii* and *P. cuspidata* showing the allopatric *P. drummondii* range in blue, the sympatric range outlined in red and the *P. cuspidata* range in green. (b) The genetic basis of flower color variation in *P. drummondii* involves the intensity locus (*I*) and the hue locus (*H*) (Hopkins & Rausher, 2011). (c) Results from common-garden field experiments showing equal fruit production of all flower color genotypes (top) and reduction in hybridization in dark genotypes (bottom) (Hopkins & Rausher, 2012). These data indicate that variation in hybridization causes selection on flower color. Bars ± SE.

Further experiments investigating pollen limitation and stigma clogging are necessary to distinguish between these two hypotheses. Research in *P. drummondii* has found no evidence of ECD influencing flower color evolution. The relative survival and seed set for each of the *P. drummondii* flower color genotypes do not vary in the presence of *P. cuspidata* (Hopkins & Rausher, 2012). Disregarding hybridization, there is no evidence that the presence of *P. cuspidata* affects the fitness of *P. drummondii*.

Local adaptation hypothesis Local adaptation to abiotic and biotic variation between allopatry and sympatry can either cause or contribute to the divergence of reproductive traits in sympatry. For example, variation in available pollinators or optimal flowering time could cause divergence in traits associated with reproduction, and mimic patterns caused by reinforcement (Fig. 2). A widely cited alternative hypothesis to reinforcement is the differential fusion hypothesis, a variant of the local adaptation hypothesis, which states that divergence caused by local adaptation may occur before range overlap of the two species, and only because of this divergence can the two species co-exist in sympatry (Templeton, 1981). A number of studies in plants have estimated selection acting on trait divergence in the absence of the second sympatric species in order to distinguish between local adaptation and reinforcement (Fishman & Wyatt, 1999; Smith & Rausher, 2008; Hopkins & Rausher, 2012). In *P. drummondii*, the local adaptation hypothesis posits that flower color divergence in the eastern, sympatric, area of the range is advantageous because of an abiotic or biotic factor unrelated to hybridization with *P. cuspidata* (e.g. herbivore defense or drought tolerance). Common-garden experiments found no evidence supporting this hypothesis; in the absence of *P. cuspidata*, the flower color varieties of *P. drummondii* show statistically similar survival and seed production (Hopkins & Rausher, 2012).

The published case studies of reinforcement in plants find evidence to support the reinforcement hypotheses, although, in many systems, alternative hypotheses explaining trait variation cannot be rejected (Table 1). Further investigations are necessary to determine the role of competition and local adaptation in reproductive trait divergence in sympatry.

Case studies – other areas of investigation

The investigation of the above four criteria can determine whether reinforcement causes divergence, but there are other interesting areas of research that can provide insight into the process. Specifically, the investigation of the level of gene flow during the process of reinforcement and the genetic basis of divergence can enhance our understanding of reinforcement.

Gene flow

An estimation of the level of RI existing before and during reinforcement can help shape our understanding of how gene flow constrains reinforcement. As discussed in Box 1, one of the arguments against the plausibility of reinforcement is that the homogenizing effect of gene flow between sympatric incipient species could prevent the evolution of RI mechanisms (Butlin, 1987). Theoretical work indicates that, under some conditions, gene flow and recombination between species can prevent the spread of a sympatric RI mechanism. However, under many theoretical values of gene flow, and pre-existing post-zygotic and pre-zygotic RI, increased RI can evolve in sympatry (Servedio & Kirkpatrick, 1997; Kirkpatrick & Servedio, 1999; Kirkpatrick, 2000; Bank *et al.*, 2012). This theoretical work can suggest when

reinforcement is plausible, but only extensive empirical investigations can identify the conditions under which reinforcement actually occurs. In plants, there appears to be variation in the amount of RI before reinforcement, but there are very few systems for which we have complete data. For example, in *P. drummondii*, pre-zygotic RI mechanisms other than flower color are unknown, although hybrids are nearly, but not completely, sterile (Ruane & Donohue, 2008). In another system, pre-zygotic RI is very strong, but it appears that post-zygotic RI is relatively weak (Kay, 2006). In many plants, selection against hybrids is unknown or assumed, but not measured (McNeilly & Antonovics, 1968; Whalen, 1978; Zeng *et al.*, 2011). More accurate estimates of the pre-existing levels of pre-zygotic and post-zygotic RI are necessary in order to better understand how the homogenizing effect of gene flow limits evolution via reinforcement.

Genetics of reinforcement

One of the interesting outcomes of theoretical research on reinforcement is that the genetic architecture of the RI mechanisms may influence the success of reinforcing selection (Felsenstein, 1981). If a mutant allele increases RI when it exists in either or both of the sympatric species, reinforcement is likely to be more successful (discussed in Servedio & Noor, 2003). This is called a one-allele mechanism of reinforcement. Recombination of a one-allele mechanism into the second sympatric taxon will not hinder the success of reinforcement, and may further decrease hybridization between the diverging taxa. The alternative scenario, the two-allele mechanism, is when the two sympatric taxa have *different* alleles at the same locus and this difference causes RI. Reinforcement can fail if recombination causes individuals of different taxa to possess the same allele at this locus.

Flower color variation in *Phlox* is an example of a two-allele mechanism. *Phlox cuspidata* plants have the 'light' flower color allele and sympatric *P. drummondii* have the mutant 'dark' allele. Because the two species have different alleles at the intensity locus, RI increases (Hopkins & Rausher, 2011). If this dark allele recombines into a *P. cuspidata* background, the two species would have the same flower color intensity and RI would not increase.

Although a one-allele mechanism has not been characterized in plants, there are possible RI mechanisms that evolve in this way. For example, an allele that increases self-fertilization, as in *Arenaria uniflora* (Fishman & Wyatt, 1999), could also cause greater self-fertilization in a sympatric species. The novel allele would increase RI in either or both of the sympatric species. Similarly, if two sympatric species have different mean flowering time, but overlapping flowering time variation, an allele that decreases variance in flowering time could increase RI in either or both species.

The dissection of the genetic basis of reinforcement is important for a number of additional reasons. First, we can begin to understand how many and what types of mutations underlie reinforcement. Second, this information can inform the creation of genetic lines that can be used to measure selection acting on each mutation (or closely linked genetic variation). To date, there is very little information on the genetic basis of traits causing

reinforcement in plants. The exception is *P. drummondii* (Fig. 3), in which the two genes underlying flower color variation have been identified (Hopkins & Rausher, 2011). One of the loci controls color hue, causing flowers to change from blue to red, whereas the other changes color intensity from light to dark. Using experiments designed to measure selection on each of the flower color loci in *Phlox,* it was shown that the 'dark' allele decreased hybridization between the two species, and is therefore involved in reinforcement, but there was no apparent selective advantage of the hue locus (Hopkins & Rausher, 2012).

Experimental evolution

A second method of investigation of the process of reinforcement is to artificially impose reinforcing selection on an experimental population and measure the response. Only one experiment of this type has been performed in plants (Paterniani, 1969). Two varieties of *Zea mays*, which produced easily recognizable hybrid kernels, were interspersed and allowed to pollinate naturally in fields. Ears were selected to contribute to the next generation based on their hybridization rate, with the level of hybridization allowed to decrease annually from 20% to 0%. A response to selection was detected immediately and, by the end of the fifth generation, the hybridization rate was < 1%. This experiment demonstrates that selection can result in increased pre-zygotic RI when post-zygotic RI is complete (hybrid lethality). Future experiments with plants should allow some hybrid success in order to investigate how reinforcement occurs with gene flow.

Comparative studies

The final method of investigation of the process of reinforcement is the use of large-scale comparative studies to find patterns consistent with natural selection acting to increase pre-zygotic RI in sympatry. The detection of these patterns requires detailed information on RI for many related taxa. The classical pattern predicted to result from reinforcement is based on the hypothesis that RI increases as genetic divergence increases (Coyne & Orr, 1989). Allopatric species pairs should show equivalent rates of increase for pre-zygotic and post-zygotic RI with increasing genetic distance. If reinforcement is occurring in sympatry, pre-zygotic RI is predicted to increase at a faster rate than post-zygotic RI as the genetic distance increases. This is precisely the pattern detected in *Drosophila* (Coyne & Orr, 1989). There have been a number of studies in plants investigating this pattern, or similar patterns (Table 3), with the two most comprehensive studies finding evidence of reinforcement (Van der Niet *et al.*, 2006; Grossenbacher & Whittall, 2011). Unfortunately, there are major caveats in the comparative studies (Table 3), such as approximate and incomplete measurements of RI, which creates uncertainty in the importance of reinforcement in plant speciation.

A study examining sister species from a number of genera in the Cape Floristic Region found that sympatric species living on different edaphic soil types almost always used different pollinator species (Van der Niet *et al.*, 2006). The prevalence of pollinator shifts was not found in sympatric pairs on the same soil type or in allopatric pairs with or without edaphic shifts. The authors argue that edaphic differences correlate with post-zygotic RI because hybrids will be unfit on either soil type. This study relies on indirect estimates of RI, but the pattern is consistent with selection favoring an increase in pre-zygotic RI between sympatric species that produce unfit hybrids.

A second study, in the genus *Mimulus*, found that sympatric sister species had greater floral morphological divergence than allopatric species pairs, whereas vegetative morphological divergence did not differ (Grossenbacher & Whittall, 2011). Instead of using post-zygotic RI as a control for the rate of divergence, this study utilized vegetative divergence. Given that some studies find that post-zygotic RI does not necessarily increase with genetic distance in plants (Moyle *et al.*, 2004; Scopece *et al.*, 2007), this alternative method can provide further insight into reinforcement.

A study by Moyle *et al.* (2004) did not find evidence supporting the reinforcement hypothesis in two genera. Specifically, the rate of pre-zygotic RI and post-zygotic RI increase with increasing genetic distance did not differ between allopatric and sympatric species. Only post-mating pre-zygotic RI was measured in this study and not pollinator isolation, flowering time isolation or morphological isolation. Additional comparative studies addressing all components of RI may help to determine why reinforcement occurs in some lineages and not in others.

Future directions

Although more research has been performed on animals, in many ways plants can provide a better system to study reinforcement. Plants are stationary, allowing for easier monitoring of survival and reproductive success in natural and experimental populations across a season or multiple seasons. Many plants are easier than animals to grow and mate for many generations in captivity (glasshouse), allowing for the creation of artificial hybrids and the evaluation of relative performance under many conditions. The modularity of plants allows direct comparison, in the same plant and at the same time, of seed development, pollen tube germination and growth from different donors. Given these advantages, and the existence of classical works on this topic (Grant, 1966; Levin & Kerster, 1967; McNeilly & Antonovics, 1968; Paterniani, 1969; Levin, 1970; Whalen, 1978), we should be able to increase our depth and breadth of knowledge on reinforcement in plants.

More case studies of reinforcement in plants may enable us to draw general conclusions about why certain types of plant are affected and why particular traits evolve. One hypothesis is that the type of RI to evolve is more strongly dependent on the type of pollination system than on the plant. Interestingly, all the wind-pollinated plants and none of the animal-pollinated plants in which reinforcement has been investigated have evolved variation in flowering time. Could there be a constraint on flowering time evolution, making it less likely to evolve in animal-pollinated plants? Is the type of RI likely to evolve in plants affected by the type of animal that pollinates them? Future research should strive to test alternative hypotheses more rigorously and to quantify RI more directly.

Table 3 Comparative studies of reinforcement in plants

Plant group	Life cycle	Comparison	Evidence for reinforcement	Caveats	Citation
Burmeistera	Perennial	Logical arguments about level of RI between sister species in allopatry and sympatry, no sympatric sister species	Maybe, or ECD	No direct measure of RI	Armbruster & Machhala (2009)
Cape Floristic Region, South Africa	Perennial	Pollinator shifts in sympatric sister species with edaphic shifts vs allopatric sister species with and without edaphic shifts and sympatric species without edaphic shifts	Yes	No direct measure of RI	Van der Niet *et al.* (2006)
Collinsia	Annual	Logical arguments about level of RI between sister species in allopatry and sympatry, one sympatric sister species	Maybe, or ECD	No direct measure of RI	Armbruster & Machhala (2009)
Dalechampia	Mostly perennial	Logical arguments about level of RI between sister species in allopatry and sympatry, very few sympatric sister species	Maybe, or ECD	No direct measure of RI	Armbruster & Machhala (2009)
Fragaria	Perennial	Post-zygotic RI in sympatry vs post-zygotic RI in allopatry	No	No pre-zygotic RI measured	Nosrati *et al.* (2011)
Gilia	Annual	Early-acting post-zygotic RI in sympatric species vs late-acting post-zygotic RI in sympatric species, early and late post-zygotic RI in allopatric species	Yes	Unknown phylogenetic relationship between species	Grant (1966)
Glycine	Perennial	Rate of pre-zygotic RI accumulation in sympatry vs rate of post-zygotic RI in sympatry, rate of pre- and post-zygotic RI in allopatric species	No	Did not include pre-mating RI	Moyle *et al.* (2004)
Mimulus	Annual	Floral divergence in sympatric sister species vs allopatric sister species and vegetative traits in both pairs	Yes	No direct measure of RI	Grossenbacher & Whittall (2011)
Orchids, food deceptive	Perennial	Rate of pre-zygotic RI accumulation in sympatry vs rate of post-zygotic accumulation in sympatry	Yes	No allopatric comparison as a control	Scopece *et al.* (2007)
Orchids, sex deceptive	Perennial	Rate of pre-zygotic RI accumulation in sympatry vs rate of post-zygotic accumulation in sympatry	No	No allopatric comparison as a control	Scopece *et al.* (2007)
Silene	Perennial	Rate of pre-zygotic RI accumulation in sympatry vs rate of post-zygotic RI in sympatry, rate of pre- and post-zygotic RI in allopatric species	No	Did not include pre-mating RI	Moyle *et al.* (2004)
Stylidium	Annual	Logical arguments about level of RI between sister species in allopatry and sympatry, not many sympatric sister species	Maybe, or ECD	No direct measure of RI	Armbruster & Machhala (2009); Armbruster *et al.* (1994)

ECD, ecological character displacement; RI, reproductive isolation.

When studying reinforcement, it is important to consider that hybridization plays diverse roles in the evolution of adaptation and speciation in plants. As discussed above, one scenario is that adaptations and genetic incompatibilities accumulate between diverging taxa and costly hybridization causes reinforcement. Hybridization can also result in immediate polyploid speciation or rapid homoploid hybrid speciation (Rieseberg & Willis, 2007; Abbott & Rieseberg, 2012). The role of reinforcement in hybrid speciation is an unexplored area of research and may reveal interesting patterns about how pre-zygotic RI accumulates after such abrupt speciation events. Finally, hybridization between previously isolated plant populations may actually be beneficial as a result of hybrid vigor (Rieseberg & Willis, 2007). Some plant populations, particularly in species with high rates of self-fertilization, have been shown to harbor deleterious recessive alleles at high frequency, causing inbreeding depression. If two inbred populations hybridize, hybrid vigor can slow or negate the process of reinforcement. An understanding of the importance of reinforcement in plant speciation may require the recognition of the multiple roles played by hybridization in plant diversification and adaptation.

Reinforcement in plants has been studied for over 50 yr and yet we still have a very cursory understanding of how selection can favor the increase in RI as a result of costly hybridization and mating in plants. More case studies and comparative studies may lead to a better understanding of whether and how the process of reinforcement differs between animals and plants, and between plants with different life history strategies or pollination mechanisms.

Acknowledgements

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