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Opinion

The Past and Future of Experimental Speciation

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Speciation is the result of evolutionary processes that generate barriers to gene flow between populations, facilitating reproductive isolation. Speciation is typically studied via theoretical models and snapshot tests in natural populations. Experimental speciation enables real-time direct tests of speciation theory and has been long touted as a critical complement to other approaches. We argue that, despite its promise to elucidate the evolution of reproductive isolation, experimental speciation has been underutilised and lags behind other contributions to speciation research. We review recent experiments and outline a framework for how experimental speciation can be implemented to address current outstanding questions that are otherwise challenging to answer. Greater uptake of this approach is necessary to rapidly advance understanding of speciation.

Highlights

Experimental speciation is an excellent complement to snapshot studies of natural populations because it can disentangle recurring problems that confound studies of natural populations.

Experimental speciation made early significant contributions to understanding evolutionary processes mediating the evolution of reproductive isolation.

Over the past decade, speciation genomics has provided better predictions on how barrier loci spread in the genome and how speciation-with-gene-flow can occur.

These developments remain difficult to test in natural populations and have not been widely adopted in experimental speciation research.

Future integration of genomic tools in an experimental speciation framework will provide a step-change to understanding these outstanding speciation questions.

Forward and Reverse Approaches to Study Speciation

The progression and outcome of **speciation** (see Glossary) depend on interactions between evolutionary forces [1] that act with varying importance over space and time to either facilitate or impede the evolution of reproductive isolation (RI) [2]. RI may arise through the action of **genetic drift** and/or divergent natural selection, may depend on **gene flow** via continuous migration or **secondary contact**, is impacted by population size and structure, and influenced by genomic properties such as mutation and recombination rates [3]. Understanding the relative contributions of these processes to the evolution of RI is the focus of speciation research. A classic and highly successful approach to studying speciation involves identifying a phenotypically divergent trait and testing its association with the level of RI between extant populations [4–6]. The increasing application of high-throughput genomic data to address speciation genomics questions (Box 1) is used to reconstruct population history (e.g., demography) and infer the evolutionary processes leading to speciation, often over a long timescale [1,3,7]. This approach is analogous to the use of forward genetics to study the function of a gene, but applied to the study of RI. Here, the study of speciation begins with a phenotype (RI) and proceeds to identify the potential evolutionary processes that caused RI to build up between diverged populations. Many studies support the success of this approach [1,4–7]. However, this forward method of studying speciation is actually backward looking, reflecting a static snapshot of the processes that contributed to divergence. Realistically, signals of early barriers to gene flow are likely erased or overwritten as speciation progresses. Thus, such studies are challenged to deduce the action of multiple evolutionary processes impacting phenotypic and genomic factors that influence speciation, either sequentially or simultaneously, either in the same or different directions, inferred over long evolutionary histories.

Laboratory **experimental evolution (EE)** experiments can address these challenges by manipulating evolutionary processes thought to generate RI over many generations and then testing the outcome on the evolution of RI. **Experimental speciation (ES)** is analogous to the use of reverse genetics to study gene function. It begins with the putative evolutionary processes and proceeds to identify the conditions leading to and maintaining RI. This approach is experimental and therefore directly identifies the evolutionary processes and circumstances for the evolution of RI.

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Box 1. Speciation Genomics

The reduced cost of genomics has expanded the ability to address outstanding questions in speciation [1,4,6,25]. Of interest is how barrier loci are distributed across genomes and how they evolve during population divergence. Predicted genomic patterns are based on whether speciation proceeds between geographically separated populations without gene flow, or with gene flow occurring either during initial divergence or following secondary contact. In allopatry, divergence is not substantially constrained by the extent of genetic linkage and recombination relative to the strength of either selection or drift producing RI. In contrast, during speciation-with-gene-flow, selection for divergence is opposed by the processes of both gene flow and recombination that erode associations between genes under selection [82]. The genic view of speciation-with-gene-flow posits that speciation is initiated by selection acting against gene flow at specific targets of selection, and speciation genomics is interested in how barriers to gene flow initiate and facilitate (through the build-up of linkage disequilibrium) RI, including subsequent genomic divergence that is dependent on **genomic architecture** [25,26,83,84]. Patterns of divergence are predicted to be different depending on whether gene flow is primary or secondary [85].

Speciation genomics has begun to address these issues by identifying barrier loci evolving in response to selection or drift, their effect sizes, genomic distribution, and associations, and how this builds up as RI increases, along with inferring demographic history and gene flow [86–89]. However, there are well-reviewed confounding factors influencing genome heterogeneity that are unrelated to speciation (e.g., population history, gene flow over time, variation in strength, and timing of selection [7,9,10]), and disentangling these factors remains challenging in studies of natural populations. Models of the rate, direction and magnitude of gene flow through time tend to rely on summary measures or comparing limited sets of hypothesised scenarios. Additionally, the impact of selection on divergence can sometimes be clearly identified [5,90–92], but it is frequently challenging to characterise selection pressures – increasingly so the further selection is traced back through history. Thus, understanding the role of ecological differentiation, isolation, and genomic differentiation in response to specific evolutionary processes is difficult to reconstruct [93]. Alongside the development of models which can coestimate demography and selection, the ability to directly observe these processes during experiments designed to track such interactions will provide powerful data to apply to natural systems where direct observation during the evolution of RI is unavailable.

ES complements snapshot studies (Table 1) but is also a stand-alone powerful approach because it reveals speciation processes in real time. ES has been implemented for several decades and when its influential contribution was last reviewed, 10 years ago by Fry [8], the technique seemed poised to exponentially accelerate understanding the evolution of RI. Fry also outlined neglected speciation questions that ES was well suited to answer. Since Fry's review, speciation theory has advanced to incorporate more sophisticated ideas on genomic conditions and constraints impacting the evolution of RI. Snapshot studies have widely adopted a genetic approach to identifying signatures of RI. However, these conventional studies are vexed with inference problems, limiting understanding of speciation [9–11]. ES provides a potent method to test speciation theory by controlling and/or testing genomic factors and environmental conditions thought to influence speciation, factors that forward speciation approaches cannot disentangle (see 'A Selection of New Challenges That Experimental Speciation Can Address').

Here, we review ES studies over the past decade to examine progress on Fry's original neglected speciation questions. We identify areas of speciation research that have progressed since that review, such as speciation-with-gene-flow models and genomic conditions impacting speciation, but which ES studies have not been applied. We provide a framework for using ES combined with genomics to enable rapid advances in understanding speciation.

Another Decade of Experimental Speciation

Fry's review suggested ES could address: the relative efficacy of selection and drift in generating RI; the relative rates of evolution of different types of reproductive barriers; the feasibility of sympatric and parapatric speciation; and the feasibility of **reinforcement** [8]. We summarise the limited progress on these topics in the past decade, identify new areas in which ES has been used, and argue that since Fry's review, two fundamental shifts in speciation theory and approach have occurred that have been ignored in an ES framework.

Glossary

Allopatry: geographic isolation resulting in two or more populations' ranges being nonoverlapping.
Barrier loci: genomic loci that experience lower effective migration rate than actual migration occurring between populations.
Cascading reinforcement: process in which reinforcement between two species indirectly strengthens RI between conspecific populations.
Coupling: co-occurrence of different barriers to gene flow, producing a stronger overall barrier effect.
Destroy all the hybrids: a moniker for a series of artificial selection experiments in which hybrids between divergent lineages were removed to select for RI.
Dimensionality: number of traits or loci impacted by selection.
Dobzhansky–Muller incompatibility (DMI): epistatic interactions between alleles that have become independently fixed in different populations, that have a deleterious effect on fitness when brought together in the same individual. DMIs are thought to be an important cause of barriers to gene flow between species.
Evolve and resequence (E&R): process of sequencing population genomes before and after EE for purposes of comparison.
Experimental evolution (EE): study of evolutionary processes under highly controlled experimental conditions.
Experimental speciation (ES): EE which directly tests for RI between diverging populations.
Extrinsic isolation: RI dependent upon environmental effects.
Founder flush: process whereby a small founder population rapidly grows to carrying capacity, typically under relaxed selection.
Gene flow: movement of alleles from one population to another.
Genetic drift: changes in allele frequency due to stochastic effects in finite populations.
Genomic architecture: genetic structure of the genome underlying traits.
Hill–Robertson effect: interference between selection at linked loci.
Hybrid speciation: hybridisation between two species that produces offspring which are reproductively isolated from the parent species.
Intrinsic isolation: RI independent of environmental effects.

Table 1. ES and Studies Using Natural Populations Are Highly Complementary[a]

Laboratory-based ES	Comparative methods with natural populations
Rare (but important) serendipitous events are likely to be missed unless the experiment is large	**Better represents the importance of a given process, rather than just its occurrence**
Starting population characteristics and genome are defined or quantified *a priori* by the researcher	In most cases, it is challenging to reconstruct ancestral populations and their genomes
Typically reliant upon standing variation alone	**Greater potential for *de novo* mutation or introgression from other populations to play a role**
Environment is controlled and can be kept constant or manipulated in a controlled manner, throughout	Often difficult to determine ancestral environment required to delineate the role of geography in restricting gene flow
Many initial effects may be due to laboratory adaptation. If laboratory adaptation has occurred pre-EE, genetic diversity will be lower	**Populations are typically close to equilibrium in the wild**
Evolutionary responses are replicated over a series of lines to robustly link conditions to responses	No true replication. Lack of parallelism may create uncertainty that a phenotypic change is a direct response to a given variable
Evolution of traits is limited to what can be performed in culture conditions. Low niche dimensionality means only simple contrasts can be made	**A much wider range of traits can be selected upon or arise**
Gene flow can be more accurately and reliably determined from highly controlled migration levels, and measures of local adaptation and RI	Difficult to determine level of ongoing gene flow
Limited to a subset of organisms suitable for EE	**Can study any diverging populations**
Easy to separate intrinsic and extrinsic forms of RI	Difficult to disentangle intrinsic from extrinsic RI
Laboratory settings may exclude many of the ecological aspects that separate species	**Can assess the full range of isolating mechanisms found in the wild**
Phenotypic and genomic data can be collected with high temporal resolution providing estimates of phenotypic change and evolutionary hindsight of underlying genomic changes	Even if ancestral genomes can be reconstructed, phenotype data is typically only a single snapshot, so cannot be matched to genomic data
Experiments can only cover short timescales and subsets of the speciation process	**Long timescales of divergence can be studied (although histories must be inferred)**

[a]Several advantages (bold) and limitations of each approach have been matched to illustrate their complementary nature.

Linkage disequilibrium: nonrandom association between alleles at different loci (whether physically linked or not).
Local adaptation: adaptation in response to selection that varies between environments.
Matching traits: mechanism of assortative mating in which individuals find mates based on communal traits or alleles.
Multifarious selection: selection on multiple environmental axes.
Multiple-effect trait: trait that contributes to more than one component of RI.
Preference/trait: mechanism of assortative mating in which both signalling trait and preference for it must diverge between populations.
Reinforcement: adaptive strengthening of prezygotic RI due to selection against hybrids (when hybrids have non-zero fitness), in a zone of secondary contact.
Secondary contact: reintroduction of two or more populations' ranges after a period of geographic isolation.
Snowball effect: greater than linear increase in RI with time because genetic incompatibilities between populations lead to reduced gene flow, further divergence and ever-greater numbers of incompatibilities.
Soft sweeps: reduction in the genomic variation of a region due to linkage with a previously neutral allele which becomes beneficial and increases in frequency.
Speciation: origin of distinct, reproductively isolated species.

The Relative Efficacy of Selection and Drift

To maintain differences between populations, barriers to gene flow must emerge and generate RI. Barriers can act at the prezygotic (premating and postmating, prezygotic) and/or postzygotic stage, and can be influenced by **extrinsic isolation** and/or **intrinsic isolation**. Initial ES studies found relatively strong support for divergent natural selection generating RI in **allopatry**, even on arbitrary traits with no clear link to an isolating mechanism [8]. However, under sympatric conditions, disruptive selection did not generally lead to RI, likely because many of the divergently selected traits had little relevance to fitness [8]. Since Fry's review, few ES studies have altered conditions for **local adaptation** and then tested for the evolution of RI. Most studies tested the role of sexual selection and sexual conflict in generating RI [12,13]. Fry found equivocal support for sexual selection generating RI [8]. Subsequent work on sexual selection and speciation continues to fail to find significant RI [14–17], even when manipulating genetic variation and population size to increase the likelihood of response [14] and assessing different RI barriers [15]. One species, *Drosophila melanogaster*, has been tested independently in two laboratories but only one study found RI [18,19]. Theory suggests that different components of sexual selection may interfere with the evolution of RI [20] and one ES study supports this interpretation. In *Drosophila*

pseudoobscura, experimental sexual selection drove divergence in female choice for divergent male courtship traits [21], which should generate assortative mating. However, males from the high sexual selection lines always outcompeted males from the enforced monogamy lines [16]. Overall, surprisingly, experimental sexual selection by itself does not seem to generate RI.

ES studies have tested the impact of either natural or sexual selection, but evolution of RI may require both and so their relative contribution should be studied [22,23]. No ES study has done this, although one study manipulated natural selection and then tested for RI that could have arisen via sexual selection [24]. Strong prezygotic RI was observed but it was independent of local adaptation. Additionally, no ES study has manipulated multiple axes of natural selection to test patterns of speciation under strong unidimensional versus **multifarious selection**, despite this being a long standing speciation question [25,26] (see 'How Can Selection Overcome Gene Flow?').

Genetic drift may generate RI but Fry found little ES evidence [8]. In the past 10 years, two further studies have manipulated population size to assess the contribution of drift. One study created 1000 bottlenecked, inbred 'founder' populations of *Drosophila yakuba*, and although weak RI was occasionally produced, extinction was overwhelmingly the most common outcome [27]. Furthermore, when population size constraints were lifted (**founder flush**), RI was diminished, suggesting that inbreeding effects, not drift alone, were responsible [27]. Another study used a bottleneck treatment combined with divergent selection, but found it did not affect RI [24]. Overall, ES studies indicate that drift is not a strong evolutionary force promoting speciation.

While generally studied separately, selection and drift interact in complex ways. Strong selection reduces effective population size, which can increase the role of drift. In turn, genetic drift may restrict genetic diversity, diminishing the effect of selection. Since Fry's review, one ES study has addressed the joint influence of selection and drift. Using an experimental niche shift to produce asymmetric strengths of selection and drift between ancestral and derived populations of the flour beetle, *Tribolium castaneum*, both premating and postzygotic RI evolved [28]. Due to strong selection and therefore reduced population size during the niche shift, RI likely arose via fixation of deleterious alleles as a consequence of drift. However, only one line of each of the ancestral and derived populations was generated and we found no other similar studies, limiting understanding of joint selection and drift effects.

Evolution of Different Types of Reproductive Barriers

Previous ES studies focused on premating barriers using patterns of assortative mating to measure RI [29]. Although this remains true for ES studies post-Fry [16,24,27,30–34], some have included postmating, prezygotic [18,32,35], and postzygotic [24,36–38] forms of RI. However, more ES studies comparing the speed of evolution, the traits targeted, and relative magnitude of extrinsic and intrinsic RI are necessary to understand mechanisms by which RI evolves. Fry [8] suggested that ES has been underutilised to test the origin of **Dobzhansky–Muller incompatibilities (DMIs)** [39,40]. Some recent ES studies, where postzygotic RI has been identified, have used analyses such as microarray-based mapping to identify candidate DMIs [41–43]. However, characterising DMIs and distinguishing these from signatures of extrinsic postzygotic RI (e.g., low hybrid fitness in a given environment) requires additional experiments, including exploring the consequences of DMIs segregating within a population via synthetic engineering [44].

Feasibility of Speciation-with-Gene-Flow

Testing for speciation under sympatric and parapatric conditions was frequent in earlier ES studies [8,29], and strongly contributed to understanding the importance of **multiple-effect traits** [45] in overcoming gene flow [8,29]. While early ES efforts showed conditions for

speciation-with-gene-flow, Fry noted models of speciation-with-gene-flow as a neglected area [8]. Over the past decade, a fundamental shift in speciation research is the acceptance that gene flow frequently occurs at some point before the completion of RI [2,39] but ES studies incorporating varying levels of gene flow have not been published in the intervening years. Gene flow in the context of **hybrid speciation** has been tested recently using ES, expanding upon similar work in yeast species [46]. The number of hybridising *Drosophila* species, and their genetic divergence, affected RI between parental and hybrid lineages. Higher RI occurred when hybrids were derived from three, rather than two species, and when parental species had intermediate levels of divergence [34].

Feasibility of Reinforcement

Gene flow during cases of secondary contact after initial divergence in allopatry can generate reinforcement. While initially controversial, evidence for reinforcement has accumulated [47–49]. Previous ES reinforcement studies were "**destroy all the hybrids**" experiments [8] which removed all gene flow between populations and thus tested for increasing isolation between already reproductively isolated species. Post-Fry, Matute addressed this criticism and manipulated amounts of migration and hybridisation (and therefore effective gene flow) between sister species of *Drosophila* [32,33]. He found premating and postmating prezygotic isolation increased but only when the numbers of migrants were low and selection against hybrids strong. Reinforcement between nascent species could also have indirect effects that generate RI between conspecific populations, known as **cascading reinforcement**. Using ES, conditions for cascading reinforcement were demonstrated in *Drosophila* (using a "destroy all the hybrids" approach [35]). Although these ES studies demonstrate that reinforcement can occur, the mechanism by which reinforcement is generated has yet to be explored; linkage of genes for local adaptation with those for assortative mating [50], or via multiple-effect traits conferring local adaptation and assortative mating through pleiotropy [51]. No study has examined the genomics of ES reinforcement, which could test how **linkage disequilibrium** is generated.

Coevolution

Antagonistic coevolution between species (e.g., hosts and parasites) can potentially drive RI [52] but Fry did not mention any ES study examining this process. Subsequently the use of EE for testing coevolution has been emphasised, but outside of the speciation context [53]. We identified one ES study that found higher postmating RI between *T. castaneum* populations that had coevolved with the parasite *Nosema whitei* than between the nonparasitised controls [38]. Another ES study tested populations of *D. melanogaster* adapting to different diets in the presence of commensal organisms that may generate RI, and found premating isolation evolved in as little as one generation [30,31]. RI was attributed to the mere presence of different microbiota and did not vary significantly over time, thus it is difficult to conclude these effects were evolutionary, rather than plastic. Attempts to replicate these results have been mixed [54,55]. Overall, despite coevolution being a potential powerful driver of speciation, ES studies have not tested this.

That Was Then, This Is Now

ES continues to be underutilised even after Fry's promotion of its use. We provide ideas for future research drawing on his suggestions. Perhaps more importantly, since Fry's review, two major developments in speciation research have occurred for which ES is highly suited but for which ES has lagged behind. First, speciation-with-gene-flow is now thought to be a dominant mode of speciation, but ES studies have manipulated gene flow in only very specific conditions: hybrid speciation and reinforcement. Second, Fry's review [8] was published on the cusp of the genomic revolution. Subsequent EE studies addressing other evolutionary problems have adopted genome sequencing, including **evolve and resequence (E&R)** [56,57] which allows tracking of

genetic changes during evolution, revolutionising EE studies [58]. However, we found surprisingly few new ES studies testing for RI and none incorporated tests of speciation theory using genomics. Given the importance of gene flow during speciation, ES design should include this, as expanded upon in Boxes 2 and 3, and genomic approaches must be used to test fundamental and

Box 2. Importance of Gene Flow in Experimental Speciation Genomics

As barrier loci can only be detected when populations are or have recently been exchanging genes [1], the degree of gene flow between diverging populations in an experimental speciation study using E&R is crucial for genomic analysis (Figure I). Without gene flow (divergence in allopatry), **soft sweeps** are predicted to produce large blocks of genomic differentiation around differentially selected alleles. This makes barrier loci hard to pinpoint, a problem which is likely to be particularly pronounced since experimental speciation studies must often use much stronger selection than would be found in nature to generate reproductive isolation within the experimental timeframe. Furthermore, experimental populations are more susceptible to the effects of drift due to their typically small population size. Without gene flow, large genomic regions may drift to differentiation.

As such, gene flow is necessary to detect barrier loci, as it homogenises background genomes, counteracts the effects of selective sweeps and drift, and allows regions of differentiation to be identified. However, too much gene flow will swamp selection and obstruct population divergence. Guidelines on the design of E&R studies focus heavily on detecting signatures of selection in allopatric populations [56,57,94]. When designing future E&R speciation experiments, it will be important to consider these in the context of gene flow, distinguishing the detection of regions under selection from that of barrier regions.

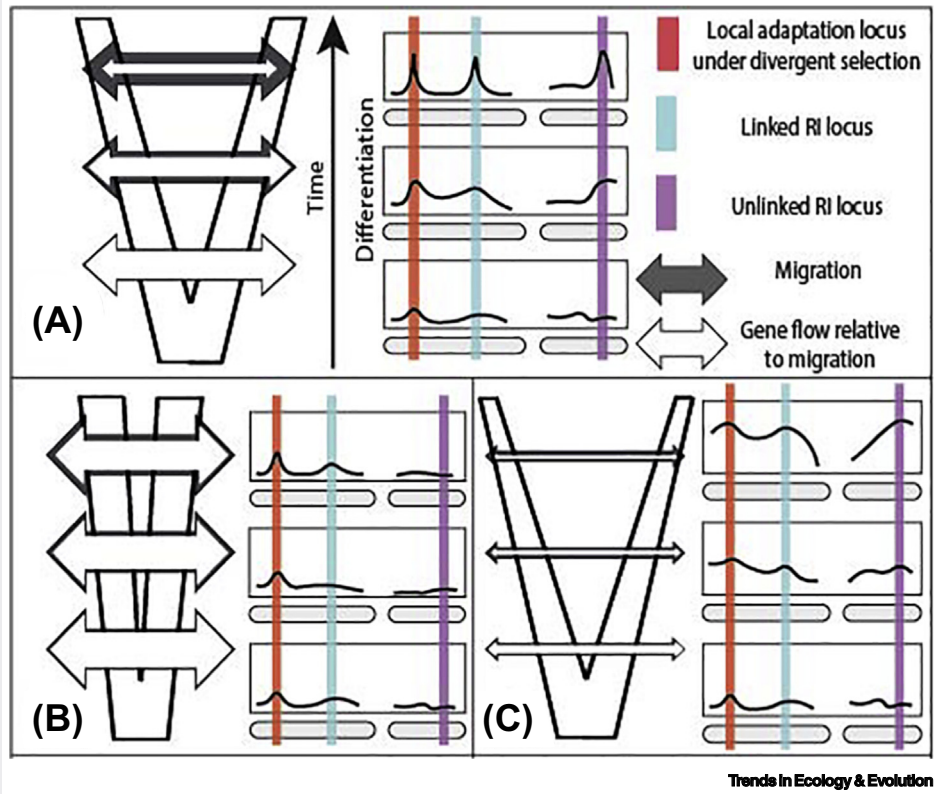

Figure I. Three Hypothetical Illustrations of the Consequences Stemming from Different Levels of Gene Flow, as a Guide to Considering the Consequences of Experimental Population Size and Migration Levels. (A) Populations have diverged, gene flow relative to migration has decreased substantially with time, and the genomic signatures of all three barrier loci are clear, allowing identification of markers and further investigation. (B) Gene flow is problematically high, selection struggles to overcome gene flow and there is little phenotypic or genomic divergence. (C) Populations with low levels of gene flow have diverged in near-allopatry, but the identification of barrier loci is difficult because populations have lost genetic variation and the background genomes are strongly differentiated due to drift and linkage. Adapted, with permission, from [1]. Abbreviation: RI, reproductive isolation.

Box 3. Blueprint for Experimental Speciation Design.

Experimental speciation (ES), in combination with genomics, provides the ability to jointly infer phenotypic responses to, and genomic signals of, selection, and should be a high priority for speciation research. We present a blueprint for the design of future ES studies investigating the impact of a process or condition on the evolution of RI in the face of gene flow (Figure I). We particularly focus on gene flow and selection manipulations, and the use of E&R. In this design, the pair of populations serves as the unit of replication; all measures of divergence (e.g., RI, F_{ST}) describe the paired metapopulation. This differs from designs in which experimental lines radiate from a single ancestral population, which typically involve no gene flow. Demography and migration rate, and the strength of natural and/or sexual selection can be controlled or manipulated. Subsequent consequences on the initiation or elevation of RI can be estimated directly and assessed across different types of reproductive barriers. The time course nature of ES allows both phenotypes that contribute to local adaptation [95], assortative mating [55], or hybrid viability [24,36,37] to be assayed from the outset. By using E&R, effective gene flow and consequences for genomic architecture can be determined.

By archiving populations throughout the experiment, a researcher can build a valuable cache of DNA data that can be analysed post-E&R with evolutionary hindsight. Having identified candidate barrier loci, the trajectory of allele frequencies of these selected loci can then be examined in detail across the course of the experiment by targeted sequencing of archived populations at selected time points. This can pinpoint how and when changes relating to RI arise and spread in populations. E&R is a potent way to identify genetic signatures of RI but the power to detect these signatures is affected by demography (population size and number of founding haplotypes), strength of selection, and number of replicate populations (as is the success of ES generally) [56,57,94,96]. While these constraints need to be kept in mind, so should the limitations of detecting signatures of selection in non-ES speciation studies [9–11].

Furthermore, if individuals can be "resurrected" (e.g., yeast, rotifers, and *Daphnia*), a suite of genomic, metabolomic, transcriptomic, or fitness-related assays could be performed post-EE at time points of interest. Replication within each treatment tests for parallel evolution and identifies strong (consistent) candidate barrier loci arising due to selection. Replicates responding similarly allows distinguishing a selective response from other evolutionary processes such as mutation and drift, the latter of which are predicted to affect replicates differently.

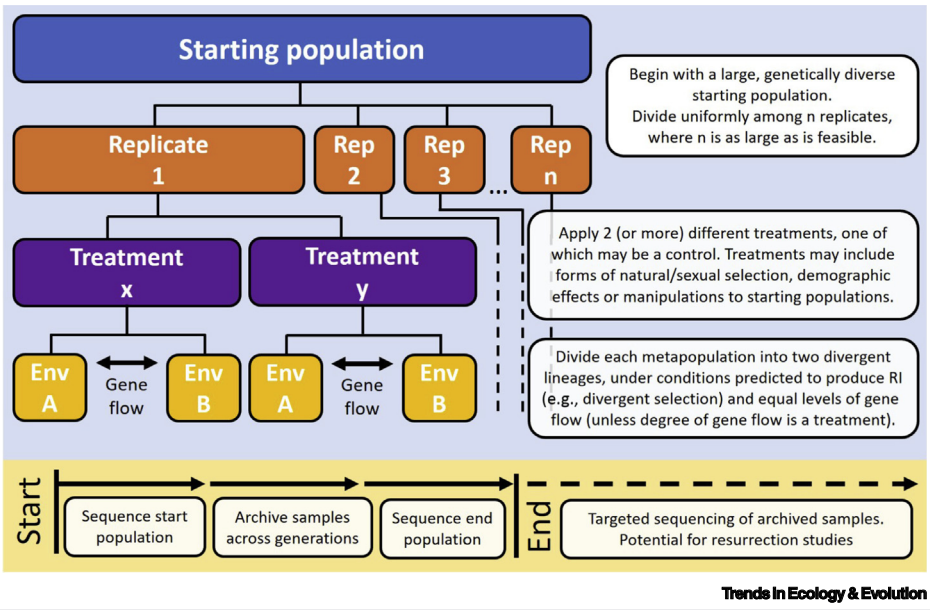

Figure I. Blueprint for ES Experimental Setup. Abbreviations: Env, environment; Rep, replicate; RI, reproductive isolation.

increasingly sophisticated speciation genomics theory (Box 1). This combination will dramatically increase the ability to directly test how RI is either initiated between individuals within a population or intensified between partially reproductively isolated populations and help fulfil the promise of ES as a powerful approach to understanding speciation. To facilitate this aim, we highlight how ES

combined with genomics can address speciation research developments in the past 10 years. Our list, below, is not exhaustive but is designed to inspire and stimulate ES speciation research.

A Selection of New Challenges That Experimental Speciation Can Address

What Genomic Conditions Promote Speciation?

Variation in mutation rate, recombination rate, and gene density, are all predicted to impact progression towards RI [1,3,11]. These genome properties can only be assessed *post hoc* in natural populations, making it difficult to disentangle current genome properties as causes or consequences of the speciation process. For instance, suppressed recombination among genes inside chromosomal inversions can generate the linkage disequilibrium required for promoting divergence and speciation. In many species, inversions have been found containing genes important for speciation. However, in natural populations it is difficult to infer whether an ancestral inversion containing **barrier loci** facilitated speciation or arose after several loci were already in linkage disequilibrium. Furthermore, these properties can shape the genomic landscape independently of the evolution of RI, complicating the identification of barrier loci [1,7]. In an ES context, these genomic features can be characterised prior to applying EE and their behaviour tracked across time via E&R. Moreover, manipulating genomic properties of starting populations is possible, allowing direct tests of their effects on the evolution of RI in the absence of confounding differences.

Taking recombination rate as an example, low recombination increases linkage around a barrier locus. Clusters of barrier loci are more likely to evolve in low recombination regions, potentially but not necessarily producing **coupling** [59]. Reduced recombination regions could therefore evolve because they enhance clustering [60]. For example, inversions that reduce recombination between barrier loci are expected to be promoted by divergent selection in the face of gene flow [61]. Conversely, high recombination can counteract the **Hill–Robertson effect**, increasing the likelihood of bringing together otherwise competing beneficial alleles in a single individual. So high recombination might speed up local adaptation and divergence during speciation, but could also slow the build-up of RI by uncoupling barrier loci in the genome. The overall effect of recombination rate on RI could be examined by experimentally evolving populations with different patterns of genome-wide recombination rates, known to vary between populations [62,63], using genetic mapping to show the differences between populations. If a facultatively sexually reproducing organism is used, then manipulations in recombination rate could be achieved by varying the proportion of time during selection spent in the asexual and sexual phases [64]. Alternatively, artificially created inversions via CRISPR/Cas9 [65] might be propagated within a population to explore their effects. We use recombination rate as an example, but these approaches could be applied similarly to genomic features such as mutation rate, gene density, or genetic diversity.

How Does Gene Flow Impact Speciation?

Gene flow is thought to be involved in most cases of speciation at some point before completion of RI [39]. However, its role in both opposing and facilitating speciation is theoretically complex. Gene flow has similar consequences to speciation as recombination. Gene flow opposes divergence under selection, but also makes recombination possible between gene combinations in diverging populations. The latter can promote local adaptation and potentially rescue diverging populations with small founding sizes [66]. Gene flow also impacts the landscape of genomic divergence. In the presence of gene flow and recombination, strength of selection and linkage are expected to influence the establishment of barrier loci, and are predicted to lead to clustered genetic architecture [67]. In natural populations, correctly inferring gene flow is challenging given uncertainty about demographic history. For instance, modern-day genomic patterns may be due to past gene flow, varying recombination rates, and/or bottlenecks [10].

In contrast, using ES allows gene flow to be either controlled or manipulated throughout an experiment and this can be confirmed directly via sequencing. Gene flow can be manipulated, singly or in combination with other factors of interest, to test conditions under which speciation-with-gene-flow is feasible. Moreover, the phenotypic and genomic patterns produced are directly determined and can then be applied to understanding these patterns in natural systems.

Experiments manipulating the amount of gene flow, with and without recombination, can be done by varying the proportion of migrants between diverging populations at the start of each generation. This would allow testing predictions about how gene flow might oppose RI but facilitate local adaptation, and about the predicted clustering of loci within the genome. For instance, Fry emphasised speciation-with-gene-flow in certain conditions (e.g., finite stepping stone [68,69], or Bush's sympatric speciation model [70]) that have not yet been tested. This basic setup could be expanded to include how sexual selection impacts speciation-with-gene-flow, to test how it may either enhance or impede the evolution of RI depending on factors such as geography, and mechanisms of assortative mating [71].

ES is probably best placed to examine the role of gene flow early in speciation. However, it could also be used to test two hypotheses for more divergent populations: reinforcement and the Genome Wide Congealing hypothesis [72]. ES has demonstrated reinforcement but how linkage disequilibrium is generated to promote reinforcement remains unresolved. Sequencing starting populations, identifying markers for barrier loci, and then employing targeted sequencing of the markers on archived ES samples allows reconstruction of the **genomic architecture** of populations as reinforcement occurs, testing mechanisms of linkage. This approach also addresses the importance of tight linkage between loci and the likelihood of speciation depending on the basis of assortative mating [73]. Speciation-with-gene-flow is theorised to be more feasible when assortment results from **matching traits**, whereas assortative mating arising from **preference/trait** mechanisms requires maintenance of linkage disequilibrium between a larger set of loci, thereby decreasing its likelihood in the face of gene flow.

The Genome Wide Congealing hypothesis posits a tipping point of linkage disequilibrium and adaptive divergence. Crossing this threshold transitions from a number of weakly selected barrier loci accumulating between diverging populations, to RI at specific genes, to a switch of RI across the whole genome [72]. Whether this threshold exists, and at what point during speciation this theoretical tipping point is reached, depends on how many loci are targets of selection, how strong selection on each locus is, and the genome-wide recombination landscape. ES could empirically test the impact of these factors by taking divergently adapted but not very isolated populations and then manipulating conditions and/or genome properties to test for a tipping point from weak to strong RI.

How Can Selection Overcome Gene Flow?

Fry reviewed ES studies testing whether selection on multiple-effect traits could overcome gene flow to generate RI [8,74,75]. However, many other facets of selection remain unexplored which, while being relatively minor in allopatry, can have major consequences in the presence of gene flow. One example is the **dimensionality** of selection – how are the components of RI affected by whether a finite quantity of selection is spread over many, or concentrated onto few, traits and/or loci? To what extent is speciation promoted when selection is strong on a single trait compared to multifarious selection? Strong divergent selection, concentrated on a single trait, may overcome gene flow more successfully, leading to greater and more rapid local adaptation, but with lower effects overall on RI and genomic differentiation. In contrast, multifarious selection

may accelerate the build-up of RI [8,25,26] by impacting linked barrier loci, impacting multiple-effect traits, or producing a **snowball effect** [40] of DMIs [76–79]. If selection is spread too thinly across many dimensions, however, then it may fail to overcome gene flow [80]. Amount of gene flow is also critical in whether uni- versus multidimensional selection facilitates complete speciation [25]. No ES study has addressed this speciation theory. While not suggested in an ES framework, Figure 3 of Nosil *et al.* [25] provides an excellent guide for ES researchers for testing the contribution of uni- and multidimensional selection on the evolution of local adaptation and RI.

Concluding Remarks

Despite early ES success, the approach has lain relatively fallow in addressing unresolved speciation questions (see Outstanding Questions; note that these are general issues in speciation research which remain general because conventional speciation studies are challenged to answer them). This is particularly true when incorporating genomic techniques. It is the combination of ES with high-throughput genomics that can provide a step-change in understanding the origin of RI by directly testing competing hypotheses on processes suggested to impact speciation. Such tests are challenging in natural populations. While ES is typically used to reveal the evolution of early RI, its use on partially reproductively isolated taxa can test how existing patterns of RI and the underlying genomic architecture impacts progression to more complete speciation. ES combined with E&R can both disentangle and test confounding demographic and genetic processes, and elucidate the conditions under which speciation is impeded or accelerated. As it is these signals that get erased or overwritten during the speciation process in natural populations, such experimental insights can be used to help interpret patterns of divergence in natural populations whose selection and demographic history are unknown. In this way, ES, while perhaps oversimplifying real-world conditions, is a powerful tool complementing forward (static) speciation studies. As ES studies accumulate, questions about the role of certain types of genes and other types of phenotypic variation, such as gene expression, in speciation can be addressed. All experiments risk failure but given how time consuming ES is, researchers may be hesitant to adopt this approach for fear RI will not be generated. Rare events can still be important [81], so modelling approaches enabling the testing of many more variables over many more replicates than feasible experimentally would be a helpful complement to ES. Furthermore, an additional benefit of taking on the ES challenge is that, even if RI does not evolve, the approach can address other fundamental questions (e.g., how gene flow and recombination impacts the genomic architecture of local adaptation), themselves outstanding evolutionary problems. Unlike our update of ES in the past decade, we anticipate that the next decadal ES review will attest to the power of this approach and its application in interpreting divergence in natural populations.

Outstanding Questions

How does the interaction between different sources of selection impact on the build-up of RI? What role does the dimensionality of selection play in generating divergence? Does the interaction between sexual and natural selection impact on the outcome of speciation? How do drift and selection interact to affect the speciation process?

What demographic and environmental effects might aid selection in overcoming gene flow? Can small amounts of gene flow accelerate speciation, through reinforcement or by augmenting genetic variation (i.e., hybrid speciation)? Can coevolution drive speciation-with-gene-flow? How might sexual selection impact speciation-with-gene-flow?

What genomic conditions facilitate or impede speciation? How do areas of suppressed recombination, such as inversions, contribute to RI? How does recombination affect the build-up of RI in the presence of gene flow? Are barrier regions typically gene rich or gene poor? Is there a tipping point of linkage disequilibrium and adaptive divergence moving from RI at specific barrier loci to RI across the whole genome, and if so, how does this change depending on how many loci are targets of selection, how strong selection on each locus is, and the landscape of recombination across the genome?

Are there general patterns of speciation we can test with experimental speciation? Are there optimal levels of standing genetic variation for speciation to occur? Do we see the same results when comparing different populations, subspecies or species?

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