

Review

Diverse pathways to speciation revealed by marine snails

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Speciation is a key evolutionary process that is not yet fully understood. Combining population genomic and ecological data from multiple diverging pairs of marine snails (*Littorina*) supports the search for speciation mechanisms. Placing pairs on a one-dimensional speciation continuum, from undifferentiated populations to species, obscured the complexity of speciation. Adding multiple axes helped to describe either speciation routes or reproductive isolation in the snails. Divergent ecological selection repeatedly generated barriers between ecotypes, but appeared less important in completing speciation while genetic incompatibilities played a key role. Chromosomal inversions contributed to genomic barriers, but with variable impact. A multidimensional (hypercube) approach supported framing of questions and identification of knowledge gaps and can be useful to understand speciation in many other systems.

Ecotype formation and speciation

Adopting the Biological Species Concept [1], speciation is a process progressing by the accumulation of **barriers** (see [Glossary](#)) to gene flow [2], until a point where it is no longer reversible [3]. Studying this process essentially amounts to investigating what types of barriers to gene flow are established between pairs of diverging populations, how they establish and in what order. Since it is normally not possible to observe the transition from panmixis to complete speciation, a common approach is to instead place pairs of populations along a one-dimensional **speciation continuum** as a framework for comparing the barriers that separate them [3–6]. The increased use of genomic approaches has boosted such studies [7], applied to population pairs representing related taxa (e.g., *Drosophila* [8], three-spine stickleback [9], stick insects [10]), or spanning multiple groups of organisms [5,11]. The main mechanisms supporting the movement of population pairs along this continuum are divergent selection [12,13], the **mutation-order** mechanism [14], and potentially genetic drift although it can also oppose divergence [15]. **Ecotypes** within a species are formed by divergent selection that generates barriers to gene flow, and this can be seen as an early step in the speciation process [12,16]. However, it is unclear how often ecotypes evolve into separate species, for example, by initial genomic barriers being reinforced by **coupling** processes [17,18].

The marine snail genus *Littorina* has recently emerged as a model system for speciation studies (Box 1) [19]. It contains pairs of taxa overlapping in geographic distribution but with various degrees of **reproductive isolation**; from ecotypes to strongly, but not completely, isolated nominal species. Some pairs have evolved repeatedly and all pairs form **contact zones** in different geographic and ecological contexts [20,21]. Studies of natural and sexual selection, genetic clines, and the **genomic architecture** of these contact zones have generated data describing divergent selection [22,23] and barriers to gene flow [24–27]. Such data have also supported mathematical models of divergence and speciation [24,28,29]. Now, we combine data from

Highlights

Marine snails (genus *Littorina*) have recently emerged as a model for speciation research, with repeated formation of different types of barriers to gene flow in contact zones between ecotypes and taxa.

Inversion polymorphisms are found in an increasing number of species, including *Littorina* snails, and have been suggested to promote both local adaptation, ecotype formation and speciation, but their roles can vary. In *Littorina*, their role in later stages of speciation remains particularly unclear.

A one-dimensional speciation continuum is frequently used to frame our understanding of the speciation process. However, a number of recent studies have argued that no single measure adequately summarises reproductive isolation and, therefore, the speciation process is best viewed in multiple dimensions. The many *Littorina* contact zones provide empirical data to illustrate how this principle can be applied.

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multiple population pairs to improve our understanding of speciation in *Littorina*, asking: which barriers to gene flow are essential to increase reproductive isolation, what are the roles of spatial isolation and divergent selection, and is ecotype formation a first step in speciation? Our synthesis shows that speciation is more complex than what can be represented with a one-dimensional continuum. A multidimensional approach was more useful and we believe it can inspire studies of many other groups of organisms, answering questions about routes and barriers of speciation, and not least identify knowledge gaps for further research.

The nature of the speciation continuum

The conceptual idea of a speciation continuum has been used to indicate how close to, or far from completion of speciation is a pair of diverging taxa [5,7,10,30,31]. Some authors consider that a one-dimensional continuum adequately represents the degree of speciation [32]. For example, Stankowski and Ravinet [3] suggested that the speciation continuum represents an axis from weak to strong reproductive isolation. Reproductive isolation is, however, not easily measured or compared among taxa [33], and how it should be defined is still a topic of debate [34]. Others have acknowledged that speciation can progress along multiple paths and be measured by different aspects of divergence [35,36]. These aspects include phenotypic, ecological, and genetic differentiation [35], but also a multitude of other measures, such as time since divergence, level of **genetic incompatibility**, fraction of the genome involved in barriers, segregation of **inversion** arrangements that hinder gene flow [37,38], and extent of gene flow over contact zones [33,36]. This complexity suggests representing the positions of population pairs in a

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Box 1. Marine snails are useful models in speciation biology

Species of *Littorina* (Table 1) are tractable targets for research on adaptation, ecotype formation, parallel evolution, and speciation [19,21,39,94,95]. These marine snails are distributed between low- and high-tide areas in a heterogeneous environment with steep physical and biotic gradients (Figure 1). They all have low adult dispersal, a roughly year-long generation time, and extensive reproductive periods. *L. fabalis* and *L. obtusata* dwell on the fucoid seaweeds, while *L. saxatilis* and *L. arcana* are rock dwellers [66]. Ecologically relevant environmental variables can be assessed in the field or simulated in the laboratory [73–75,96]. Reproduction varies from planktonic larvae (not in species included in this study), to direct development with benthic egg masses or brooding [66]. Tagging, tethering, or transplantation can be used to assess phenotype-associated survival and habitat-linked selection [22,23,74,75]. Reproductive systems are simple with separate sexes, no self-fertilisation, and obligate sexual reproduction. Most direct-developing *Littorina* species form ecotypes [39,66] with hybrids produced in contact between ecotypes [20,27,29,57]. Such contact zones are ideal contexts for assessing barriers and processes involved in reproductive isolation and speciation [97,98]. In the snails, these contact zones are replicated at various levels of population independence, from zones in the same geographic area where populations share the same gene pool [25], to replicate zones between similar ecotypes in different geographic regions [41].

Phylum Mollusca is the second most species-rich animal phylum but only around 50 species have an assembled genome [99] and the annotation level is generally poor compared to crustaceans (incl. insects) and vertebrates. A genetic map and a first, highly fragmented draft genome of *L. saxatilis* [29] boosted investigations of gene flow barriers between ecotypes and sister species and revealed multiple polymorphic chromosomal inversions [49]. A new, chromosome-level reference genome [100] places 90% of the genome on 17 superscaffolds, and will support much more detailed characterisation of genomic regions involved in ecotype formation and speciation, including inversions and their break-points.

Table 1. Basic life-history and genome characteristics of *Littorina*

Genome size	1.2 Gb
Number of chromosomes	17
Generation time	0.5–1 yr
Effective population size (N_e) estimated from demographic inference	9000–12 000 (<i>L. saxatilis</i> Sweden) [48] 26 000–37 000 (<i>L. saxatilis</i> Spain) [48] 4900–5200 (<i>L. fabalis</i> Sweden) [58]
Current number of identified putative inversions	22 (<i>L. saxatilis</i>) [25,49,83,101] 12 (<i>L. fabalis</i>) [58]
Sex chromosomes	No, but sex-determining genomic regions [101,102]

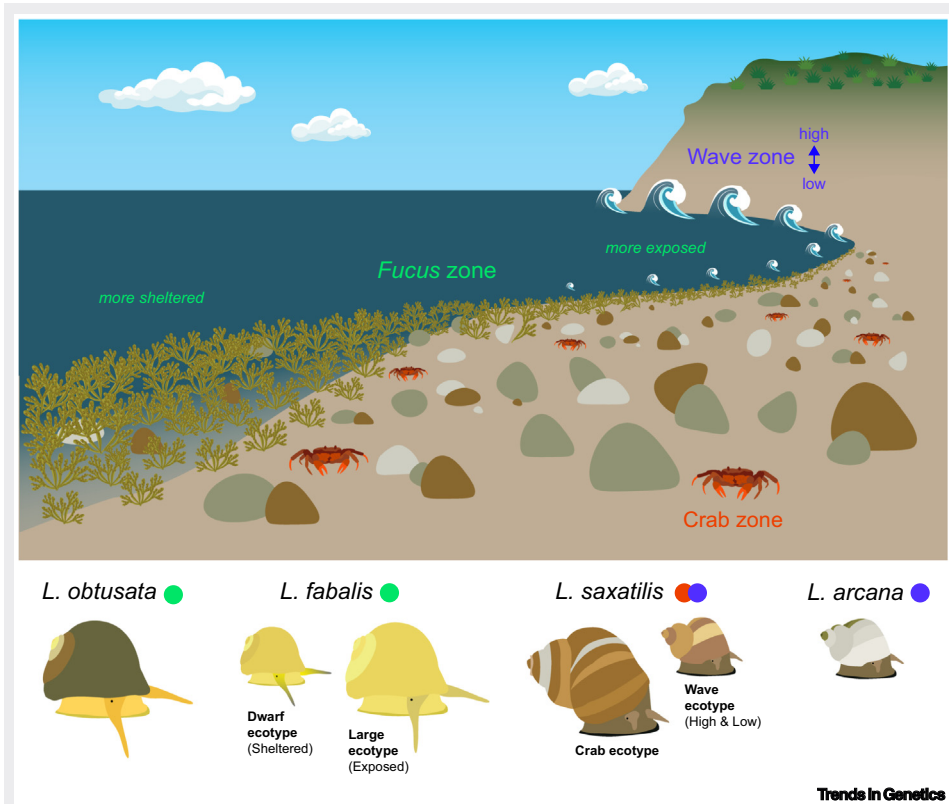


Figure 1. Schematic distribution of *Littorina* pairs on a rocky shore. The example is a NW European shore although similar environmental heterogeneity and formation of contact zones are also found along the Atlantic coast of the Iberian Peninsula. In tidal areas, the Crab and Wave *L. saxatilis* ecotypes have a vertical distribution (see [Figure 3](#) in main text), and in atidal areas (e.g., Sweden) *L. arcana* is absent. In NW Europe, *L. obtusata* overlaps with the dwarf ecotype of *L. fabalis* in more sheltered parts of the *Fucus* seaweed zone, while in Iberia the presence of the two *L. fabalis* ecotypes has not yet been investigated, and other ecotypes may be present. The coloured circles near the species names match the colours of the habitats in the main image.

multidimensional space rather than on a one-dimensional continuum. Multiple dimensions can be illustrated using a **speciation (hyper)cube**.

Two different cubes have been designed previously, defined either by axes that emphasise routes towards speciation [35] or by axes describing different aspects of divergence [36] ([Figure 1A,B](#)). We propose another, not mutually exclusive possibility; a barrier cube ([Figure 1C](#)). Because there is no single definition of reproductive isolation that comprehensively describes the reduction of interbreeding and gene flow [33], a barrier cube can be used to reflect alternative useful measures. For example, an organismal view of reproductive isolation [33], focused on the factors that prevent successful interbreeding, leads to the measurement of different components of isolation such as **immigrant inviability**, assortative mating or hybrid sterility. These components can reasonably be combined into a single measure and an axis representing organismal barriers. This axis can, however, be different from an axis describing the reduction of gene flow at unlinked neutral loci, which is one genetic measure of reproductive isolation [33]. However, this genetic measure does not capture all aspects of barriers to gene flow [34]; for example, it does not encapsulate the proportion of the genome experiencing a barrier or the reduction in gene flow

Glossary

Allozyme genes: enzyme coding genes used in early population genetic studies to detect genetic variation.

Barrier: anything that reduces gene flow between populations either across the genome (e.g., spatial or physical barriers) or in specific parts of the genome (e.g., barriers that reduce interbreeding or fitness of hybrids) ([Box 2](#)).

Collinear loci: loci in the genome outside inversions.

Contact zone: an area where two genetically distinct populations are in contact and potentially hybridise.

Coupling of barriers: joint action of barriers, often with linkage disequilibrium between underlying loci, that will usually strengthen the overall barrier to gene flow.

Demographic analysis: here referring to DNA sequence-based estimation of parameters describing variation in effective population size over time, gene flow between populations, and periods of isolation.

Ecotype: a phenotypically and genetically distinct form of a species that is adapted to a particular habitat.

Genetic incompatibility: negative interactions between alleles of the same, or of different loci, usually evolved under isolation but appearing together in the same individual following hybridisation.

Genomic architecture: the effect sizes of genes influencing a trait or barrier, their number, and their spatial organisation along the genome, including their presence in chromosomal rearrangements, such as inversions.

Immigrant inviability: the lower viability of an immigrant, relative to individuals of a resident population, due to adaptation to spatial environmental variation.

Inversion: sequence of the DNA molecule that has been reversed in direction due to a chromosomal mutation. Rare recombination takes place in individuals carrying one copy of each arrangement by double cross-over or gene conversion (gene flux).

Mutation-order mechanism: independent fixation of different mutations in isolated populations while adapting to similar fitness peaks, generating incompatible allele combinations that cause genetic barriers following secondary contact.

Reproductive isolation: reduction in successful interbreeding between populations or the resulting reduction in

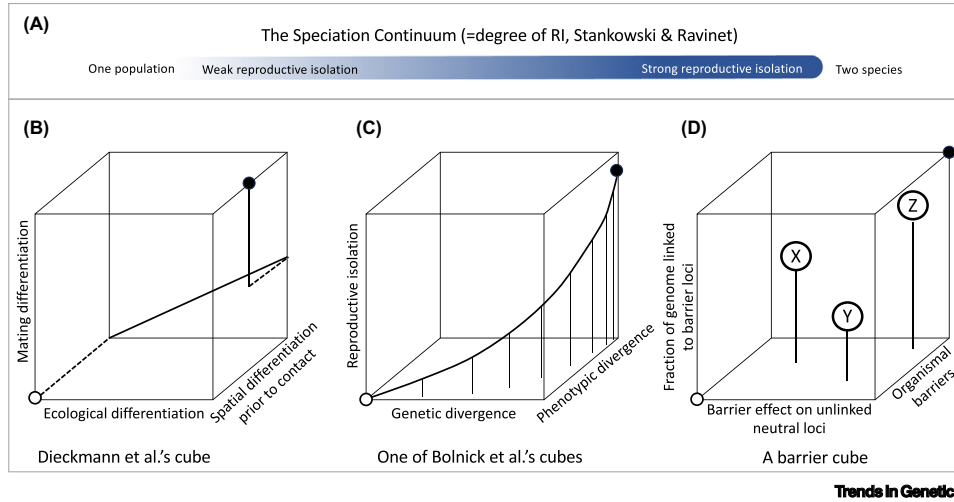


Figure 1. Different conceptual ways to illustrate speciation. (A) The one-dimensional speciation continuum illustrating increasing levels of reproductive isolation between pairs of populations from left to right. In its plain form, this does not make any predictions about the directionality of divergence and/or speciation processes over time (i.e., reproductive isolation may remain constant, decrease or increase), and simply illustrates variation in the degree of reproductive isolation [3]. (B) Different pathways involved in speciation illustrated as a ‘speciation cube’ which combines axes of organismal differentiation, ecological divergence, and spatial isolation [35]. In this example, a possible route of speciation is initial spatial isolation followed by accumulation of ecological and mating differences. Next, secondary contact reinforces assortative mating, and finally completes speciation. (C) One of many alternative multidimensional cubes [36] with different axes illustrating various components of speciation that can (ideally) be measured across many taxa. (D) A cube inspired by Westram *et al.* [34] with axes indicating measurable aspects of reproductive isolation. Inside the cube, a given population pair might have very different values on the barrier axes. For example, pair Y has a strong barrier in unlinked neutral loci but a weak organismal barrier, while the opposite occurs in pair X. In pairs close to completion of speciation (e.g., Z), the different barrier measurements will tend to converge towards their upper limits. Open circles indicate the start of speciation and black circles are where speciation is completed. Illustrations B and C are modified from [35,36], respectively.

gene flow. Reproductive isolation can be conceptualised and measured in different ways.

Secondary contact: contact and potentially hybridisation between two populations following a period of geographic isolation.

Speciation continuum: traditionally, a conceptual model placing pairs of populations on a single axis from early stages of divergence to completed speciation.

Speciation (hyper)cube: a conceptual model to illustrate a multidimensional speciation continuum by describing different routes to speciation, ways of assessing divergence or aspects of barriers to gene flow on different axes.

Standing genetic variation: polymorphisms at different levels of genome organisation (e.g., SNPs, alleles of the same gene, inversion arrangements) from which selection can fish out alleles that are favourable in a new habitat.

at barrier loci themselves, which might need additional axes. For these reasons, we argue that multiple axes are needed for a full description of reproductive isolation (Box 2). Here, we make use of knowledge accumulated in the snail study system to assess the utility of a multidimensional view in describing and understanding speciation. A largely open question related to this is, how, and to what extent, the different axes of a hypercube are related (see Outstanding questions).

Marine snails as models for studying adaptation and speciation

Intertidal snails of the genus *Littorina* offer opportunities for combining ecological and genomic studies over repeated contact zones formed between diverging taxa. These snails live in high densities in areas that are easy for us to access (shores) and are characterised by steep environmental gradients in temperature, desiccation risk, predation, and wave action. The heterogeneous environment promotes small-scale adaptation, resulting in ecological [39], physiological [40], and genetic differentiation [20], replicated both at a local geographic scale and over latitudinal gradients [25,27,41]. Furthermore, these stress-tolerant snails are tractable organisms for field manipulations, laboratory experiments and crossbreeding, and the first assembled genome and a genetic linkage map for *Littorina saxatilis* [29] have boosted studies of the genomic architecture of divergence (Box 1).

We start by placing the best studied pairs along a continuum of genetic differentiation (F_{ST} , which ranges from 0 to 1), because this is easily quantified (Figure 2, Key figure). We give details on the divergence of each of the six pairs and fill a matrix of other observations relevant to speciation, such as time since divergence, phenotypic distinctness at contact, and rate of occurrence of

Box 2. Axes of isolation

Reproductive isolation (RI) is fundamental to speciation, but there is no single definition of RI that comprehensively describes the reduction in gene flow and interbreeding relevant to the speciation process [33]. We focus on three axes that will not always be strongly correlated, and so help to illustrate the value of considering multiple dimensions: (i) the strength of organismal barriers (RI as described in [103,104]); (ii) the fraction of the genome that is linked to barrier loci (one way of reflecting the reduction in gene flow), and (iii) the barrier effect at neutral loci that are unlinked to barrier loci (RI as described in [33]). Each axis might be subdivided, or expressed differently, and other axes are possible.

Organismal barriers are underpinned by barrier phenotypes that reduce the production and/or viability of offspring in between-population crosses relative to within-population crosses. In the illustrated example (Figure 1A), two organismal barriers are underpinned by the difference in size. First, small and large snails are locally adapted to contrasting environments, with migrants showing reduced survival (and thus fitness) in the alternative habitat. Second, the phenotypic difference drives assortative mating, such that fewer matings occur between snails that differ more in size. The strength of each organismal barrier can be calculated using various formulae and combined into a total measure [103]. When barrier traits are caused by genetic differences, the organismal barrier causes a reduction in gene flow at barrier loci (in this case, at the loci that cause variation in size), illustrated below as a reduction in the effective migration rate between populations (Figure 1B). These barrier effects extend to linked loci, meaning that a fraction of the genome experiences local barriers. However, barrier loci also cause a genome-wide barrier effect that impacts unlinked neutral loci (shown for chromosome 2 in Figure 1B). In theory, this effect can be quantified for an unlinked neutral locus as the ratio of the effective migration rate (m_e) to the rate of migration in the absence of a reproductive barrier (m) (or $1-m_e/m$; [33]). In practice, however, such estimates can be difficult to obtain. Other non-genetic factors can cause a genome-wide barrier effect between populations (e.g., spatial separation or physical barriers to dispersal).

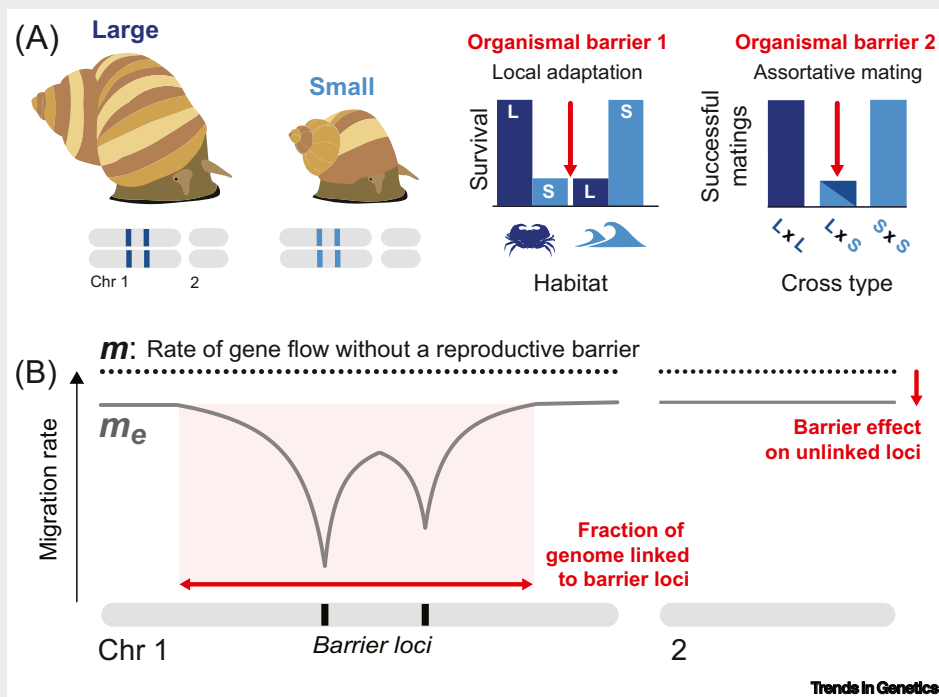
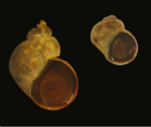

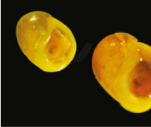

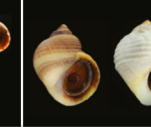
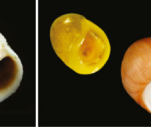


Figure 1. Relationships between barriers to gene flow. (A) Organismal barriers (local adaptation and assortative mating) between two hypothetical populations of large and small snails. (B) Two chromosomes, one containing two barrier loci, and the other without. The dotted line shows the rate of gene flow due to migration at rate m in the absence of a reproductive barrier. The grey line shows the reduced 'effective migration rate', m_e , caused by barrier loci.

hybrids. We distinguish divergence under (near) continuous gene flow from divergence with periods of isolation as alternative routes, and the role of divergent selection in potentially driving divergence. We compile available information on reproductive barriers, including the proportion of

Key Figure

A matrix of speciation components for six population pairs of *Littorina*

		Ecotypes: High - Low shore <i>L. saxatilis</i> Sweden	Ecotypes: Crab - Wave <i>L. saxatilis</i> Sweden	Ecotypes: Dwarf - Large <i>L. fabalis</i>	Ecotypes: Crab - Wave <i>L. saxatilis</i> Spain	Sister species: <i>L. arcana</i> - <i>L. saxatilis</i>	Sister species: <i>L. fabalis</i> - <i>L. obtusata</i> Sweden
							
Progress	Genetic differentiation (F_{ST})	~0.02	~0.04	~0.09	~0.10	~0.10 - 0.2	~0.4
	Divergence time	< 100 years	~15 k years	>60 k years	~57 k years	~60 - 1700 k years	~800 k years
	Phenotypic divergence	Size & shape	Size, shape, behaviour	Size	Size, shape, behaviour	Female anatomy	Size, shape, penis shape
	Hybridisation rate	High	High	Moderate	Low	Zero? (No hybrids found)	Low (hybrids rare)
Route	Spatial separation prior to contact?	No	No	Yes	No	Brief period of isolation	Unknown but likely
	Barriers	Strength of assortative mating	Weak	Moderate	Weak to none	Strong	Moderate
Ecological differentiation		Moderate	Strong	Moderate	Strong	None obvious	Minor
Strength of genetic incompatibility		Weak to none	Weak to none	Moderate	Moderate to strong	Likely strong	Likely strong
Inversions under divergent selection		3-4 show frequency differences	7-8 show frequency differences	9 fixed different	12-14 show frequency differences	None	No data
Fraction of genome linked to a barrier locus		Small	Moderate	Moderate	Large	Very large	Very large

Trends in Genetics

Figure 2. The ecotype pairs are distributed in different shore habitats with extensive contact zones overlapping with environmental gradients (as illustrated in Box 1). Sister species pairs overlap in distribution and occupy, at least partly, the same shore habitat. All taxa are direct developing without larval dispersal. For convenience, pairs were ordered based on genome wide F_{ST} but it is clear that other measures of divergence or barrier effects do not always follow this order. Divergence time and geographic isolation were assessed from demographic inferences, but for the first pair, a natural recolonisation event indicated a very brief divergence time [45]. In this case, much of the adaptive variation that selection acted upon may be much older than the demographic divergence. The fractions of the genome linked to barrier loci are based on genome-wide analyses. Local adaptation was assessed from reciprocal transplants of ecotype populations that occupy separate shore environments. Genetic incompatibilities were assessed from embryo abortion rates or from deficit or absence of hybrids at contact despite the occurrence of between-population mating. Inversions have been identified using population genomic tools, linkage maps, and an independent pool-seq approach. References to data entries are in the main text under the descriptions of each snail pair, and further specified in Figure S1 in the supplemental information online.

the genome experiencing a barrier effect, strength of assortative mating (size- or habitat-based), and genetic incompatibilities inferred from postzygotic barriers. Finally, we describe the barrier role of inversions for which different arrangements (the ancestral and the derived) segregate. While we acknowledge the role of genetic drift, order of selected mutations, and coupling of barriers, information on their role is currently largely missing and they are therefore left out. Synthesising all information, we highlight how the same pair may have vastly different locations on different speciation axes, demonstrating the multidimensionality of speciation.

Pair 1. High- and low-shore *L. saxatilis*

Closely related populations (within either the Crab or Wave ecotype, see below) present in high and low parts of rocky Atlantic shores of Europe and with a continuum of intermediate phenotypes through the mid-shore level [41,42]. Shell shapes are very similar but high-shore snails tend to be larger and have higher spires [43]. Steep vertical physical gradients in temperature and risk of desiccation select for physiological divergence [40]. Some regions of the genome show parallel patterns of strong

differentiation over a pan-European scale [44] including inversions on chromosomes 9, 11, and 12 [41,42]. In 1988, a toxic algal bloom completely removed a Swedish low-shore population but migration from the high shore, combined with strong directional selection, completely restored the genetic variation that characterises the low-shore population, within less than 10–15 generations [45]. This suggests that the vertical differentiation is slight and evolves repeatedly, from ancestral **standing genetic variation**, under divergent selection and in the face of gene flow.

Pair 2. Crab and Wave ecotypes of *L. saxatilis* in Sweden

Ecotypes adapted to sheltered crab-rich and low-predation wave-exposed habitats, respectively [46]. The large, robust, and wary Crab ecotype contrasts strongly to the small, fragile and bold Wave ecotype. In Sweden, Crab and Wave ecotypes are distributed along different parts of shores and are in contact only at the transition between habitats [29,47]. The Swedish Crab and Wave ecotypes have a postglacial history and diverged under gene flow as inferred from **demographic analyses** [20,48]. The main driver of divergence is habitat-linked divergent selection [22], with adaptation involving seven or eight polymorphic inversions [25,49]. In addition, **collinear loci** contribute to roughly half of the divergence in quantitative traits [50]. Size-assortative mating is strong in the laboratory [51], but the smooth shift in snail size over the contact zone decreases the strength of this barrier in nature [24]. Viable and fully fertile F1 and F2 generations are easily obtained by crossbreeding in the laboratory [26], and substantial admixture is detected at the contact zones in nature [47,52]. Embryo abortion rates are similar in hybrid females and pure ecotype females, suggesting few, if any, genetic incompatibilities [53].

Pair 3. Dwarf and Large ecotypes of *Littorina fabalis*

These ecotypes are common in brown seaweeds along shores of northwestern Europe [54–56]. The Large ecotype is present in moderately wave-exposed areas and the Dwarf in more protected parts of shores. Size is the only described consistent phenotypic difference, with the Dwarf ecotype being approximately two thirds the size of the Large ecotype [54,57]. Demographic analysis shows a period of isolation prior to the current geographic overlap in distribution and the formation of multiple contact zones [58]. Hybrids are found at contact, but they are at a strong deficit [57–59] despite no evidence of assortative mating [59]. This suggests that hybrid deficiency is due to genetic incompatibilities evolved during isolation. Furthermore, nine inversions are almost fixed different between the two ecotypes and remain strongly coupled where ecotypes hybridise [58].

Pair 4. Crab and Wave ecotypes of *L. saxatilis* in Spain

These ecotypes have diverged in parallel to the Swedish ecotypes [20,48]. However, in Spain crabs dwell in rockpools in the high shore, while wave action is severe in the low shore and crabs are absent here. Consequently, the Spanish ecotypes simultaneously experience a high/low-shore gradient and a crab/wave gradient which may strengthen the overall barrier, compared to Sweden where the two environmental gradients are perpendicular. Moreover, the contact zone between the two ecotypes is much more extensive than in Sweden and runs parallel to the shoreline [60]. Intermediate snail phenotypes in the contact zone and local patterns of parallelism earlier suggested gene flow between the two ecotypes [23,60,61], but recent genome-wide analyses show two largely isolated genetic clusters [62,63]. One cluster predominantly contains individuals with a Crab phenotype present in the high shore, but also phenotypically intermediate individuals found in the low shore, while the other cluster consists of pure Wave-phenotype individuals and is exclusively present in the low shore [63]. Abortion rates in crosses between Spanish Wave and Crab ecotypes are much higher (60%) (KJ, personal observation) compared to Swedish crosses of the same ecotypes (12%) [53]. Together with assortative mating in the contact zone [64,65] this likely explains the rarity of hybrids. Spanish Crab-Wave divergence also involves many chromosomal inversions. Most appear to be the same as those present in Sweden, though the frequency differences of the arrangements tend to be greater

in Spain [42]. Like the Swedish Crab-Wave ecotypes, the Spanish clusters have diverged without obvious periods of isolation [20].

Pair 5. Sister species *Littorina arcana* and *L. saxatilis*

These species have extremely similar phenotypes, with female reproductive mode (egg-laying in *L. arcana*, and brooding in *L. saxatilis*) being the only diagnostic trait [66]. The species have undifferentiated proteomes [67], and phenotypic separation of males is unreliable, but males and juveniles can be separated using some species-diagnostic SNPs [68]. Despite this very close relationship, no hybrids were found analysing diagnostic SNPs from 3000 snails from the British Isles and France, although whole-genome sequencing revealed evidence of a low rate of hybridisation [68]. Furthermore, demographic analysis showed divergence with gene flow, although a short period of isolation could not be ruled out. Currently, where present, *L. arcana* are largely overlapping on the shore with *L. saxatilis* [66], and thus, habitat-related divergent selection is unlikely to be strong. Preliminary data suggest that assortative mating by both microhabitat choice, direct mate choice, and partial seasonal separation may contribute to isolation, but that is insufficient to explain the lack of hybrids observed in the field (AMW and SS personal observations). Loci associated with differences between the two reproductive modes (egg-laying in *L. arcana* and brooding in *L. saxatilis*) are distributed throughout the genome [69], and 75% of all loci are impacted by barriers to gene flow [68]. This suggests that genetic incompatibilities are important in maintaining species integrity, possibly supported by assortative mating and micro-geographic separation.

Pair 6. Sister species *L. fabalis* and *Littorina obtusata*

The two species have overlapping phenotypic traits, and the only strictly diagnostic character is the shape of the male penis [66]. Current distributions are largely overlapping on the European side of the Atlantic while *L. fabalis* is absent on the American side [66]. Sharing of common mtDNA haplotypes and demographic analysis based on mtDNA and two nuclear genes suggest that divergence occurred with some gene flow [56,70]. Currently, however, hybridisation is rare across the species' distributions apart from one locality in Portugal where the proportion of hybrids is around 50% [71]. Males of both species prefer the larger females of *L. obtusata* (S.H. Saltin, PhD thesis, University of Gothenburg, 2013), but differences in penis shape might contribute to prezygotic barriers [72]. Divergent selection is unlikely to be strong in places where the species share the same environment (e.g., northern Europe), and so genetic incompatibilities are likely important to species isolation.

Position on the speciation continuum and routes to divergence

Three metrics show highly consistent patterns among the pairs: genetic differentiation, time of divergence, and hybridisation rate (Figure 2). This might suggest that the process of speciation can be represented along just one dimension. However, adaptive phenotypic divergence strongly deviates from this pattern, despite the expected role of divergent selection in initiating and driving speciation. In Spanish *L. saxatilis*, for example, divergence was probably initiated by local adaptation to different microhabitats resulting in ecotype formation, and habitat choice and assortative mating supported the barriers. Such progress can, however, be context dependent since in Sweden the same two ecotypes remain in contact with no deficiency of heterozygotes in hybrid zones. Sadedin *et al.* [28] modelled speciation from spatial data on fitness and mate selection in the Swedish ecotypes of *L. saxatilis*. Overall, ecotypes evolved readily, while conditions for the ecotypes to evolve towards completely isolated species were more restricted largely due to hybrid superiority in the contact zone, as observed in nature [22].

The routes of divergence in all six snail pairs have at least partly taken place under periods of gene flow, and three of the ecotype pairs show no evidence of any earlier period of geographical isolation [20,45,48]. Also, the sister species *L. saxatilis* and *L. arcana* likely diverged with only a short

period of isolation [68], while a more extensive period of isolation supported the divergence of the two *L. fabalis* ecotypes [58]. Thus, geographical isolation is not necessary for divergence in these snails, although it may facilitate establishment of certain barriers.

Barriers to gene exchange

Seehausen *et al.* [5] suggested that speciation can be initiated by prezygotic or extrinsic postzygotic barriers due to environmentally related divergent selection and assortative mating, or by intrinsic postzygotic barriers caused by genetic incompatibilities. We can use the snails to study the nature and evolution of barriers to gene flow in more detail.

In the four ecotype pairs of *Littorina*, a major barrier is immigrant inviability, an extrinsic prezygotic barrier due to adaptation to different local environments [22,23,73–75]. These barriers are replicated at hundreds of places over large geographic areas [20,25,41], but are context dependent and can evolve rapidly. As described above, a high-shore population of *L. saxatilis* could evolve and adapt to the low shore under directional selection within <15 generations [45]. Another example is a transplanted Crab ecotype population that evolved into a Wave ecotype population in <30 generations [76]. Both the extensive parallelism and the examples of rapid evolution are due to available standing variation coding for adaptive traits present in polymorphic inversions and in the collinear genome [26,50].

Assortative mating is an important barrier between the Spanish *L. saxatilis* Crab and Wave ecotypes [64]. It likely emerged as a result of divergent selection on size, coupled to size-assortative mating, wherein size-assortative mating is an ancestral trait shared among many snails [77]. The clear size difference between ecotypes associated with microhabitat choice in Spain [65] strengthens this mating barrier [24]. In contrast, the more gradual changes in snail size in Sweden over the Crab-Wave contacts result in much weaker barriers [24]. Thus, the realised effect of this barrier trait can be strongly context dependent.

Genetic incompatibilities are generally thought to evolve during periods of geographical isolation (but see [78]), because mutations fixed by drift, new adaptive mutations, and mutations that compensate for deleterious mutations, will likely differ between populations [79,80]. These derived alleles may be incompatible, resulting in lower fitness of hybrids upon **secondary contact** [81]. Divergence between spatially isolated populations in similar environments can also result in other types of barriers, such as divergence in penis shape generating prezygotic isolation [72]. In the sister-species pairs of the snails, the strongest barriers most likely include the effects of genetic incompatibilities. Such barriers are also likely present, in addition to extrinsic isolation, between the two most divergent ecotype pairs (Figure 2). For example, strongly inflated abortion rates have been observed in crosses between Spanish Wave and Crab ecotypes (details given earlier). Hybrid deficits in contact zones between Dwarf and Large *L. fabalis* ecotypes [58] suggest genetic incompatibilities evolved during isolation prior to contact. The absence of hybrids between overlapping populations of *L. fabalis* and *L. obtusata* in most sites, despite frequent mating attempts (S.H. Saltin, PhD thesis, University of Gothenburg, 2013), also suggests incompatibilities between these species. Finally, egg laying versus brooding seems to provide a strong barrier to gene flow between *L. arcana* and *L. saxatilis* [68], and illustrates how a single trait can isolate species that largely overlap in other traits [69]. Overall, genetic incompatibilities seem more powerful and persistent barriers than habitat-linked divergent selection in the snails, while assortative mating is context-dependent and of variable importance.

The architecture of barriers

Inversions restrict recombination between ancestral and inverted arrangements. This can maintain linkage disequilibrium among barrier loci and extend their effects to neutral loci over large

genomic regions. Thus, inversions can contribute strongly to the barrier effect between taxa with substantially different arrangement frequencies [82]. Multiple large and moderately large inversions are found in *L. saxatilis*, and many large inversions are also polymorphic in *L. fabalis*, *L. arcana* and *L. obtusata* [29,42,49,58,83]. In *L. saxatilis*, specific inversions contribute to adaptive divergence at least throughout the whole European range [83], and consistently separate ecotypes along two different environmental axes (Figure 3 and [41]). In this way, inversions are key barriers to gene flow in four of the five pairs for which we have data (Figure 2), but the barriers vary in strength. For example, moderate differences in arrangement frequencies between Swedish *L. saxatilis* Crab and Wave ecotypes are associated with lower genome-wide differentiation (F_{ST}) compared to differentiation between *L. fabalis* Dwarf and Large ecotypes that have almost all inversions fixed different (Figure 2). In the Swedish *L. saxatilis* ecotypes, roughly half the divergence emerges from selection on collinear parts of the genome [50], while in *L. fabalis* almost all divergence is linked to the 20% of the genome that is covered by the inversions [58]. The origin of these barriers is also different; the Swedish *L. saxatilis* barriers were established under gene flow from divergent selection on standing variation of inversion arrangements with as yet unknown histories. The *L. fabalis* barriers, in contrast, evolved to a large extent during isolation, either due to divergence in different habitats, or perhaps following mutation-order divergence. In the contact zone, inversion arrangements are strongly coupled in the *L. fabalis* ecotypes and hybrids are much less common than expected at the centres of the hybrid zones [58]. The Spanish Crab-Wave ecotype divergence presents yet another contrast to the Swedish ecotypes, with additional arrangements having high frequency differences between ecotypes [42]. We infer that initial divergent selection on both inversion polymorphisms and collinear parts of the genome was followed by divergence in size, habitat selection, and assortative mating. This reduced gene flow and promoted even stronger differences in inversion arrangement frequencies, and potentially also more incompatibilities accumulating inside inversions [84]. Overall, the snail data corroborate earlier theoretical and empirical conclusions that inversions, when present, play an important role in local adaptation and ecotype formation [85–89].

Many of the inversion polymorphisms are shared widely and probably old [83], and so they contribute to standing variation and are used repeatedly [41,58]. A major unknown in the snails is the role of individual genes, for example, key **allozyme genes** found inside inversions with alleles differing in several non-synonymous mutations [27,44]. This suggests they are targets of selection, but we need more data to fully reject neutrality due to their tight linkage to other potential candidate genes inside the inversion.

We also expect population-specific mutations and combinations of alleles to generate similar phenotypes contributing to ecotype differentiation in different locations. In sticklebacks, for example, sharing of SNPs contributing to local adaptation is low among populations from the same watershed [90]. In the snails, 36–55% of divergent loci (18–25% upon excluding inversions) were shared among island populations a few kilometres apart [25], which calls for studies on the contribution of new mutations in the divergence of the snails.

The snail pairs demonstrate the difficulty of finding a single measure for the overall strength of the barrier. The barrier effect for unlinked neutral alleles and the proportions of the genome experiencing barriers of different strengths are both useful measures of isolation (Box 2). Between the two ecotypes of *L. fabalis*, for example, inversion regions experience a strong barrier that covers ~20% of the genome and corresponds to 93% of the divergent SNPs [58], while the barrier experienced by the remaining 80% of the genome is weaker. In contrast, in the Swedish Crab-Wave *L. saxatilis* pair, only small parts of the genome experience a barrier as strong as the barrier for inversion regions in *L. fabalis*, but significant clines are established in a large number of loci (~50%) across the whole genome [29], indicating the presence of a genome-wide barrier.

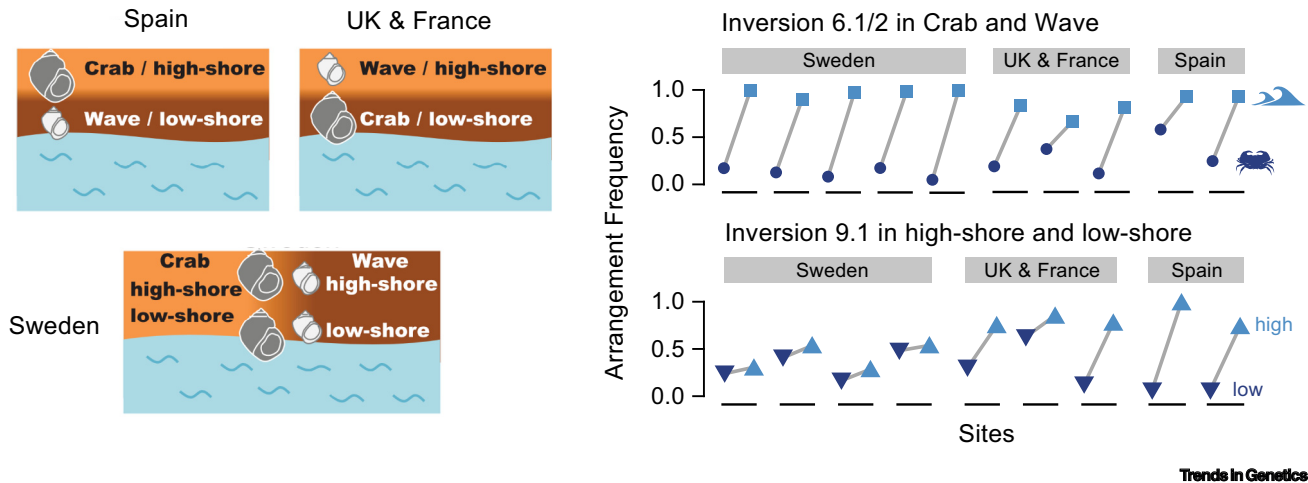


Figure 3. Spatial distribution of *Littorina saxatilis* ecotypes and variation in chromosomal inversions. The transition between wave exposed, crab-free microhabitats and wave-protected areas with crabs constitutes a major selection gradient [22,23] resulting in a Wave ecotype and a Crab ecotype, respectively. A second selection gradient runs from low to high shore with different risks of desiccation and extreme temperatures. Size, shape, and behaviour traits are under divergent selection over the crab-wave axis [39], and physiological traits are selected over the high–low shore axis [40]. Left panel: gradients are parallel on the Atlantic coast of Spain with extreme wave action at low-shore and high-shore rockpools with crabs. In UK and France, waves hit the vertical high-shore rocks while crabs are hiding among low-shore boulders, inverting the crab-wave axis. In Sweden, crab-rich boulder shores are interrupted by wave-exposed rocky outcrops perpendicular to the high–low shore axis. These variable conditions allowed the separation of the genetic basis of adaptation to the different environmental axes [41,42]. Right panel: divergence along the crab-wave axis is linked to segregation of arrangements of an inversion on chromosome 6. Wave ends of transects having higher frequency of one of the two arrangements compared to crab ends in five Swedish, three UK/France, and two Spanish localities. Lines connect crab and wave ends of the same transect. Similarly, divergence between high and low shore ends of transects is consistently associated with variation in an inversion on chromosome 9. Figures reproduced, with permission, from [41,42].

Thus, we cannot say whether the *L. saxatilis* or *L. fabalis* ecotypes are closer to complete speciation: one is closer on one dimension of speciation; the other is closer on another dimension. Between *L. arcana* and *L. saxatilis* the genome-wide barrier is strong, affecting 75% of the genome, but inversions do not contribute at all to this barrier [69].

Speciation is multidimensional

This summary of *Littorina* studies demonstrates the multidimensionality of both ecotype formation and speciation, and the need to go beyond a one-dimensional speciation continuum. By using multiple axes we can describe different dimensions of reproductive isolation that do not necessarily evolve in a coordinated fashion (Box 2). Speciation (hyper)cubes help to visualise this multidimensional view of speciation [35,36]. Translating the semiquantitative data from the speciation matrix (Figure 2) into example cubes, we use the snails to illustrate how a multidimensional approach can aid in our understanding of the process of speciation (Figure 4) and help in framing questions for further research. The barrier effect on unlinked neutral loci is challenging to measure, see Box 2, and we lack estimates for these *Littorina* pairs and instead use estimates of incompatibilities.

The cube illustrating routes to speciation (Figure 4, left) shows some of the many different paths that divergence seems to take in the snails; three pairs initially diverged by ecological differentiation (divergent selection and local adaptation; pairs 1, 2, and 4), while in the other three (3, 5, and 6), spatial isolation led to the accumulation of barriers. This cube also illustrates how divergence in mode of reproduction (transition from egg laying to brooding) drove the separation into two incipient species, *L. arcana* and *L. saxatilis* (pair 5), without obvious habitat separation. By outlining the paths taken towards speciation, this cube helps to frame questions about the order of appearance of different barriers.

The barrier cube (Figure 4, right) uses semiquantitative estimates (based on the matrix data; Figure 2) to outline the relationships between multiple aspects of the barriers to gene flow that exist for each population pair. Despite extensive work on this system, better quantification of barriers is still needed. In the barrier cube, the *Littorina* pairs fall into two different clusters: one with generally weak barriers (pairs 1–3) and the other with stronger barriers (pairs 4–6). Within each cluster, there is only a weak correlation among the three measurements of barriers, and even for the pairs with stronger barriers, values on the axes do not yet converge towards their maxima, showing that speciation is still not completed. The Dwarf–Large *L. fabalis* (pair 3) is surprisingly far from the upper-right corner (speciation) despite being almost fixed different in nine coupled inversions. This contrasts with the Spanish *L. saxatilis* ecotypes (pair 4) with weaker inversion differences overall than the *L. fabalis* ecotypes, but a larger general reduction in neutral gene flow (Figure 4). The *L. arcana*/*L. saxatilis* pair has no inversion barrier but is closer to complete speciation than the other two pairs. These results show that in the snails the evolution of strong barriers does not correlate with the recruitment of chromosomal inversions, contrary to

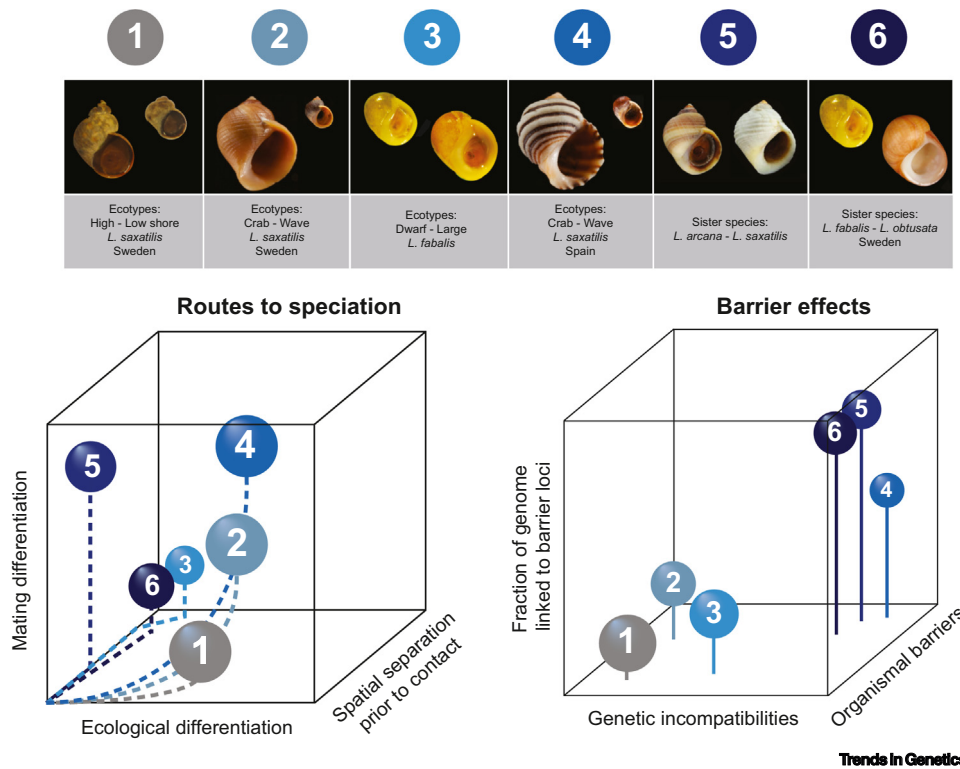


Figure 4. The snail pairs fitted into speciation cubes. The routes depicted for each pair (left cube) are consistent with speciation theory, demographic inference, and ecological data, but the order of events might differ from the sequence shown. Nevertheless, it is possible to compare the current position of different pairs: for example, both pairs 4 and 5 have a high degree of mating differentiation but have followed different routes to this stage of speciation. Another contrast is the rather similar magnitude of ecological differentiation among three of the pairs (1, 2, and 4) while at the same time they are substantially different in mating differentiation. Pair 3 is the only pair that has differentiated somewhat along all three axes. The different axes of the barrier cube (right cube) capture strength of genetic incompatibilities, fractions of genome linked to a barrier locus, and organismal barriers based on the semiquantitative data in Figure 2. The last axis integrates estimates of local adaptation and assortative mating, but also needs to consider hybrid fitness which we have not measured. However, hybrid fitness will strongly relate to strength of genetic incompatibility. The barrier cube shows how the different axes are only weakly correlated, and pairs do not line up along a diagonal axis from the left bottom corner to the top right corner which would be expected if divergence could be expressed along a one-dimensional speciation continuum. Note that both cubes show just three out of many possible dimensions.

what has been suggested [38,91–93]. Inversions are key players in ecotype formation in the snails, but their role, if any, for the later stages of speciation is far from obvious.

Concluding remarks

Combining speciation data from four closely related *Littorina* species, we show large variation in routes of divergence, drivers of divergence, and the genomic architecture of barriers to gene flow. A speciation matrix and a multidimensional approach (speciation cubes) helped us to synthesise and integrate the multifarious data. However, we acknowledge that additional dimensions of divergence and speciation in *Littorina* will be important to approach in future studies by incorporating allopatric populations, pairs with no current gene flow, and broadcast-spawning species that do not form ecotypes [39]. The multidimensional views, both of routes and of barriers, can be helpful in studying speciation in other systems. Moreover, to generalise about speciation, we need to gather comparable data on many other clades, and also fill knowledge gaps in well-studied cases. This is particularly true for some of the more difficult things to measure, like the reduction in gene flow at neutral loci. Nevertheless, we are convinced that a good way forward is to combine genomic analysis, including across contact zones, demographic analysis, and a strong understanding of the ecology and the selection pressures on diverging taxa.

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Declaration of interests

The authors have no conflicts of interest to declare.

Supplemental information

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Outstanding questions

Is coupling of various barriers to gene flow (e.g., divergent adaptation, assortative mating, genetic incompatibilities) necessary, or does one type of barrier (e.g., genetic incompatibility) commonly dominate the completion of speciation?

What are the relationships between different axes in a barrier cube, and do they differ among taxa? For example, how does the reduction in gene flow at unlinked neutral loci relate to the proportion of the genome experiencing barrier effects?

How do organismal and genomic barriers relate, i.e., how do barriers we can observe at the organismal level translate into a reduction in gene flow?

Are there empirical cases where the effects of different barriers to gene flow on reproductive isolation are so strongly correlated that the speciation process can essentially be delineated in terms of a single effective barrier axis (i.e., speciation axis), and consequently viewed as a one-dimensional process?

Under what circumstances do inversions not only promote ecotype formation but also make a key contribution to the completion of speciation?

How commonly is the formation of ecotypes a first step in the process of speciation?

In the snails we found divergent ecological selection playing little role in the evolution of strong reproductive isolation and inversions playing a minor role in the completion of speciation. Are these general patterns or idiosyncrasies of *Littorina* species?

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