Reproductive isolation in relation to population differentiation and local adaptation to environmental unpredictability

Doctoral Thesis by

Ivana Jezkova

Supervised by

Dr. Manuel Serra Galindo

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Doctoral Programme in Biodiversity and Evolutionary Biology

Valencia, July 2023

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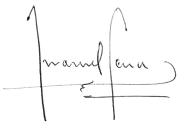
certify that **Ivana Jezkova** has carried out under our direction and with the greatest use, the research work included in this doctoral thesis, and entitled: "Reproductive isolation in relation to population differentiation and local adaptation to environmental unpredictability", to obtain the degree of Doctor in Biodiversity and Evolutionary Biology.

And for the record, in compliance with current legislation, we issue this certificate in València on July 14, 2023.



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*The numbers of figures and tables, together with the citation style of the articles have been modified to adapt to the thesis as a whole.

Abstract

Reproductive isolation is one of the crucial phenomena that allow the maintenance of within species variability and species richness, and has strong implications for the evolutionary dynamics of populations, including speciation. Reproductive isolation implies intrinsic and extrinsic factors that reduce or prevent gene flow between populations. The barriers to reproduction can act at different stages of the reproductive process, from male-female encounter probability and mate recognition in animals, through zygote development, to viability and fertility of the offspring.

Populations of the zooplankter *Brachionus plicatilis*, *a* cyclically parthenogenetic rotifer, have been intensively studied in the Iberian Peninsula. They present a high degree of population differentiation even at small geographical scale, and three main phylogeographic clades has been described in the region. Additionally, these populations inhabit ponds with different environmental conditions (e.g., the predictability of the flooding regime) and local adaptation has been found for such conditions. In this thesis, after sampling natural populations, we took advantage of these features to investigate factors involved in the evolution of reproductive isolation. With this aim, three approaches were used in laboratory studies: (1) Behavioural reproductive isolation via mate recognition; (2) Reproductive isolation assessed by the production of diapausing eggs (sexually produced) of laboratory cultures; and (3) Genetic differentiation of the Mating Recognition Protein (MRP), a surface glycoprotein that drives male-female recognition in *B. plicatilis*.

Signatures for ongoing reproductive isolation between natural populations diverging phylogeographically and ecologically have been found, the latter divergence having a stronger effect.

The behavioural study, as well as the diapausing eggs approach, showed similar tendencies, namely a lower propensity for between population crosses in population with higher adaptive divergence between them. This result is consistent with the finding of significant correlation between the divergence in the MRP gene and adaptive divergence. This correlation, together with the lack of correlation between genetic divergence in neutral markers and adaptive divergence, supports the hypothesis that the adaptive divergence, and not genetic drift, drives the evolution of reproductive isolation in the studied populations. Cues for stabilizing selection acting on the MRP gene were also found. Thus, the observed divergence seems to have evolved despite the differentiation in the mating system tends to be eroded by its expected evolutionary stabilization.

Exceptions for within-population reproductive preferences were also observed. Although not studied mechanism of isolation might be at work in the wild, these cases may account for an actual lack of reproductive isolation. Occurrence of exceptions was not unexpected, as populations belongs to the same species.

In this thesis, we shed light on the incipient reproductive isolation in the zooplankter *B. plicatilis*. As reproductive isolation is a rare historical event in nature, finding partial isolation within some of the populations studied seems to us biologically significant. Our results suggest that intrinsic

isolation may arise associated with population differentiation and might play a role in the diversification of rotifers.

Resumen

El aislamiento reproductivo es uno de los fenómenos cruciales que permite el mantenimiento de la variabilidad intraespecífica y la riqueza de especies, y posee implicaciones importantes para la dinámica evolutiva de las poblaciones, incluida la especiación. El aislamiento reproductivo implica factores intrínsecos y extrínsecos que reducen o evitan el flujo génico entre poblaciones. Las barreras reproductivas pueden actuar en diferentes estadios del proceso reproductivo, desde la probabilidad de encuentro macho-hembra y el reconocimiento de pareja en animales, al desarrollo del cigoto, y a la viabilidad y fertilidad de la descendencia.

Las poblaciones del rotífero partenogenético cíclico *Brachionus plicatilis*, que forma parte del zooplancton, han sido estudiadas intensamente en la península ibérica. Presentan un alto grado de diferenciación incluso a pequeña escala geográfica, y se han descrito tres clados filogeográficos en la región. Además, estas poblaciones habitan lagunas que difieren en sus condiciones ambientales (por ejemplo, en la predictibilidad de sus periodos de inundación), y se conoce que existe adaptación local para tales condiciones. En esta tesis, a partir muestras de poblaciones naturales, se explotan estas propiedades para investigar los factores implicados en la evolución del aislamiento reproductivo. Para ello se han utilizado tres aproximaciones en estudios de laboratorio: (1) aislamiento reproductivo etológico a través del reconocimiento de pareja; (2) aislamiento reproductivo evaluado en la producción de huevos diapáusicos, que son los producidos sexualmente; y (3) diferenciación genética en la *Mating*

Recognition Protein (MRP; proteína de reconocimiento en el apareamiento), una glicoproteína que condiciona el reconocimiento macho-hembra en *B. plicatilis*.

En esta tesis se han encontrado indicios de aislamiento reproductivo entre poblaciones naturales que divergen filogeográficamente o ecológicamente, con un efecto más notable en el caso de la divergencia ecológica. El estudio etológico y el centrado en la producción de huevos diapáusicos muestran tendencias semejantes, consistentes en una menor propensión a los cruces entre poblaciones con mayor divergencia adaptativa entre ellas. Este patrón está de acuerdo con la observación de que la divergencia genética en MRP se incrementa con la divergencia adaptativa. Esta correlación, junto a la falta decorrelación entre la divergencia genética en marcadores neutros y la divergencia genética adaptativa, apoya la hipótesis de que la divergencia adaptativa —y no la deriva genética— es la responsable más importante de la evolución del aislamiento reproductivo en las poblaciones estudiadas. Además, se han encontrado indicios de selección estabilizante para el gen codificante de MRP. Así, la divergencia en el gen parece haber evolucionado a pesar de que la diferenciación en el sistema de apareamiento tiende a ser erosionada por selección estabilizante, selección que, por otro lado, es esperable que actúe sobre un sistema de reconocimiento.

Las regularidades señaladas tienen algunas excepciones. Ello no excluye concluyentemente el aislamiento, pues no todos los mecanismos potenciales han sido estudiados aquí. No obstante, cabe señalar que estas excepciones al aislamiento, de ser reales, no serían sorprendentes si se

considera la pertenencia a una misma especie de las poblaciones estudiadas.

Globalmente esta tesis contribuye a la comprensión de un aislamiento reproductivo incipiente en el invertebrado planctónico *B. plicatilis*. Dado que el aislamiento reproductivo es un fenómeno histórico infrecuente en la naturaleza, haber encontrado aislamiento reproductivo parcial posee relevancia biológica. Los resultados de la tesis apoyan la idea de que el aislamiento reproductivo por factores intrínsecos puede estar asociado a la diferenciación de las poblaciones y tener implicaciones en la diversificación de los rotíferos.

Resum

L'aïllament reproductiu és un dels fenòmens crucials que permet el manteniment de la variabilitat intraespecífica i la riquesa d'espècies, i posseeix implicacions importants per a la dinàmica evolutiva de les poblacions, inclosa l'especiació. L'aïllament reproductiu implica factors intrínsecs i extrínsecs que redueixen o eviten el flux gènic entre poblacions. Les barreres reproductives poden actuar en diferents estadis del procés reproductiu, des de la probabilitat de trobada mascle-femella i el reconeixement de parella en animals, al desenvolupament del zigot, i a la viabilitat i fertilitat de la descendència.

Les poblacions del rotífer partenogenètic cíclic *Brachionus plicatilis*, que forma part del zooplàncton, han sigut estudiades intensament en la península ibèrica. Presenten un alt grau de diferenciació fins i tot a petita escala geogràfica, i s'han descrit tres clades filogeogràfics a la regió. A més, aquestes poblacions habiten llacunes que difereixen en les seues condicions ambientals (per exemple, en la predictibilitat dels seus períodes d'inundació), i es coneix que existeix adaptació local per a tals condicions. En aquesta tesi, a partir mostres de poblacions naturals, s'exploten aquestes propietats per a investigar els factors implicats en l'evolució de l'aïllament reproductiu. Per a això s'han utilitzat tres aproximacions en estudis de laboratori: (1) aïllament reproductiu etològic a través del reconeixement de parella; (2) aïllament reproductiu avaluat en la producció d'ous diapausics, que són els produïts sexualment; i (3) diferenciació genètica en la Mating Recognition Protein (MRP; proteïna de

reconeixement en l'aparellament), una glicoproteïna que condiciona el reconeixement mascle-femella en *B. plicatilis*.

En aquesta tesi s'han trobat indicis d'aïllament reproductiu entre poblacions naturals que divergeixen filogeogràficament o ecològicament, amb un efecte més notable en el cas de la divergència ecològica. L'estudi etològic i el centrat en la producció d'ous diapausics mostren tendències semblants, consistents en una menor propensió als encreuaments entre poblacions amb major divergència adaptativa entre elles. Aquest patró està d'acord amb l'observació que la divergència genètica en MRP s'incrementa amb la divergència adaptativa. Aquesta correlació, amb la manca de una correlació entre la divergència genètica en marcadors neutres i la divergència genètica adaptativa, la qual cosa dona suport a la hipòtesi que la divergència adaptativa —i no la deriva genètica— és la responsable més important de l'evolució de l'aïllament reproductiu en les poblacions estudiades.

A més, s'han trobat indicis de selecció estabilitzant per al gen codificant de MRP. Així, la divergència en el gen sembla haver evolucionat a pesar que la diferenciació en el sistema d'aparellament tendeix a ser erosionada per selecció estabilitzant, selecció que, d'altra banda, és esperable que actue sobre un sistema de reconeixement.Les regularitats assenyalades tenen algunes excepcions. Això no exclou concloentment l'aïllament, perquè no tots els mecanismes potencials han sigut estudiats ací. No obstant això, cal assenyalar que aquestes excepcions a l'aïllament, de ser reals, no serien sorprenents si es considera la pertinença a una mateixa espècie de les poblacions estudiades.

Globalment aquesta tesi contribueix a la comprensió d'un aïllament reproductiu incipient en l'invertebrat planctònic *B. plicatilis*. Atés que l'aïllament reproductiu és un fenomen històric infreqüent en la naturalesa, haver trobat aïllament reproductiu parcial posseeix rellevància biològica. Els resultats de la tesi donen suport a la idea que l'aïllament reproductiu per factors intrínsecs pot estar associat a la diferenciació de les poblacions i tindre implicacions en la diversificació dels rotífers.

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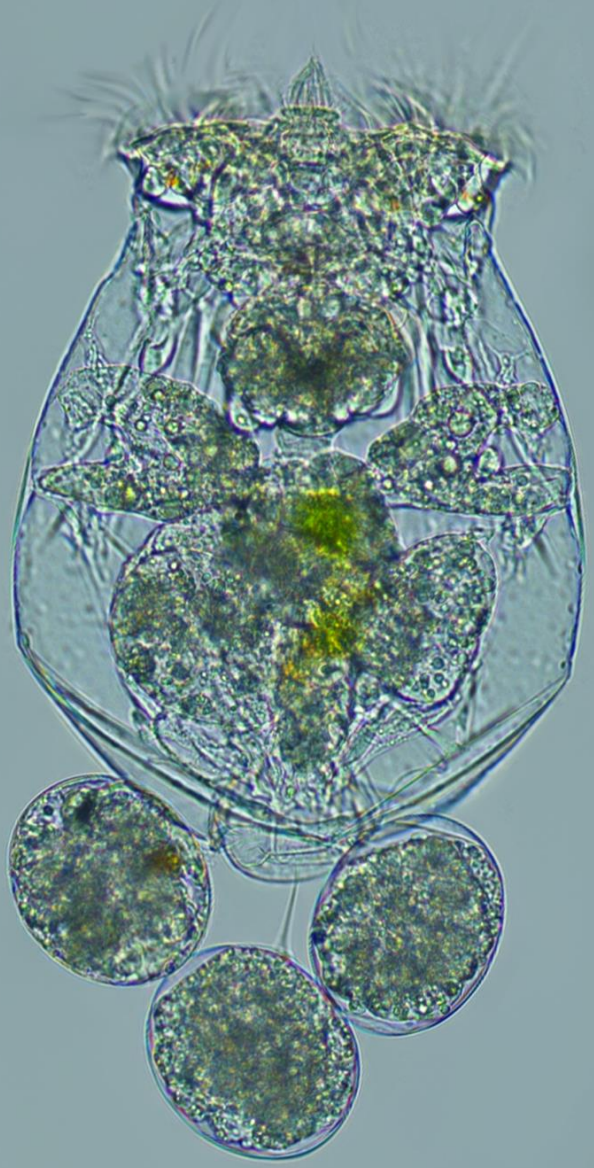

Chapter 1

General introduction

Population differentiation

Populations of a single species are often able to inhabit a variety of diverse environments and localities. Therefore, understanding how evolutionary processes and environmental factors influence their fate in different environments represents one of the key challenges in evolutionary biology. Diverging forces, both adaptive and non-adaptive, affect populations over time (Dobzhansky, 1977; Holderegger et al., 2006). The outcome of these forces is continually wiped off by factors that maintain or promote homogeneity within a species. Depending on which factors prevail, the evolutionary history of a population may take a variety of directions. Population differentiation allows populations to explore and make use of different environments, playing a crucial role in maintaining biodiversity, species persistence, and is usually required in speciation (Nosil & Feder, 2012; Wolf & Ellegren, 2016).

Studying population differentiation has practical implications in conservation biology, helping to understand the processes of adaptation and population persistence in changing environments (Wang et al., 2016; Guo et al., 2019; O'Malley et al., 2019). Additionally, it provides insights into invasions and the challenges associated with the spread of non-native species (Roux, 2021). Population differentiation also has direct applications in medicine, such as understanding disease evolution, its response to different treatments or possible resistances (Gilabert & Wasmuth, 2013; Weng et al., 2022; Zhu et al., 2022). Furthermore, the findings from population differentiation research can be applied in agriculture and breeding (Gurung et al., 2013; Wang et al., 2023).

The history of population differentiation dates back to the first half of the 20th century when two influential figures, Theodosius Dobzhansky and Ernst Mayr, shaped the field of population differentiation research and modern evolutionary studies (Coyne, 1994). Historically, studies on population differentiation relied on investigating differences in morphological, physiological or behavioural traits (Hosken & Balloux, 2002; Wootton, 2009). With the improvement of molecular biology, the focus shifted to investigating primarily molecular markers, both neutral and under selection (Martiny, 2001; Holderegger et al., 2006).

Neutral and adaptive differentiation

Population differentiation can be neutral or non-neutral. Neutral, non-adaptive population differentiation refers to genetic variation among populations that is ultimately caused by genetic drift and has no direct effect on fitness (Dobzhansky, 1977; Holderegger et al., 2006). Neutral differentiation can be promoted by extrinsic factors, such as geographic distance, which protects the differentiation from the homogenizing effect of effective migration (i.e., gene flow), or environmental fluctuation affecting population sizes. The effect of neutral factors is particularly noticeable in populations with rapid dynamics, highly fluctuating population sizes, or those displaced from their original sites and colonizing new niches (Kawecki & Ebert, 2004). Given enough time, these processes can result in genetic changes throughout the genome, not specifically concentrated around adaptive traits. As full genome sequence data are rather scarce for most diverging populations and incipient species, neutral

differentiation must be inferred from variable markers, such as mitochondrial DNA, microsatellites, or SNP loci in non-functional genomic regions (Berrieman et al., 2005; Holderegger et al., 2006; Zimmerman et al., 2020).

Alternatively, non-neutral population differentiation implies selection, and is typically associated with the divergent adaptation to local conditions. When facing different environments, local adaptation can be one of the evolutionary results. Adaptive divergence, even when caused by natural selection, is not a contingency-free phenomenon. When populations get exposed to similar selective environments, they may take dissimilar evolutionary trajectories, as they may solve similar evolutionary challenges through different strategies (Endler, 1986; Arendt & Reznick, 2008). This arises from the interaction between genetic drift and adaptive evolution. Adaptive divergence, however, does not have to be the only response to environmental variation. Individuals can show phenotypic plasticity or bet hedging providing their lineages a way to adjust to that variation, and this flexibility makes adaptive divergence less likely (Botero et al., 2015; Perry et al., 2018).

Signatures of both non-adaptive (i.e., neutral, due to genetic drift) and adaptive (i.e., non-neutral, mediated by disruptive selection) population differentiation have been found in different taxa, from prokaryotes (e.g., Vos & Velicer, 2008; Luo et al., 2021), to plants (e.g., Arroyo et al., 2008; Wang et al., 2023) or vertebrates (e.g., Gascon et al., 1998; Hatanaka et al., 2006; Friesen et al., 2007). Small invertebrates inhabiting continental waters are particularly convenient systems for studying different factors

influencing population differentiation for several reasons (see e.g., De Meester, 1996; Martens & Horne, 2000; Louette & De Meester, 2005; Peijnenburg & Goetze, 2013; Serra et al., 2019): (1) their short generation times and large population sizes provide a high evolutionary potential in both natural and laboratory environments, (2) the organisms often occupy patchy habitats along environmental gradients even at a relatively small geographic scale, and (3) their reproductive system spans from fully sexual to fully asexual, including facultative parthenogenesis, which allows to investigate the effect of different reproductive strategies on population differentiation.

Factors involved in population differentiation

Within-population genetic variability supplies raw material for evolutionary changes and population differentiation. It is one of the factors that creates opportunities for adaptation and provides resilience to populations when facing recurrent environmental changes or environmental novelties (Kassen, 2002; Raeymaekers et al., 2017; Chaturvedi et al., 2021). Genetic diversity is a result of the evolutionary history of the populations, mutation and recombination rates, the level of fluctuation and heterogeneity of the environment, and the effective population size and population density, among other factors (Booy et al., 2000; Hughes et al., 2008).

Different intrinsic characteristics of a single species may influence the capacity to create and maintain within-population diversity. Mutation rates

vary among taxa and environments and can be promoted by a series of factors such as the efficacy of repair mechanisms or exposure to certain environmental triggers (Lynch et al., 2016; Melde et al., 2022). The mode of reproduction can also influence the genetic diversity of populations (Bengtsson, 2003; Zierold et al., 2009). Sexual reproduction increases variation through recombination. Moreover, it has been shown in different phyla that bouts of sex in dominantly asexual lineages have the ability to promote large genetic diversity (Bengtsson, 2003; Tang et al., 2014).

Effective population size influences the ability to preserve a certain amount of genetic diversity. At the same time, small populations are relatively more affected by genetic drift, which can result in the random loss of some alleles and fixation of others. Reduction in population size and the evolutionary consequences of that can occur after an abrupt environmental change, habitat fragmentation, or colonization of a new habitat by a reduced number of individuals (i.e., founder effect) (Leimu et al., 2010; Slatkin & Excoffier, 2012). Genetic drift promotes directly differentiation and might be involved in rapid adaptive evolution (Chaturvedi et al., 2021).

Gene flow –i.e., the effective migration among populations– has the potential to transfer alleles from one population to another and may have contrasting effects on the recipient population. It affects both neutral and adaptive traits. When the number of migrants is high, it prevents differentiation, leading to the homogenization of populations (McKay & Latta, 2002). However, if the number of effective migrants is low enough, gene flow can supply recipient populations with adaptive genetic variants

and contribute to adaptive differentiation among populations (Baker, 1981).

While genetic drift is regarded as stochastic process, natural selection is non-random, as it promotes adaptations of individuals in a predictable way, driving changes at the population level. It is the mechanism that drives the capacity to maintain adaptive changes in the form of beneficial mutations in populations. Three types of selection –directional, stabilizing and disruptive– affect the mean of a phenotypic trait and its variance. They can act as a creative force (increasing variance in case of disruptive selection, or changing the mean in case of directional selection), or as a conservative force (stabilizing selection decreasing the variance of a trait) (Dobzhansky, 1977; Endler, 1986).

Stabilizing selection purges the deviations from the phenotypic mean of populations when this mean is at an evolutionary optimum (Yair & Coop, 2022). Most mutations have effects ranging from neutral to deleterious, and stabilizing selection helps to maintain the fitness of populations. In well adapted populations inhabiting constant environments, it helps to keep beneficial combination of alleles in populations. For some traits, stabilizing selection is self-promoted because there is an intrinsic advantage for individuals in keeping their traits similar one to each other; i.e., positive frequency dependent selection. This is the case of traits involved in mate recognition because uncommon individuals incur in the risk of not being recognized, thus losing reproductive opportunities (Heisler, 1984; Wojcieszek & Simmons, 2012; Zacchello et al., 2020).

Disruptive or directional selection promotes adaptative evolution of populations and can be especially intense after strong environmental disturbance; e.g., in case of human intervention, changes in population size, or after natural environmental changes (Hansen et al., 2012; Pilot et al., 2013).

Genetic drift, gene flow, and natural selection are constantly interacting and shaping the evolutionary path of populations. While numerous studies investigate the effect of one or two of these factors on populations, studies combining all three factors are rather scarce (but see e.g., Star & Spencer, 2013; Johnson et al., 2018).

Reproductive isolation

Reproductive isolation is one of the fundamental phenomena in evolutionary biology that shapes the course and form of life on Earth (Westram et al., 2022a). The evolution of reproductive isolation promotes diversification within species and has the potential to produce new evolutionary units (Mayr, 1963; Coyne & Orr, 2004). In the Biological Species Concept (BSC), Ernst Mayr proposed reproductive isolation to be the defining feature of a "species" (Ereshefsky, 1992). Other species concepts, regardless of extensions to (i.e., Templeton, 1989) or criticisms of (i.e., Paterson, 1985) BSC, retain reproductive isolation as a feature in species delimitation (Ereshefsky, 1992; Queiroz, 1998).

Historically, reproductive isolation referred to barriers that, at least partially, prevent organisms from different species or diverging populations of the same species from interbreeding. Admittedly, the concept does not apply to delimitation of obligate asexual species. Nowadays, the set of factors with effects on reproductive isolation is still a matter of debate (see Westram et al., 2022a, 2022b and the references thence). Intrinsic isolation refers to barriers that do not depend on the distribution of species or populations, regardless of whether the distribution is due to historical geographic factors or ecological factors. These barriers result from features of the species/population reproductive system itself. Contrarily, extrinsic barriers, sometimes not considered to be barriers by some authors, typically refer to external factors or adaptations that do not directly operate on reproductive system, i.e., geographical or ecological isolation (Coyne & Orr, 2004; Matute et al., 2009).

Reproductive isolation barriers can act at different stages of reproduction, from encounter probability, mate recognition and courtship display (if applicable), through reproductive cell interaction, zygote development, to viability and fertility of the offspring (Mayr, 1963; Coyne & Orr, 2004), with a relevant distinction of isolation between premating and postmating isolation (Table 1.1).

Table 1.1 Classification of reproductive isolation barriers and its mechanisms in animals (modified from Dobzhansky, 1977; Coyne & Orr, 2004).

		mechanism	intrinsic [1]
premating	geographic	potential mates do not meet due to physical distance	NO
	ecological	potential mates do not meet due to ecological or adaptive constrains	NO
	behavioural	potential mates meet but do not mate due to incompatibilities in courtship behaviour or mating rituals	YES
postmating prezygotic	gametic isolation	female and male gametes fail to interact properly	YES
postmating postzygotic	hybrid unviability, infertility, breakdown	reduces fitness of offspring	YES

[1] Direct natural selection may act on intrinsic barriers and may not act on extrinsic barriers.

As a result of this complexity, the barriers involve a wide range of factors, e.g, spatial-temporal, for populations inhabiting different localities or the same locality in different time; ecological, for adapted populations inhabiting different ecological niches; behavioural during the mating; physiological barriers in form of structural differences of reproductive organs, or general genetic incompatibilities, among many others (Coyne & Orr, 2004). The general outcome is to impede gene exchange between species or populations and thus recombination among genomes (e.g., Orr,

2005; Matute et al., 2009; Matute, 2010). Consequently, it maintains population divergence, genetic integrity, and advantageous combinations of alleles arising through adaptive divergence. Reproductive isolation can avoid unproductive mating attempts (Coyne & Orr, 2004). However, it may also negatively influence populations by preventing intraspecific adaptive introgression (Edelaar et al., 2012; Tigano & Friesen, 2016).

Evolution of premating isolation, i.e., isolation that reduces the probability of mating, has a selective advantage in diverging populations (Coyne & Orr, 2004). It prevents the creation of unfit offspring at the earliest stages of reproduction and can be under direct selection. The evolution of postmating isolation, (i.e., decreased fertility, sterility or unviability of offspring) is regarded as a by-product of the evolutionary divergence of genetic systems. It might be selected to save resources (e.g., via lack of parental care) in unfit offspring. Moreover, postmating isolation might operate as a selective pressure on premating isolation. When populations that previously established some levels of postmating reproductive isolation (usually in allopatry) come into secondary contact, after between-population crossing their offspring may show inferior phenotypes presenting reduced fitness or fertility (Butlin, 1987b; Liou & Price, 1994; Nosil et al., 2003). This might create a selective pressure for premating isolation, a phenomenon known as reinforcement (Butlin, 1987a; Servedio & Noor, 2003).

The evolution of reproductive isolation is a tangled process influenced by a number of factors that often act together, either promoting or opposing each other. Additionally, the genetic architecture underlying reproductive

isolation is complex (Coyne & Orr, 2004). Even in fully formed species, specific components of reproductive isolation can be imperfect or “leaky” (e.g., Short, 1997; Jankowski & Straile, 2004; Gribble & Welch, 2012). The evolution of premating reproductive isolation is challenging because stabilizing selection usually acts on traits involved in mating, ensuring individuals to find mating partners and maximizing mating success.

Rare phenotypes have a low probability of finding similarly rare mates (Heisler, 1984; Wojcieszek & Simmons, 2012). However, when diversification occurs, and reproductive isolation is at least partially present, it becomes advantageous to avoid intermediate phenotypes. Thus, directional selection often takes over and can lead to rapid evolution of reproductive isolation (Swanson & Vacquier, 2002; Wilburn & Swanson, 2016).

In populations where gene flow is prevented by external factors, such as geographic distance (isolation by distance) (Wright, 1943), reproductive isolation can evolve neutrally as a consequence of gradual random changes in the genome (e.g., mutations or genetic drift) (Palumbi, 1994; Orr, 2005), and may stay incomplete or even may not always form (Nice et al., 2002; Nosil et al., 2009; Cotto & Servedio, 2017). These diverging populations can still successfully reproduce in case of secondary contact or under laboratory conditions (Abbott et al., 2013).

Objective, hypotheses and outline of the thesis

The aim of this thesis is to address reproductive isolation between populations of small aquatic zooplankter *Brachionus plicatilis* (Müller 1786) (Ciros-Pérez et al., 2001). The objective is to find patterns and relationships that can provide insights into the evolution of reproductive isolation. Previous studies of *B. plicatilis* populations in Eastern Iberian Peninsula have characterised these populations from the point of view of phylogenetic divergence (Gomez et al., 2002) and the adaptive response to unpredictability of the habitats (Franch-Gras et al., 2017). Thus, this system provides an ideal opportunity to investigate the reproductive isolation between populations and assess the potential for within-population reproductive preferences.

A population-study approach was adopted here. Typically, reproductive isolation between species or populations in rotifers have been based on many individuals belonging to a single or a few clones (e.g., Hagiwara et al., 1994; Suatoni et al., 2006; Kim et al., 2017). A small number of biological replicates per population allows robust phylogenetic inferences (Gomez et al., 2007, Gomez et al., 2002). However, such small numbers do not allow to conclude on populational patterns when the focus is on phenotypes or on within-population genetic diversity. This brought us to allocate a large part of the research effort on biological replicates; that is, studying many clones from each population.

The following hypotheses are proposed and subjected to investigation:

1. Partial reproductive isolation exists between different populations *of B. plicatilis* inhabiting waterbodies in the Iberian Peninsula.

2. The partial reproductive isolation, if present, is correlated with phylogenetic distance among populations.

3. The partial reproductive isolation, if present, is correlated with the adaptation of *B. plicatilis* populations to the level of environmental predictability.

4. The partial reproductive isolation, if present, is correlated to the divergence in locus which product, mating recognition protein (MRP), is responsible to male-female recognition in *B. plicatilis*.

After this introductory **Chapter 1**, the thesis is organized as follows:

Chapter 2 describes the general methodological approach of the thesis, introducing the model organism with its main biological features and describing the study site.

Chapter 3 deals with intrinsic reproductive isolation acting just after a random female-male encounters. To quantify the degree of behavioural reproductive isolation, mating reproductive assays were conducted in two groups of *B. plicatilis* populations: (1) populations with reduced or absent gene flow due to geographical distance and belonging to different phylogeographic groups, and (2) neighbouring populations (i.e., with

presumptive gene flow) from the same phylogeographic group with adaptive divergence.

In **Chapter 4**, the number neighbouring populations studied in Chapter 3 was broadened, as Chapter 3 showed stronger isolation signatures in this group compared to a group with substantially reduced or absent gene flow. The preference for within-population reproduction was assessed by quantifying deviation from Hardy-Weinberg proportions from the diapausing egg produced in admixed laboratory populations.

The goal of the **Chapter 5** was to assess the genetic variation of MRP, a protein responsible for male-female recognition in *B. plicatilis*. All the populations that were used previously (i.e., in Chapters 3 and 4) were genotyped for the *mmr-b*, a gene coding MRP, along with the variation in microsatellites. The latter was used to evaluate genetic diversity in neutral markers.

Chapter 6 focuses on a general discussion of the main results obtained in the thesis, discuss future perspectives and enumerates the conclusions.

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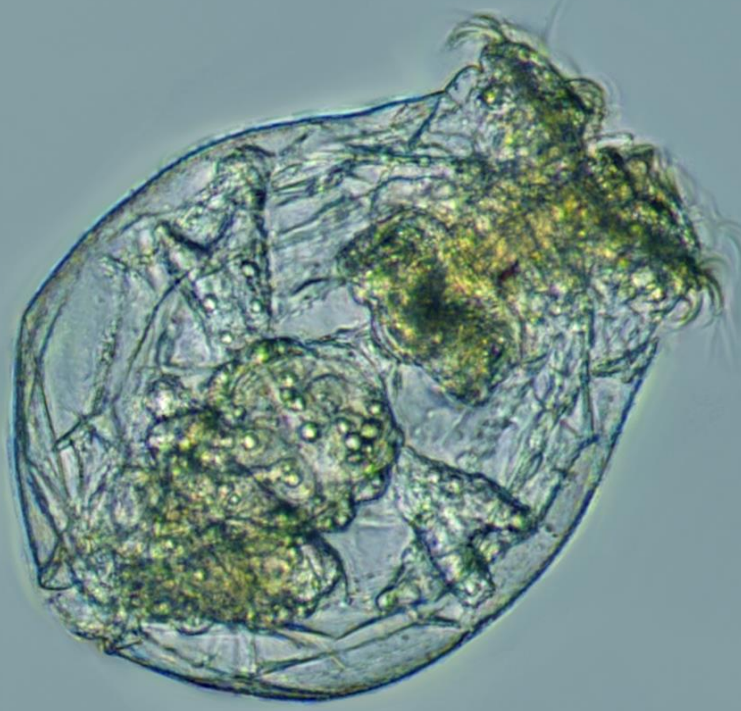

Chapter 2

Methodological approach: Rationale for the research model

Model organism: the rotifer *Brachionus plicatilis*

As stated in the first chapter, microinvertebrates inhabiting continental water are good evolutionary models to address the interplay between population differentiation and the reproductive system. In this dissertation we have used the monogonont rotifer *B. plicatilis* (Fig. 2.1) as a model organism.

The phylum Rotifera ('rotifera', meaning "wheel-bearing" organisms, due to the impression of rotation created by their ciliated corona) comprises more than 2000 named species (Wallace et al., 2015; José de Paggi et al., 2020), but the actual species richness is uncertain, as a considerable number of cryptic species complexes have been discovered (Gomez et al., 2002b; Fontaneto et al., 2008; Kordbacheh et al., 2017; Mills et al., 2017). Rotifers are diminutive multicellular eukaryotes; the body length varies between (40 µm - 2 mm). They are eutelic, i.e., the number of cells is predetermined and does not change during the rotifer life. Despite their size, rotifer individuals may be anatomically complex.

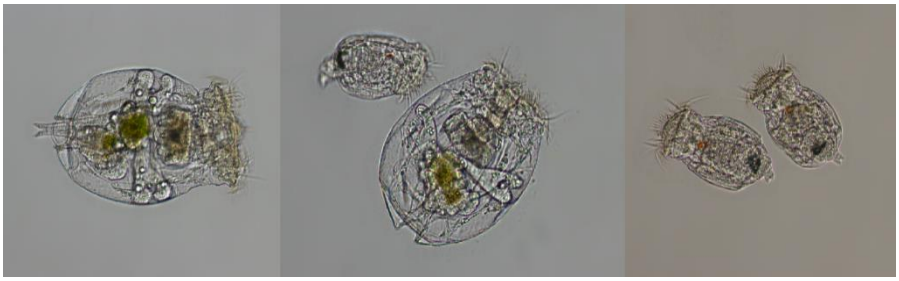

Fig 2.1 *B. plicatilis* female, female with male and two males (from left to right).

Rotifers are one of the most abundant metazoan groups in the zooplankton. They are considered to be globally expanded, although an individual species only sporadically has a cosmopolitan distribution (Dumont, 1983; Segers, 2007). They can be found in all types of continental aquatic habitats of varied sizes or environmental conditions and form part of a variety of communities. Some of them inhabit extreme habitats, such as hot springs, drops of water in mosses and lichens or localities with high radiation (Wallace et al., 2005; Iakovenko et al., 2015; Rebecchi et al., 2019). This ability to survive in wide range of environments is mediated by the production of resistant stages (Schröder, 2005), as well as their high capacity to disperse (Bohonak & Jenkins, 2003), and quickly colonize new environments (Gomez et al., 2002a; Schröder, 2005).

The phylogenetic position and major taxa of the phylum remains controversial (Mark Welch, 2000). At least two classes of rotifers have been proposed: (1) Pararotatoria, composed of a single family (Seisonidae), and (2) Eurotatoria, with two subclasses. These subclasses are Bdelloidea and the most numerous and often planktonic rotifers Monogononta (Segers, 2008; Wallace et al., 2015). In these three main groups (Seisonidae, Bdelloidea and Monogononta), three types of reproduction can be found: Seisonidae are obligatory sexual organisms, Bdelloidea are obligatory asexuals, and Monogononta are cyclical parthenogens; that is, they combine sexual and asexual generations (Wallace et al., 2015).

Typically, Monogononta populations are temporarily (e.g., seasonally) active in the water, and this period of activity is named growing season. The common life cycle of Monogononta is as follows (Fig. 2.2). At the

beginning of the growing season, diploid rotifer females hatch from the diapausing (resting) eggs in the sediment, start to reproduce clonally through parthenogenetically produced asexual subitaneous eggs and give rise to clones of diploid asexual females in the water column (Serra et al., 2018).

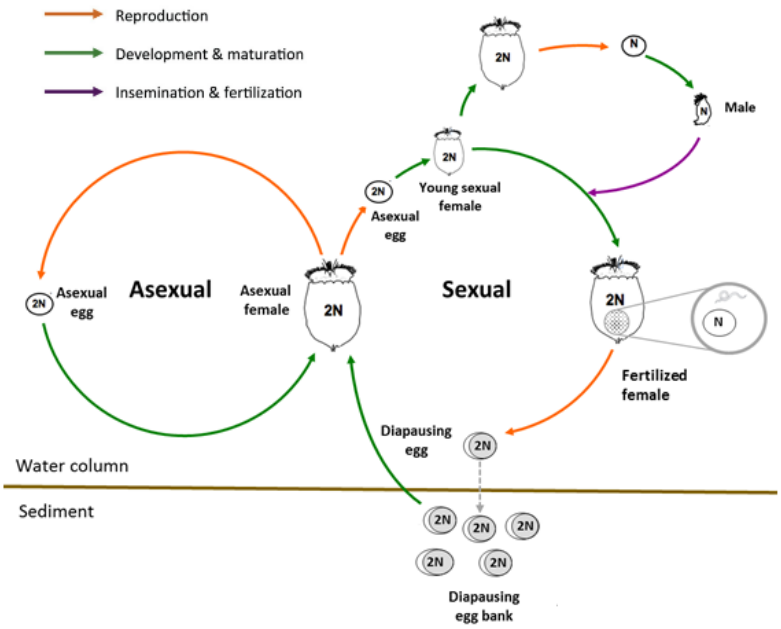

Fig 2.2 Life cycle of the monogonont rotifer *B. plicatilis* (modified from Serra et al., 2018).

This asexual reproduction allows populations to achieve high densities in a relatively short time. Sexual reproduction is induced by population density after an obligatory period of asexual reproduction (Hagiwara & Hino, 1989; Stelzer & Snell, 2003). The density threshold to induce sex is dependent on a series of factors and varies from population to population (Snell & Boyer,

1988; Franch-Gras et al., 2017b). After reaching the threshold, asexual females produce diploid sexual females (daughters) as a part of their offspring. The sexual females produce haploid eggs which, if not fertilized, give rise to haploid males. The males are dwarf and anatomically and functionally reduced compared to females. When sexual females get inseminated in the first hours of their life (Snell & Childress, 1987; Snell & Hoff, 1987), their gametes are fertilized, and they produce diploid diapausing eggs (Fig. 2.3). Despite being called "eggs", these are actually embryos. Diapausing eggs, after being carried by the mother for some time, sink to the sediment and eventually hatch in one of the next growing seasons (Wallace et al., 2015).

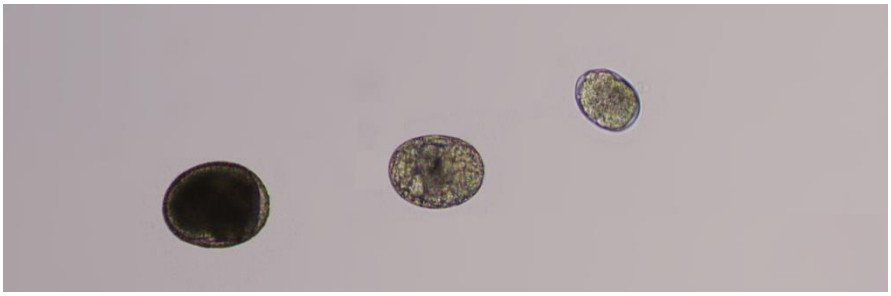

Fig 2.3 Different types of *B. plicatilis* eggs: Diapausing egg, subitaneous eggs giving rise to asexual females and eggs giving rise to males (from left to right).

Mating in rotifers is a three-step sequence: encounter between female and male, circling and copulation (Fig. 2.4). Rotifers do not release any pheromone to the environment and the encounter is random. However, mate recognition is mediated through contact chemoreception, with the mate recognition protein (MRP) as a major component of it (Snell & Morris, 1993; Snell et al., 1995; Gribble et al., 2011).

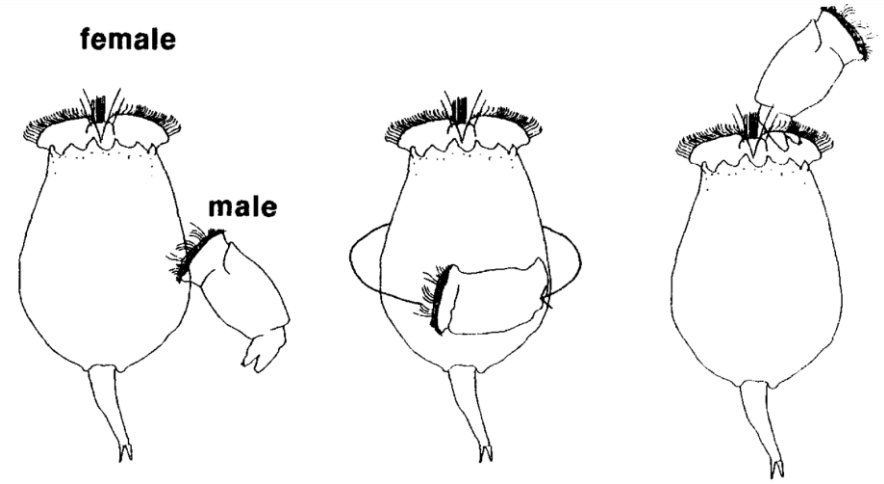

Fig. 2.4 *B. plicatilis* mating behaviour, encounter, circling and copulation (from left to right) (Gomez & Serra, 1995).

The reproductively active population is recreated every year by the hatchlings from diapausing eggs deposited in sediment. Clonal reproduction is supposed to lead to reduction of genetic variability. However, the large size of rotifer populations (Wallace et al., 2015), together with the bouts of sexual reproduction, is believed to generate and maintain genetic variability within these populations. Some diapausing eggs do not hatch in the growing season following the one in which they were produced. As a result, an egg bank exists in the sediment of the water bodies, and this is accountable for maintaining genetic variability within populations (Hairston, 1996; Montero-Pau et al., 2017).

Facultative parthenogenesis itself offers different evolutionary opportunities. Rapid reproduction without the need of a mate during the

clonal phase lowers the cost of sex –i.e., the cost of males and, in this case, the cost of diapause (Serra & Snell, 2009)– and can be beneficial in case the of colonization of a new habitat, rapid change of environmental conditions, etc. (Stelzer, 2011). Bouts of sexual reproduction creates a potential for acquiring new genetic variability, and thus a potential for new adaptations (Hairston, 1996; Goddard et al., 2005). Periods of clonal reproduction, when not followed by adequate periods of sexual reproduction that would allow the formation of resting forms in sufficient amount, may negatively affect the survival of populations during unfavourable conditions (Stelzer, 2011). Besides the production of resting stages and genetically diverse offspring via sexual reproduction, rotifers possess other strategies to lower the probability of lineage extinction during harsh periods. In environments that are characterized by predictable, though fluctuating, environments, phenotypic plasticity can evolve to respond to different environmental conditions by producing alternative phenotypes (Stelzer, 2002). In unpredictable environments, on the other hand, bet hedging may evolve (Gilbert & Schröder, 2007; García-Roger et al., 2014, 2017).

Populations of *B. plicatilis* offer a good opportunity to conduct evolutionary ecology research due to the number of features (Serra et al., 2019). As a result of previous studies we know that (1) large genetic diversity is harboured within populations (Gomez & Carvalho, 2000; Ortells et al., 2006; Montero-Pau et al., 2017), (2) historical events have shaped its phylogeographic structure (Gomez & Carvalho, 2000; Campillo et al., 2011a), (3) their populations show divergence in ecologically relevant traits (Campillo et al., 2011b), and (4) their populations show differential

adaptation to environmental unpredictability (Franch-Gras et al., 2017b), which is of particular interest to this thesis.

The Iberian Peninsula as field laboratory

The Iberian Peninsula is of major evolutionary interest, as it is one of the most important Pleistocene glacial refugia in Europe (Hewitt, 2004; Abellán & Svenning, 2014). It allowed for the survival and differentiation of populations and served as species and genetic diversity repository for post-glaciation periods, and thus drove phylogeographic pattern in Europe (Gomez & Lunt, 2007; Millette et al., 2011). In the evolutionary history of aquatic organisms inhabiting inland water bodies, the complex system of ponds made this region a "refugia within refugia" (Gomez & Lunt, 2007; Abellán & Svenning, 2014).

In the Spanish populations of *B. plicatilis* three phylogeographical clades have been described previously, based on neutral markers (COI): Northern, Southern and Coastal (Gomez et al., 2002a). Within the Southern clade, a group of populations has been studied in-depth. At a small geographical scale (240 km^2), the water bodies inhabited by rotifers substantially varied in water regime fluctuation and predictability (Franch-Gras et al., 2017a). Moreover, it was found that propensity for sexual reproduction (an important life history trait in *B. plicatilis* life cycle) was strongly correlated with the level of environmental unpredictability in these localities (Franch-Gras et al., 2017b). In less predictable environments, rotifers tend to be more prone to start sexual reproduction earlier, ensuring the production

of at least some diapausing eggs in case of an unexpectedly short growing season.

Therefore, due to (1) the phylogeographic structure affecting geographically distant areas and (2) the ecological differentiation related to environmental predictability in a single area, Iberian populations of *B. plicatilis* provide a good scenario for testing the influence of these factors on the evolution of intrinsic reproductive isolation.

The list of populations with basic information about their ponds, and affiliation to different chapters can be found in Table 2.1 The geographic location of the ponds is displayed in Fig. 2.5.

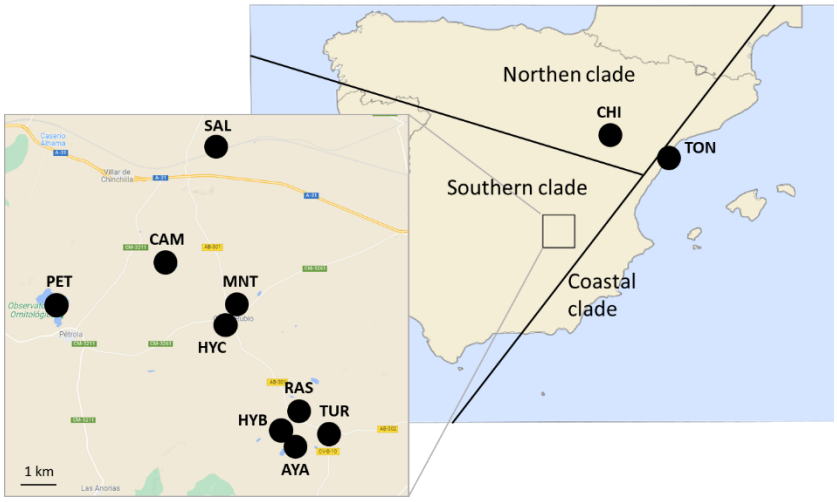

Fig. 2.5 Geographic location of the populations of *B. plicatilis* used in the thesis in Iberian Peninsula.

Table 2.1 Studied populations of *B. plicatilis*, basic characteristics of their ponds. Their use in three main chapters of this thesis is shown.

Population	Acronym	Location[1]	Pond surface area (m^2)	Clade[2]	Ch. 3[3]	Ch. 4[4]	Ch. 5[5]
Atalaya de los Ojicos	AYA	38°46'20"N, 1°25'49"W	75.000	S	×	✓	✓
La Campana	CAM	38°51'29"N, 1°29'36" W	29.000	S	×	✓	✓
Hoya Yerba	HYB	38°46'46"N, 1°26'06"W	1.060	S	✓	✓	✓
Hoya Chica	HYC	38°49'46"N, 1°27'49"W	32.000	S	×	✓	✓
Hoya del Monte	MNT	38°50'44"N, 1°26'38"W	15.800	S	×	✓	✓
Pétrola	PET	38°50'16"N, 1°33'49"W	1.190.000	S	×	✓	✓
Hoya Rasa	RAS	38°47'06"N, 1°25'37"W	40.000	S	✓	✓	✓
Salobralejo	SAL	38°54'52"N, 1°28'06"W	237.000	S	✓	✓	✓
Hoya Turnera	TUR	38°46'36"N, 1°24'37"W	26.000	S	×	✓	✓
Chiprana	CHI	41°14'20"N, 0°11'02"W	230.000	N	✓	×	✓
Torreblanca Norte	TON	40°08'54"N, 0°10'07"E	120	C	✓	×	✓

[1] Datum WGS84.

[2] Phylogeographic clades: S, Southern; N, Northern; C, Coastal (Gomez et al., 2002a).

[3] Ch. 3 Insight into incipient reproductive isolation in diverging populations of *Brachionus plicatilis* rotifer.

[4] Ch. 4 Development of reproductive barriers in sympatry.

[5] Ch. 5 Genetic Variability of the Mating Recognition Gene in Populations of *Brachionus plicatilis*.

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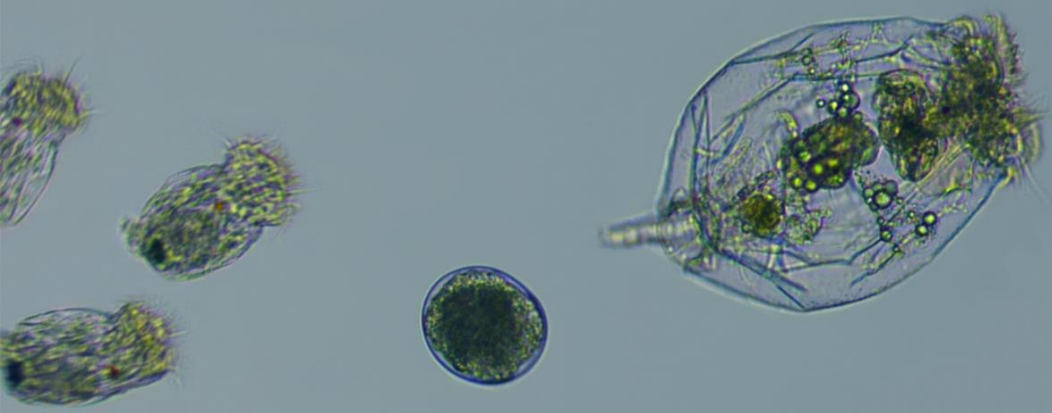

Chapter 3

Insight into incipient reproductive isolation in diverging populations of *Brachionus plicatilis* rotifer

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Abstract

The emergence of reproductive isolation is key in maintaining within- and between-species diversity and one of the initial steps of speciation. In the Iberian Peninsula, the diverging populations of the *Brachionus plicatilis* rotifer create an ideal system to shed light on the mechanisms that give rise to the emergence of reproductive isolation. Herein, we quantify the degree of behavioural reproductive isolation in two groups of *B. plicatilis* populations, namely, neighbouring populations diverging by adaptation to the local environment and populations diverging in the absence of gene flow due to geographic distance. We conduct behavioural no-choice assays to test mating reproductive isolation between these populations. The analysis shows signatures of ongoing behavioural reproductive isolation in most of the population crosses, which is more pronounced in populations with a higher level of adaptive divergence, presumably under high migration rates. Overall, this study suggests that local adaptation is associated with mating behaviour resulting in reproductive isolation.

Keywords

Mate recognition; Behavioural Isolation; Speciation; Population divergence; Reproductive barriers

Introduction

Reproductive isolation has strong implications for both genetic differentiation and evolutionary dynamics, affecting population structure, local adaptation, speciation and sister species coexistence (Coyne & Orr, 2004; Weber & Strauss, 2016). There is a wide range of intrinsic and extrinsic factors that could contribute to isolation. The causal loops that might occur among them make the dissection and testing of such a panoply complicated (Palumbi, 1994; Coyne & Orr, 2004).

One of the factors leading to reproductive isolation between populations may result from the difficulty of immigrants arriving, surviving and/or becoming sexually involved in the recipient population (Sexton et al., 2014; Turbek et al., 2018). If arrival rate is the prevalent factor, then a pattern of isolation by distance (IBD) is expected to emerge due to the combined, opposite effect of migration and genetic drift (Wright, 1943; Church & Taylor, 2002). On the other hand, if unmatched timings of dispersal/reproduction and survival are the prevalent factors, then a pattern of isolation by environment (IBE) is expected (Nosil et al., 2005; Wang & Bradburd, 2014). Local adaptation is likely to be involved in this second case (Nosil et al., 2002). IBD and IBE have been found in a broad range of organisms (IBD, e.g., Zeller et al., 2006; Mills et al., 2007; Campillo et al., 2011; Ventura et al., 2014; IBE, e.g., Shafer & Wolf, 2013; Martin et al., 2021) and may act together (Sexton et al., 2014; Weber et al., 2017). In both scenarios—IBD and IBE—low gene flow would promote behavioural reproductive isolation—i.e., based on mate preferences—due to genetic drift acting on genes that affect behaviour. Moreover, the genetic

hitchhiking of behavioural genes linked to genes for local adaptation might play a role in the case of IBE (Hawthorne & Via, 2001; Wu, 2001, but see Parchman et al., 2013). In addition, if genetic divergence, either adaptive or nonadaptive, is associated with at least some degree of post-mating isolation, then selection for behavioural isolation would occur because it results in an optimized allocation of parental resources, i.e., avoiding the production of hybrid offspring. Thus, the degree of behavioural isolation is expected to increase with the geographic and environmental distance between populations and concomitantly with the genetic distance in neutral markers. Notably, regardless of how behavioural isolation arises, it is expected to reinforce genetic divergence in neutral markers, which illustrates the two-way causal links that might be at work.

A relatively high number of studies have focused on testing premating isolation between phylogenetically close species. This includes species studies in both vertebrates and invertebrates (Schröder & Walsh, 2010; Arthur & Dyer, 2015; Carranza et al., 2017; Luo et al., 2017; Cowles & Uy, 2019; Langton-Myers et al., 2019; Maltseva et al., 2021; Turbek et al., 2021) and has sometimes been associated with identifying cryptic speciation (Gomez & Serra, 1995; Schröder & Walsh, 2007; Juárez et al., 2015; Meguro et al., 2016; Castro Vargas et al., 2017; Ismail & Brooks, 2018). However, the evolution of premating isolation between populations within the same species has received much less attention (Jiggins et al., 2004; Sobel & Streisfeld, 2015; Cruz-Yepez et al., 2020). In this sense, microscopic aquatic invertebrates inhabiting spatially fragmented habitats such as lakes, ponds and lagoons offer an ideal model

to study the effect of population isolation on reproductive isolation within species. Despite having the potential for high dispersal capability due to the small body size (< 1 mm length) (Bohonak & Jenkins, 2003; Louette & De Meester, 2005; Ventura et al., 2014), they show deep genetic divergence, which is a phenomenon known as the "dispersal-gene flow paradox" (De Meester et al., 2002). At the same time, living in a wide range of environmental conditions, local adaptation has been described in a variety of zooplankton species (Declerck et al., 2001; Declerck & Papakostas, 2016; Franch-Gras et al., 2017a).

Brachionus plicatilis Müller 1786 is a cyclical parthenogenetic rotifer that inhabits saline and brackish ponds. Its predominant type of reproduction is ameiotic parthenogenesis (Gilbert, 1963; Wallace et al., 2015; Serra et al., 2018). Its natural populations, which dwell in the water column temporarily within a year, are composed mainly of asexual (also called amictic) females. Sexual reproduction is initiated at high population densities (Stelzer & Snell, 2003) and starts with the production of sexual (mictic) daughters (Snell & Boyer, 1988). Sexual females, which produce meiotic eggs, have two mutually exclusive reproductive fates: to become male producers if not inseminated by a male while they are young or to become diapausing egg producers otherwise (Serra et al., 2018). Sexual and asexual females are morphologically identical, but they can be identified according to the eggs they carry.

Mating behaviour in rotifers is a three-step sequence: male–female encounter, circling and copulation. As rotifers do not secrete any sexual pheromones into the environment (Snell et al., 1995), male–female

encounters occur randomly (Snell & Garman, 1986). After the encounter, the ciliated corona of the male contacts a female's body (Snell & Morris, 1993; Snell et al., 1995), and the male displays a circling behaviour, i.e., swimming around the female while maintaining contact by his corona. After several circlings, the male locates either the female's corona or the foot opening, and hypodermic insemination takes place (Gomez & Serra, 1995). The whole mating behaviour can take from tens of seconds to several minutes, and the three-step sequence can be interrupted at any time (Snell & Hawkinson, 1983; Snell & Hoff, 1987). A surface glycoprotein (mate recognition protein, MRP) present on the female's body is involved in recognition of a mate (Snell & Morris, 1993; Snell et al., 1995; Gribble et al., 2011). Although mate choice is mostly driven by males, females may exhibit a reaction by varying their resistance to male circling (Snell et al., 2007). Female susceptibility to fertilization and male capacity for fertilization decline with age (Snell & Childress, 1987). Male rotifers tend to copulate more often with young females but show no preference for sexual over asexual females (Gomez & Serra, 1996).

In the Iberian Peninsula, populations of the cyclically parthenogenetic rotifer *B. plicatilis* create an ideal system for studying intrinsic factors resulting in some level of reproductive isolation. The species consists of highly divergent lineages (Gomez et al., 2000), reflecting strong founder effects with subsequent resource monopolization by the founder genotypes. Additionally, Iberian populations of *B. plicatilis* inhabit highly diversified habitats, from ephemeral—and highly environmentally unpredictable—to permanent ponds. They show local adaptation affecting

their sexual reproduction patterns, adjusting them to variation in environmental predictability (i.e., high propensity to sex in populations with low environmental predictability Franch-Gras et al., 2017a, b). A recent study on the diversity of the gene *mmr-b*, which is involved in mate recognition, has shown that despite being under stabilizing selection, a correlation is found between gene diversification and differences in environmental factors when comparing *B. plicatilis* populations (Jezkova et al., 2022). *Brachionus plicatilis* belongs to a species complex, and the divergence of *mmr-b* among the species of the complex has also been documented (Gribble & Welch, 2012).

In this research, we tested the hypothesis that evolution of premating behavioural isolation occurs between populations of the species *B. plicatilis*. We hypothesized that the ecological divergence is correlated with that isolation. We used bidirectional no-choice mating tests to investigate within-species mating preferences in populations belonging to different phylogeographic groups or showing ecological divergence. The results were used to assess the consistency of different evolutionary hypotheses, and phylogeographical and ecological divergences were found to play a role in the establishment of behavioural reproductive isolation.

Material and methods

Study populations

We selected two groups of populations of *B. plicatilis* inhabiting shallow ponds in eastern Spain. One group was based on known phylogeographic

structure, with each population belonging to a different clade, and the other was based on known differentiation in adaptation to local conditions (Table 3.1).

Table 3.1 Studied populations of *B. plicatilis* and their ponds.

Pond name	Acronym	Group	Pond location[a]	Pond surface area [km^2]
Hoya Rasa	RAS	LA	38°47'N, 1°25'W	0.047[b]
Hoya Yerba	HYB	LA	38°46'N, 1°26'W	0.001[b]
Salada de Chiprana	CHI	PS	41°14'N, 0°11'W	0.230[c]
Salobralejo	SAL	PS, LA	38°54'N, 1°28'W	0.360[c]
Torreblanca Norte	TON	PS	40°10'N, 0°10'E	0.012[c]

Populations are grouped into phylogeographically structured populations (PS) and locally adapted populations (LA).

[a.] Datum EPSG 3857.

[b.] Ortells et al., 2000.

[c.] Franch-Gras et al., 2018.

For the group of phylogeographically structured populations (PS), we chose three populations, each with a dominant haplotype belonging to divergent mtDNA clades and showing a high degree of pairwise population differentiation (Gomez et al., 2002) (Table 3.2). The selected populations were Salada de Chiprana (CHI) from the northern clade, Torreblanca Norte (TON) from the coastal clade and Salobralejo (SAL) from the southern clade. For the group of locally adapted populations (LA), we chose three populations belonging to the same phylogeographic clade but differing in

their propensity for sexual reproduction, which correlates with environmental unpredictability (Franch-Gras et al., 2017a, 2018). The selected populations were Hoya Yerba (HYB), Hoya Rasa (RAS) and Salobralejo (SAL), corresponding to low, medium and high predictability, respectively. The pairwise F_{ST} values of the populations and geographic distances between their localities are shown in Table 3.2.

Clone isolation and culture conditions

The sediment of the ponds was sampled in 2013. Diapausing eggs were extracted from the sediment in 2017 using a sugar flotation technique (i.e., Gomez & Carvalho, 2000), and rotifer clones were established from isolated, individually hatched eggs. As *B. plicatilis* belongs to a cryptic species complex, hatchlings were taxonomically identified based on restriction fragment length polymorphism (RFLP) analysis of a fragment of the mitochondrial gene COI (Campillo et al., 2005). Individual clones (30–40 per population) were maintained in the laboratory in 15 ml stock cultures and grown in a culture of the microalgae *Tetraselmis suecica* in artificial seawater (Instant Ocean® Sea Salt, Aquarium Systems) at 12 g l^{-1} salinity, f/2 enriched medium (Guillard & Ryther, 1962), and at 25°C under constant illumination (PAR: approx. 35 µEm^{-2} s^{-1}). These conditions, hereafter referred to as the standard, were also used with minor modifications in the experiments. Every week, a small volume of each culture was transferred to fresh medium, and the rest was discarded.

Table 3.2 Pairwise genetic differentiation and geographic distance between populations

Group	Population	Population		
		SAL	RAS	HYB
LA	SAL	--	0.431	0.414
	RAS	15	--	0.054
	HYB	15	1	--
Group	Population	Population		
		SAL	TON	CHI
PS	SAL	--	0.52	0.58
	TON	198	--	0.59
	CHI	280	123	--

The two upper 3x3 hemi-matrices show the FST values from six microsatellite loci obtained in Montero-Pau et al. (2017; for LA) and Gomez et al. (2002; for PS). All pairwise FST values were significant at $p < 0.001$. The lower 3x3 hemi-matrices show the geographic distance in km (see Table 3.1 for population acronyms).

Mating experiments

Pre-experimental cultures were established by placing five asexual, ovigerous females of each clone into a flask with 40 ml artificial seawater and *T. suecica* algae at a concentration of 2×10^5 cells ml^{-1}. The aim was to achieve exponential growth, to induce sexual reproduction and to obtain a high abundance of both males and females. Thus, the flasks were checked daily until sexual reproduction was observed (i.e., when the first males were detected, after 4–7 days).

In the mating assays, young individuals (not older than 3 h) of both sexes were used to maximize the potential for mating responses. To collect virgin new-borns, eggs were detached from the female's body by vigorous shaking of the flasks. This method has been proven efficient and harmless

for eggs and hatchlings (Gomez & Serra, 1996). The eggs were sorted according to their type (smaller—male—and larger—female—eggs), collected using a micropipette, placed separately in the standard conditions with no algae addition and allowed to hatch. For each mating assay, one clone provided females (25 individuals), and a different clone provided males (six individuals). The observation protocol followed the experimental setup used in Gomez & Serra (1995), but the observation time was increased to 10 min. All the females were placed together in 50 µl fresh medium at 25°C with no algae, and males were added sequentially. After the addition of each male, the mating behaviour was observed for 10 min and then replaced with another male. This was repeated until all six males were used. The experimental volume was kept constant, as individual males were added and removed in a small volume of medium of approximately 2 µl. Observations were conducted under a stereomicroscope (Olympus SZX10, Japan), and the number of encounters, circlings and copulations was recorded according to Gomez & Serra (1995) and Snell & Hawkinson (1983). We defined an encounter as any physical contact between the male's corona and the female's body, a circling (implying previous encounter) was recorded when the male swam around the female body at least once, and copulation occurred when the male penetrated the female's soft body parts (corona or foot opening). Circling always preceded copulations.

Nine population crosses were carried out for each population group (PS and LA) using males and females in all possible pairwise combinations (i.e., three intrademic and six interdemic combinations). Each population cross

was replicated five times (9 crosses × 2 population groups × 5 replicates = 90 mating assays; note that six sequential measures—one per male—were obtained for each mating assay). Replication consisted of changing the clones in the mating assays. No clone was used in more than one assay within a group (5 populations × 30 clones per population = 150 clones). The experiment lasted 4 months, and one to two, rarely three, mating assays were performed per day. A single observer (I.J.) recorded the data between March and July 2018, and a previous anonymization of the experimental cultures was adopted to prevent observation bias.

Data analysis

Variation in individual male activity within an assay, albeit included in the experimental procedure, was not considered a factor in our data analysis (i.e., the counts from six males were summed up). For each population combination, we calculated the average percentage of circlings resulting from encounters (hereafter, "circling after encounter"), the average percentage of copulations resulting from circlings ("copulation after circling") and the average percentage of copulations resulting from encounters ("copulation after encounter"), the last one being a compound of the other two.

We used a GLMM (as implemented in the lme4 package in R software, version 3.6.2) (Bates et al., 2015; R Core Team, 2019) to analyse the significance of the effects in the experimental design on copulations after encounters. Our restriction to this response was oriented to avoid

dependence in our statistical analysis while favouring the biological relevance in focus. GLMM was applied separately to the PS and LA study groups. Based on the Bayesian information criterion, we assumed a binomial error distribution (assessed against a Poisson distribution with fate—copulation or not—as an additional factor). For the selected distribution, its canonical link function (logit) was used. The factors in the GLMM were (1) male-providing population (MP), (2) female-providing population (FP), (3) their interaction (MP:FP) and (4) clone pair as a random factor. Notice that the MP:FP interaction accounts for a deviation from independence (i.e., when a specific population combination deviates from the average copulations after encounters of the two populations involved). Therefore, to find a behavioural propensity against interdemic mates, a significant MP:FP interaction is necessary; however, the MP:FP effect of intrademic crosses must also be higher (i.e., relatively more copulations vs. encounters) than in interdemic crosses. Hence, to quantify this propensity between population crosses, we estimated coefficients associated with the interaction effect. These coefficients were obtained by additively decomposing the proportion of copulations (vs. encounters) observed in a mating assay. The components were (1) a (grand) mean value, (2) a component due to the male-providing population (mean deviation of that population when providing males), (3) a component due to the female-providing population (mean deviation of that population when providing females) and (4) the residual, which retains the interaction (MP:FP) and the random variation among mating assays. The decomposition follows the approach in Tortajada et al. (2009). The 45 coefficients accounting for the MP:FP interaction for each mating assay in each study group (PS or LA)

were inspected to check whether the MP:FP of intrademic crosses was higher than that of interdemic crosses.

Additionally, to compare with other studies, we also computed the relative deficit–excess of interdemic copulations using a reproductive isolation index (RI, Sobel & Chen, 2014), although it presents caveats, such as that this index is affected by the indiscriminating mating activity, and mating activity might be largely sensitive to the experimental environment. RI, computed as follows:

$RI = 1 - 2\frac{interdemic\ copulations}{total\ copulations}$ (range from − 1 to + 1) indicates the strength of the effective prezygotic barrier (opposing gene flow) in the experimental conditions.

One-sided t test on Spearman's rank correlation coefficient using the cor.test function implemented in R software, version 4.1.2. (R Core Team, 2021) was applied to assess the correlation between our mating behaviour metrics (vectors of interaction coefficients and RI) and different variables linked to the population characteristics (genetic differentiation in neutral traits, geographic distance and differentiation in environmental predictability, the latter for LA only).

Results

From the observation of the 540 males (90 mating assays; intrademic and interdemic crosses), we recorded 4,318 encounters (LA 2,055; PS 2,263), 3,191 circlings (LA 1,610; PS 1,581) and 1,850 copulations (LA 853; PS 997).

Raw data from the mating experiment can be found in Suppl. Table 3.1. The percentages of copulations after encounters are shown in Table 3.3. They averaged 48% for intrademic crosses and 42% for interdemic crosses. When comparing pairwise intrademic and interdemic crosses that share a population, a higher percentage of copulations was found in intrademic crosses in 15 out of 24 comparisons. However, only SAL (in the two groups) and HYB (from the LA group) had a higher percentage of copulations for intrademic crosses in all comparisons (LA group: SAL intrademic 69.5% vs. interdemic 19.4–45.0%; HYB intrademic 56.2% vs. interdemic 19.4–50.8%, PS group: SAL intrademic 64.4% vs. interdemic 34.7–59.0%; for disaggregated values, see Table 3.3).

Table 3.3 Percentage of copulations after encounters for all population combinations (9 crosses × 5 replicates × 6 males per replicate) in the two study groups (SE between parentheses)

	Male	Female population		
Group	population	SAL	RAS	HYB
LA	SAL	69.50 (0.05)	38.03 (0.06)	41.15 (0.06)
	RAS	45.02 (0.09)	40.00 (0.08)	50.82 (0.08)
	HYB	19.44 (0.07)	42.45 (0.10)	56.18 (0.08)
		TON	SAL	CHI
PS	TON	50.70 (0.10)	58.99 (0.06)	50.00 (0.10)
	SAL	34.66 (0.07)	64.44 (0.07)	48.97 (0.06)
	CHI	52.36 (0.05)	39.29 (0.06)	24.39 (0.13)

In general, circling after encounters and copulation after circling had similar qualitative patterns as copulation after encounters (Suppl. Fig. 3.1). An exception was the CHI population (PS group), where the percentages of copulations after encounters were low and associated with high rates of

encounter in intrademic crosses, while subsequent circlings and copulations did not show values remarkably different from those in other populations.

The results of the GLMM for copulation after encounter showed significant effects for all fixed factors [i.e., male-providing population (MP), female-providing population (FP), as well as its interaction (MP:FP) for each study group (Table 3.4)].

Table 3.4 GLMM analysis for copulation after encounters (χ^2, df 2)

Study group	Factor	χ2	*P* value
LA	Female population	40.170	<0.001
	Male population	41.874	<0.001
	Interaction	37.197	<0.001
PS	Female population	21.842	0.001
	Male population	21.652	0.001
	Interaction	15.364	0.004

Fig. 3.1 ranks the coefficients for an interaction effect obtained from additively decomposing the percentage of copulations after encounters. For both study groups, the average coefficient is higher for intrademic crosses than for interdemic crosses, indicating a higher propensity towards intrademic over interdemic crosses. This trend was stronger in the LA study group than in the PS group. The positive interaction associated with intrademic crosses is largely due to SAL and HYB populations. At the same time, in the PS study group, crosses between males from CHI and females from TON showed rather high positive values (Table 3.5). The prezygotic isolation index, RI, was in general slightly positive for all population

combinations, pointing to weak behavioural reproductive isolation (Table 3.6).

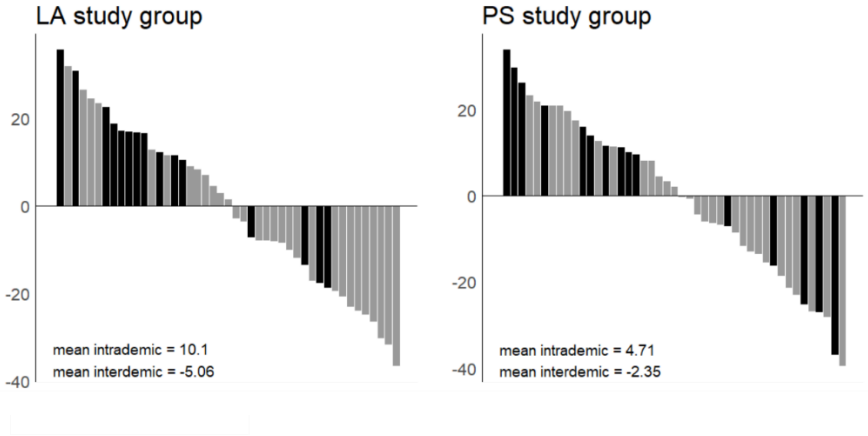

Fig. 3.1 Ranked coefficients associated with the interaction effect for the 45 population combinations in each study group: locally adapted (LA) and phylogeographically structured (PS) groups. The higher the value is, the higher the propensity for mating. Black bars: intrademic crosses; grey bars: interdemic crosses. The average values of intrademic and interdemic crosses are shown.

Ranked coefficients associated with the interaction effect for the 45 population combinations in each study group: locally adapted (LA) and phylogeographically structured (PS) groups. The higher the value is, the higher the propensity for mating. Black bars: intrademic crosses; grey bars: interdemic crosses. The average values of intrademic and interdemic crosses are shown.

Table 3.5 Average values of the coefficients associated with the interaction effect for each population combination

Group	Male population	Female population		
		SAL	RAS	HYB
LA	SAL	19.55	-7.79	-11.75
	RAS	1.36	-1.15	-0.21
	HYB	-20.9	8.94	11.96
		TON	SAL	CHI
PS	TON	2.42	-2.8	0.37
	SAL	-15.51	13.79	1.72
	CHI	13.08	-10.99	-2.09

Table 3.6 Prezygotic isolation index (RI) for all the studied population crosses in the two study groups.

Study group	Population cross	RI
LA	RAS x HYB	0.017
	SAL x RAS	0.047
	SAL x HYB	0.076
PS	TON x CHI	-0.054
	TON x SAL	0.002
	SAL x CHI	0.076

The index ranges from –1 (complete avoidance of intrademic mates) to +1 (complete propensity towards intrademic crosses).

Spearman's correlation between the interaction coefficients of the two study groups and population characteristics (predictors: difference in environmental predictability, geographic distance and F_{ST} values in neutral traits) were, except for geographic distance in the PS group, significantly negative, as expected after a preference for intrademic crosses (Table 3.7). The correlation coefficients between RI and the predictors were positive (as expected for a preference for intrademic crosses), except for F_{ST} of

microsatellites in the PS group. However, these correlations were not statistically significant.

Table 3.7 Spearman's rank correlation (ρ) between (1) reproductive isolation metrics (the interaction coefficient after an additive decomposition of the proportion of copulation after encounters and the reproductive isolation index RI) and (2) the predictor variable

		Interaction coefficient[a]		RI[b]	
Group	Variable	ρ	*P* value	ρ	*P* value
LA	F_{ST} microsatellites	-0.339	0.011	0.500	0.500
	Geographic distance	-0.410	0.003	0.866	0.167
	Difference in environmental predictability	-0.450	<0.001	1.000	0.167
PS	F_{ST} microsatellites	-0.254	0.046	-0.500	0.833
	Geographic distance	0.139	0.818	1.000	0.167

A negative correlation is expected when intrademic preference exists (H_0: $\rho = 0$, H_1: $\rho<0$)

A positive correlation is expected when intrademic preference exists (H_0: $\rho = 0$, H_1: $\rho>0$)

P value after unidirectional. [a,b] asymptotic t approximation

Discussion

Our results show that populations of *B. plicatilis* in the Iberian Peninsula evolved a low but not negligible level of behavioural mating isolation. These populations have a deep genetic divergence associated with post-glacial recolonization (Gomez & Lunt, 2007; Campillo et al., 2011) and/or ecological divergence (Franch-Gras et al., 2017b). We documented the tendency for mating preferences using two metrics: interaction

coefficients for copulations after encounters and the reproductive isolation index, RI. Both metrics are qualitatively consistent in suggesting a preference for intrademic mating. Positive interaction coefficients were mostly found for intrademic crosses, and RI had mostly positive values (five out of six comparisons), indicating a tendency for reproductive isolation. RI values are not expected to be high for populations of the same species inhabiting the same region. The RI between closely related *Brachionus* species ranges from 0.4 to 1.0 (Gribble & Welch, 2012), indicating incomplete behavioural isolation even between species. Overall, our results are compatible with the presence of partial behavioural isolation among our studied populations.

Despite showing the same trend, we regard our results on the interaction coefficients for successful encounters as more reliable than comparisons based on RI. Estimates of RI might be affected by differences in mating activity of the genotypes. As male–female encounters are random (Gilbert, 1963; Snell & Hawkinson, 1983), this activity depends on swimming speed, among other factors. Thus, an effect of the laboratory environment on RI is possible if, for instance, the environment favours the performance of individuals of some populations over others. Thus, following the approach in Tortajada et al. (2009), we used the interaction coefficients for encounters resulting in copulation because they are additive departures from the expectancy based on the general mating activity of the concerned genotypes. Additionally, we focused on copulations resulting from encounters because copulations have stronger implications for isolation than the other events.

Several mechanisms for behavioural mating isolation between genetically divergent populations have been recognized in the literature, namely, genetic drift acting on mating behaviour-coding genes (Kaneshiro, 1980; Carson & Templeton, 1984), indirect selection if those genes are physically linked to loci under disruptive selection and associated with local adaptation (Dobzhansky, 1937 in Gavrilets, 1999; Coyne & Orr, 2004) and direct selection if outbreeding fitness depression exists (Coyne & Orr, 2004). Conversely, when a preference for intrademic mating occurs, it should work as an intrinsic gene flow barrier enhancing genetic divergence. These mechanisms predict a negative correlation between genetic differentiation and behavioural mating. Our results show signatures of such a pattern in the two groups of populations studied here. The pattern is supported by our Spearman correlation analysis on the interaction coefficients, suggesting that, whichever the mechanisms at work are, their effects are stronger than the stabilizing selection expected to act on the mate recognition systems (Brooks et al., 2005; Smadja & Butlin, 2009), a selection whose benefit is to not become unrecognizable for a potential partner. Our results contrast with those of Berrieman et al. (2005), who, using a single clone for each of four populations, did not find evidence for reproductive isolation between the northern and southern Iberian clades of *B. plicatilis*.

By comparing the two experimental groups of populations, our results provide insights into the association of behavioural mating isolation with IBE and IBD. First, signatures for intrademic mating preferences are stronger in the LA group than in the PS group, despite the former

populations belonging to the same phylogeographic clade (Gomez et al., 2002) and lying in geographical proximity. This suggests a stronger association of mating behaviour with IBE than with IBD and contrasts with the strong evidence for IBD in Iberian *B. plicatilis* populations when neutral markers were studied (Gomez et al., 2002). Second, we did not find a significant correlation between geographic distance (IBD) and mating isolation in the PS group, while in the LA group, mating isolation was slightly more correlated with environmental divergence than with geographic distance. With both predictors being collinear, the correlation with geographic distance in LA is likely spurious to some extent. As another piece of evidence, genetic differentiation in the *mmr-b* gene, which codes for the receptor responsible for mating recognition, increases with ecological distance (Jezkova et al., 2022).

There are several findings worthy of note. (1) The populations with the lowest differentiation in neutral traits (RAS–HYB; Montero-Pau et al., 2017) showed no mating discrimination when considering the RI index or even preference for interdemic crosses in the case of interaction coefficients. The localities of these two populations are situated very close to each other (< 1 km) and are likely to frequently merge. This suggests the role of migration in blurring differentiation. (2) Due to the historical colonization pattern, the coastal lineage and the northern lineage are the closest lineages (Gomez et al., 2000; Serra et al., 2019). This could explain why mating between TON and CHI (from the coastal and northern clades, respectively) shows lower avoidance of interdemic crosses than shown by mating between either population with SAL (the southern clade). (3) In

both population groups, the SAL population drives the most distinguishable pattern of behavioural isolation, so the caveat is whether our argument is based only on one single population. We notice, however that behavioural isolation in the SAL–HYB pair is higher than that in the SAL–RAS pair, which is the expected ranking in an IBE scheme.

The association between IBE and mating isolation found herein makes the question of selection of isolation (Butlin, 1987) worthy of investigation in these rotifers. An important condition for selection for premating isolation is that the intensity of the potential gene flow must be intermediate, meeting a balance between the homogenizing effect of extensive gene flow and lack of gene flow (no need for selection for isolation; (Nosil, 2013; Yukilevich, 2012). Even if direct measures of migration rates for rotifers are scarce (Lopes et al., 2016; Moreno et al., 2019), a gradient of rates is most likely in populations such as those in the LA group.

In our research, we focused on behavioural mating isolation as a premating (i.e., low-cost) intrinsic barrier. As anticipated above, other intrinsic barriers not captured here might be involved in premating isolation in the wild. In *Brachionus* rotifers, the beginning of sexual reproduction is triggered by the accumulation of a crowding chemical signal in the environment (Snell et al., 2006; Snell, 2017). While it was shown that the signal is rather conservative and sex can be cross-induced between species (Stelzer & Snell, 2006; García-Roger et al., 2009), the threshold concentration varies even among genotypes of a single species (Franch-Gras et al., 2017a). These differences could affect the timing of sex, thereby

promoting reproductive isolation between immigrants and residents via their allochronic sexual periods.

How populations differentiate, become locally adapted and eventually diverge into distinct species are persistent questions in evolutionary biology, and their answer depends upon a panoply of processes acting in opposing directions, with complex interactions due to causal feedbacks and having different strengths in relation to organisms and habitat features. The taxon of cyclically parthenogenetic rotifers harbours high species richness, and the few well-studied species in the taxon are clusters of genetically differentiated and locally adapted populations. Our results suggest that isolation may arise associated with genetic divergence and might play a role in the diversification of rotifers. We nevertheless remark that behavioural isolation is just a piece of the premating isolation puzzle.

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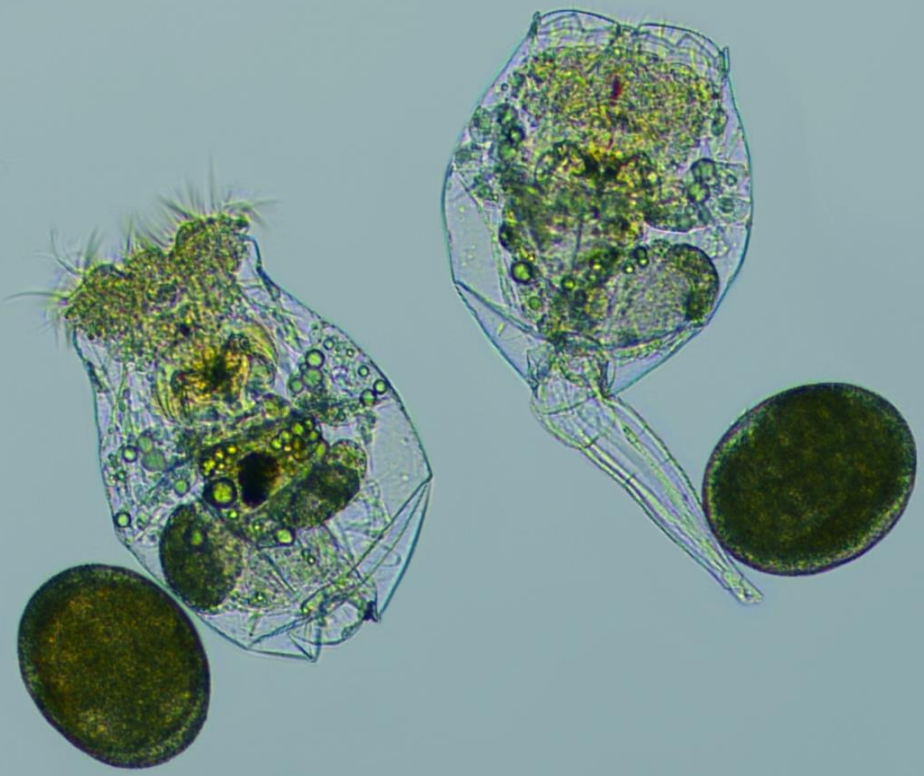

Chapter 4

Development of reproductive barriers in sympatry

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Abstract

Freshwater zooplankter *Brachionus plicatilis* is able to inhabit different habitats and locally adapt to their environmental conditions. It also shows a high degree of population structuring in small geographical regions. Here we try to shed light on the evolution of reproductive isolation in populations of *B. plicatilis* with presumptive gene flow among locally adapted populations. We have conducted laboratory experiments on admixed pairwise populations that differ in predictability of the water regime. We have assessed the potential for within-population reproductive preferences as a deviation of genotypes from Hardy–Weinberg equilibrium in diapausing eggs, a product of sexual reproduction. We expected heterozygote deficit to increase with environmental distance. We have found signs for incipient reproductive isolation in one third of our admixed populations, however no correlation with environmental distance was found, nor with genetic or geographic predictor variables. The overall inbreeding coefficient showed a tendency for within-population crosses preferences to decrease over time.

Keywords

Brachionus plicatilis; Local adaptation; Population differentiation; Speciation; Zooplankton

Introduction

Understanding the factors and mechanisms that underline population diversification and speciation remains to this day one of the challenges in current biology, due to the complexity of the processes involved. The evolution of reproductive isolation is crucial for the maintenance of population differentiation and, in some cases, subsequent species formation (Mayr, 1963; Coyne & Orr, 2004; Westram et al., 2022). Reproductive isolation arises as a result of different processes or events. The main factors involved in the development of reproductive isolation are sufficiently long periods in allopatry, adaptation to local environments with subsequent selection for reproductive isolation, and historical processes related to range expansion and colonization (Tregenza, 2002; Coyne & Orr, 2004).

In populations separated by large geographical distances, where low or no exchange of individuals occurs, reproductive isolation is expected to arise randomly, as a part of overall population differentiation and processes such as genetic drift or genetic hitchhiking (Mayr, 1963; Coyne & Orr, 2004; Feder et al., 2013). The situation is different without geographical isolation, where exchange of individuals occurs, and subsequent gene flow could continuously disrupt diverging evolution among populations. Here, selection for reproductive isolation can follow incipient local adaptation in order to avoid interpopulation breeding if outbred offspring show lower fitness due to disruption of locally adapted genomes (Butlin, 1987; Coyne & Orr, 2004). Reproductive isolation is in this case likely to occur in earlier phases of species formation (Coyne & Orr, 1989; Yukilevich, 2012; Nosil,

2013). Despite the abundance of theoretical work studying evolution of reproductive isolation, relatively little experimental studies are investigating this topic in its breadth (but see Chin et al., 2019).

Environmental gradients in geographical proximity are convenient to study evolution of reproductive isolation arising due to local adaptation. In the Iberian Peninsula, the facultative sexual zooplankter *Brachionus plicatilis* (Müller, 1786) inhabits brackish ponds ranging from ephemeral puddles to permanent lakes, embracing wide scales of salinity, temperature, food quality, etc. The Iberian populations of *B. plicatilis* have been found possessing genetic differences in their traits for timing for sexual reproduction in order to match local environmental unpredictability, as assessed by non-regular fluctuations in the length of annual periods a pond is flooded (Franch-Gras et al., 2017a, b). Populations of *B. plicatilis* consist of clones of asexual females reproducing by parthenogenetic propagation until sexual reproduction is induced in response to population density (Carmona et al., 1993; Stelzer & Snell, 2003; Gilbert, 2007). As a result of sexual reproduction, diapausing (resting) eggs are produced, and they sink to the sediment, forming a reservoir that allows rotifers to survive adverse periods of the annual cycle. The timing of sexual reproduction (i.e. switching to sexual reproduction earlier or later) is a relevant trait for local adaptation, as switching too early or too late has respectively costs in terms of decreased clonal proliferation or not producing stages able to survive adverse periods. This within-species ecological divergence occurs in a geographical area limited to a few tens of kilometres. Despite the advantages of rotifers as model organisms in micro-evolutionary studies

(Declerck & Papakostas, 2016), and contrary to numerous studies on between-species reproductive isolation (Gomez & Serra, 1995; Suatoni et al., 2006; Schröder & Walsh, 2007; Kordbacheh et al., 2019, 2023; Zhang & Declerck, 2022a, b) to date, little attention has been devoted to study the diversification and reproductive isolation within a single species. In order to address the processes involved in the evolution of the reproductive isolation, we advocate for a population approach, with studies including within –and among– population genetic variation which are scant (but see, e.g., Jezkova et al., 2022a, b). A population approach accounts for the genetic variability harboured in local populations, which is important when dealing with quantitative and polygenic traits, as presumably involved in partial reproductive isolation.

With the aid of mating choice experiments, Jezkova et al. (2022a) reported mating behaviour that promotes reproductive isolation between Iberian populations of *B. plicatilis*, a trend stronger in populations with a higher degree of adaptive divergence to unpredictability. However, this study tested for isolation (1) using high male and female densities (2) in absence of mating competition (i.e., females of one single clone are assayed with males of one single clone), (3) simulating the synchronic timing of sex of the assayed clones. Here, we approach a more natural setup by using laboratory cultures that mix genotypes from two different populations at lower population densities and, unlike in Jezkova et al. (2022a), who studied copulation rates, we look for evidence of reproductive isolation assessed by genetic marker inspection in the diapausing eggs produced. Focussing on this stage results in a composite measure that includes a

variety of barriers like assortative mating, fertilization success or even early abortion of embryos. We classified these eggs as produced by intrademic and interdemic crosses. Mixed pairs of natural populations covered a range of ecological divergence. Our hypotheses are that genotypes are prone to intrademic reproduction, and that this tendency is stronger when populations are more distant in the predictability of their environment.

Materials and methods

Study area

The nine studied populations of *B. plicatilis* inhabit in a small area of Eastern Spain (Table 4.1). They differ in the length and predictability of the flooding season of the habitats (Franch-Gras et al., 2017a) and are locally adapted to these conditions by having different propensities to initiate sexual reproduction (Franch-Gras et al., 2017b). Additionally, they have been characterized in terms of genetic differentiation with neutral (i.e., microsatellites) and non-neutral markers (i.e., the gene coding for the mate recognition protein, *mmr-b*, which is responsible for the female-male encounter; Jezkova et al., 2022b).

Table 4.1 Studied populations of *B. plicatilis* and their pond characteristics

Population	Acronym	Location[a]	Pond surface area (m^2)	Environmental predictability[b]	Group
Atalaya de los Ojicos	AYA	38°46'20"N, 1°25'49"W	75.000	0.75	A
La Campana	CAM	38°51'29"N, 1°29'36" W	29.000	0.11	A
Hoya Yerba	HYB	38°46'46"N, 1°26'06"W	1.060	0.34	A
Hoya Chica	HYC	38°49'46"N, 1°27'49"W	32.000	0.12	A
Hoya del Monte	MNT	38°50'44"N, 1°26'38"W	15.800	0.19	A
Pétrola	PET	38°50'16"N, 1°33'49"W	1.190.000	1.00	A
Hoya Turnera	TUR	38°46'36"N, 1°24'37"W	26.000	0.70	A
Hoya Rasa	RAS	38°47'06"N, 1°25'37"W	40.000	0.66	B
Salobralejo	SAL	38°54'52"N, 1°28'06"W	237.000	1.00	B

"Group" refers to the two population groups relevant in the experimental design.

[a] Datum WGS84.

[b] Environmental predictability values based on constancy and contingency metrics from satellite images, range from 0 (highly unpredictable) to 1 (highly predictable environmental conditions) according to Franch-Gras et al. (2017a).

Culture conditions and clone isolation

Rotifers were cultured at 12 g l^{-1} artificial sea water (Instant Ocean® Sea Salt, Aquarium Systems), maintained at 20°C with moderate aeration, constant illumination of approx. 35 µmol quanta m^{-2} s^{-1}. They were fed ad libitum once a week with the microalgae *Tetraselmis suecica* at $1 \cdot 10^6$ cells

ml^{-1} which was cultured under the same laboratory conditions as the rotifers.

In order to establish single-clone cultures, diapausing eggs were extracted from the pond sediments using a sugar flotation technique (Gomez & Carvalho, 2000) and placed individually to hatch at 6 g l^{-1} artificial sea water and kept at 25°C and constant illumination. Hatchlings were isolated individually, and single-clone cultures were then established by allowing parthenogenetic proliferation. These clonal cultures were taxonomically identified as *B. plicatilis* using restriction fragment length polymorphism analysis (RFLP) on a fragment of the mitochondrial gene cytochrome oxidase I (COI) (Campillo et al., 2005). A total of 30–40 clones per population were established in 2017 except for CAM, which were established in 2019.

Experimental cultures

We created multiclonal laboratory cultures for each population by placing 5 females carrying asexual female eggs by clone in 2 l glass containers (30 clones × 5 = 150 individuals). These cultures were kept in the same standard conditions as the single-clone cultures but fed with microalgae at 2×10^5 cells ml^{-1}. The resulting density ensures that sexual reproduction does not occur.

After 4 days the multiclonal laboratory cultures were filtered through a 30-µm Nytal mesh sieve, and 150 egg-bearing females were picked up. The collected females from two multiclonal laboratory cultures were combined

in 6 l of medium and cultured under the same conditions as above but with a microalgae concentration of 1×10^6 cells ml^{-1}. A total of 28 admixed experimental cultures, resulting from the combination of seven populations (Franch-Gras et al., 2017b) referred as "A" populations) with SAL and RAS (referred as "B" populations) was obtained (Fig. 4.1, Table 4.2). SAL was selected as inhabiting the most predictable environment and RAS in the intermediate level of unpredictability. Two replicates per combination were set up.

Sampling of the experimental cultures

Admixed experimental cultures were sampled on day 15, 22, and 29 (hereafter called "temporal samples"). We used a vacuum pump to extract 5 l of the water column taking care of not disturbing the bottom of the container, where most of the diapausing eggs accumulate. The remaining volume was filtered through a 30-µm Nytal mesh sieve and diapausing eggs were collected and stored in 60 g l^{-1} artificial sea water in the dark at 4°C in order to prevent spontaneous hatching.

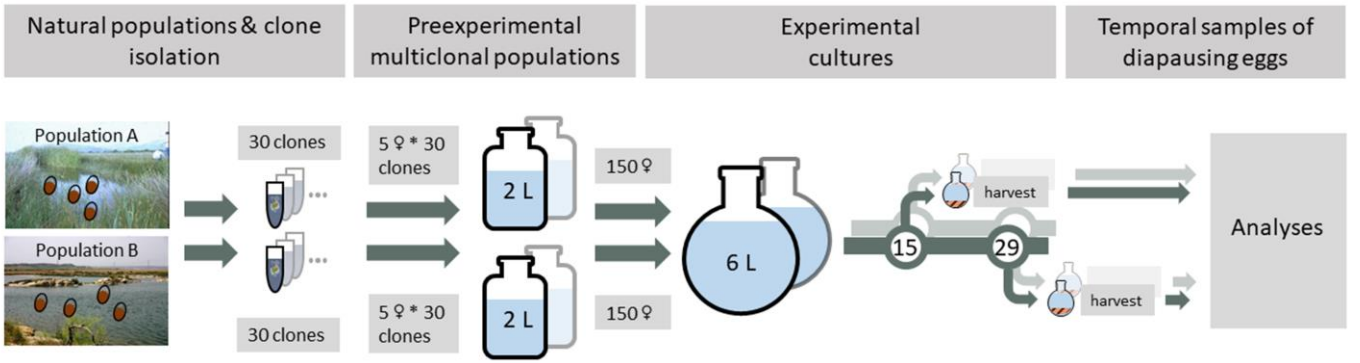

Fig. 4.1 Experimental setup, illustrated for the combination of two natural populations. Each pair of the two 2-l cultures correspond to a natural population. Each of the two 6-l cultures correspond to a replicate.

Table 4.2 Experimental combinations of natural populations

Population combination		Geographical distance (km)	Predictability distance [a]	F_{ST} micro-satellites [b]	ϕ_{ST} *mmr-b* [b]
B	A				
SAL	AYA	16	0.25	0.414	0.130
SAL	CAM	7	0.89	0.207	0.433
SAL	HYB	15	0.66	0.270	0.561
SAL	HYC	9	0.88	0.202	0.140
SAL	MNT	8	0.81	0.176	0.566
SAL	PET	12	0.00	0.148	0.154
SAL	TUR	16	0.30	0.491	0.000
RAS	AYA	1	0.09	0.091	0.000
RAS	CAM	10	0.55	0.239	0.166
RAS	HYB	1	0.32	0.083	0.303
RAS	HYC	6	0.54	0.123	0.000
RAS	MNT	7	0.47	0.071	0.311
RAS	PET	13	0.34	0.143	0.000
RAS	TUR	2	0.04	0.221	0.000

For each population combination, geographical distance (Km), pairwise differences in pond predictability (Franch-Gras et al., 2017a) and FST and ϕST values for neutral markers and *mmr-b* gene, respectively, (Jezkova et al., 2022b) are shown.

[a] Franch-Gras et al., 2017a

[b] Jezkova et al., 2022b

We then returned the 5 l of the water column to the glass container, adding 1 l of medium with microalgae at 1×10^6 cells ml^{-1}. Only diapausing eggs from the temporal samples of day 15 and 29 were used for subsequent analysis. Density of diapausing eggs of day 15 and 29 was estimated in order to cover different phases of the population dynamics. This density was estimated using a particle counter (Coulter Counter, Beckman) by counting an aliquot of the sample.

DNA extraction and egg genotyping

We selected 46 healthy-looking diapausing eggs from each replicate from the temporal samples collected on days 15 and 29. DNA was extracted from each egg separately. To do this, the diapausing eggs were washed in distilled water and placed separately in 0.2 ml Eppendorf PCR tubes with 20 µl of 5 mM TE buffer and sonicated during 60 s in an Ultrasonic bath FB 15,047, Fisherbrand™ at room temperature. As the rigid chitin-like double external layer covering rotifer diapausing eggs (García-Roger et al., 2005; Denekamp et al., 2010) is sometimes resistant to breakage, the success of the sonication was inspected visually under a stereo microscope (Olympus SZX10, Japan) by searching for an open egg envelope without visible inner content of the eggs. If failed, we manually disrupted the external layer using a pipette tip and repeated the sonication. DNA was stored at − 20°C until further use.

For the identification of the diapausing eggs to inbred (AxA, BxB) or outbred (AxB) crosses we used Kompetitive allele specific PCR (KASP™, LGC Genomics, Teddington, Middlesex, UK) genotyping assays. Using available genomic information (Franch-Gras et al., 2018) for the same natural populations, we designed a set of SNPs with private or quasi-private alleles to discriminate between pairs of populations (Suppl. Table 4.1). DNA samples from single diapausing eggs were analysed by KASP™ genotyping assays by Biosearch Technologies (UK). We analysed 92 samples for each population combination from two temporal samples (days 15 and 29).

Due to the low amount of DNA in a single *B. plicatilis* egg, KASP™ genotyping often had problems in identifying the genotypes. For this

reason, each sample was run twice and the following genotyping rules were adopted: (1) if both runs produced the same genotype, the resulting genotypes was assigned to the diapausing egg; (2) if only one run produced results, it was assumed as the egg genotype; and (3) in case of a discordant genotyping—either (a) different homozygotes or (b) heterozygote plus homozygote—between runs, it was assumed as an effect of allele dilution due to the low concentration of DNA and the heterozygote genotype was assigned to the egg. Notice that these rules might result in an overestimation of heterozygotes, therefore being a conservative assumption in relation to the hypothesis of reproductive isolation.

Data analysis

We calculated the percentage of all three genotypes (AxA inbred, BxB inbred, and AxB outbred) in the diapausing eggs for each temporal sample separately (14 combinations of natural populations × 2 replicates × 2 temporal samples). Fixation index (F_{IS}) and its significance for heterozygote deficiency (uni-directional tests) was calculated using GenePop on the web (Raymond 1995, Rousset 2008). F_{IS} ranges from − 1 (complete outbreeding) to + 1 (complete inbreeding). Before testing for heterozygote deficiency, in order to increase the power of subsequent statistical tests, we performed a chi-square test of homogeneity for the two temporal samples and the two replicates of each population combination, using the chisq.test implemented in R software (ver. 4.1.2) and pooled those samples that resulted homogeneous. Additionally, several average F_{IS} were computed after weighting with egg abundance (see "Results"). The correlation

between F_{IS} values of the two replicates within-population combinations, as well as correlation between F_{IS} and the different predictors (geographic, environmental and genetic pairwise distance, Table 4.2) was calculated using *cor.test* function implemented in R software (Pearson correlation, one sided). In these correlations, environmental distance was computed as the difference between the unpredictability values from each pond according to Franch-Gras et al. (2017a). Genetic distance was based on the F_{ST} value of microsatellites and ϕ_{ST} value for the terminal repeat of the mate recognition gene *mmr-b* (Jezkova et al., 2022b).

Results

All 28 admixed experimental cultures produced diapausing eggs. Egg densities varied between replicates and mostly along the experimental time course. Based on these egg densities, the daily egg production rate ranged from 0.04 to 1.54 eggs ml^{-1} day^{-1} on day 15, and from 0.02 to 0.46 eggs ml^{-1} day^{-1} on day 29. In all cases, accumulated egg density on day 15 (which integrates two weeks of production) was much higher than the corresponding day 29 of sampling (one week of production) (Suppl. Table 4.2).

A total of 4539 SNPs were previously identified for the different populations (Franch-Gras et al., 2018), from those, only 276 SNPs had the capability to differentiate between natural populations in at least one of the 14 population combinations. We were able to design a KASP™ assay for all population combinations in our experimental design except in two cases

(RASxHYB and SALxMNT), where only one and two SNPs respectively were able to differentiate between the two populations involved in the cross but gave no result in the KASP™ assays. These two population combinations were excluded from further analysis. Genotypes could be assigned to 2005 eggs (between 27 and 46 eggs per replicate and population combination). Frequencies of genotypes are shown in Fig. 4.2. Genotyping rules (see "Material and Methods") had to be applied to 50.4% of the genotypes as they presented inconsistencies among KASP™ runs. From those, 72.0% were classified as heterozygotes. The percentage of manually assigned heterozygotes varied among population combinations (interquartile range: 49.9 and 90.7%). Deficiency of heterozygotes was observed in 26 out of 48 samples. Temporal samples and replicates were pooled in 7 combinations (RASxAYA, RASxHYC, RASxMNT, RASxPET and SALxHYB, SALxPET, SALxTUR) after testing for homogeneity of the genotypic frequencies. After pooling homogeneous samples, significant deficiency of heterozygosity was found in four population combinations (RASxMNT, RASxPET, SALxHYB, SALxTUR; pooled replicate and dates), and in the 15-day temporal samples of both replicates of RASxTUR. That is, we found significant heterozygote deficiency in 6 out of 27 tests, involving 5 out of 12 population combinations.

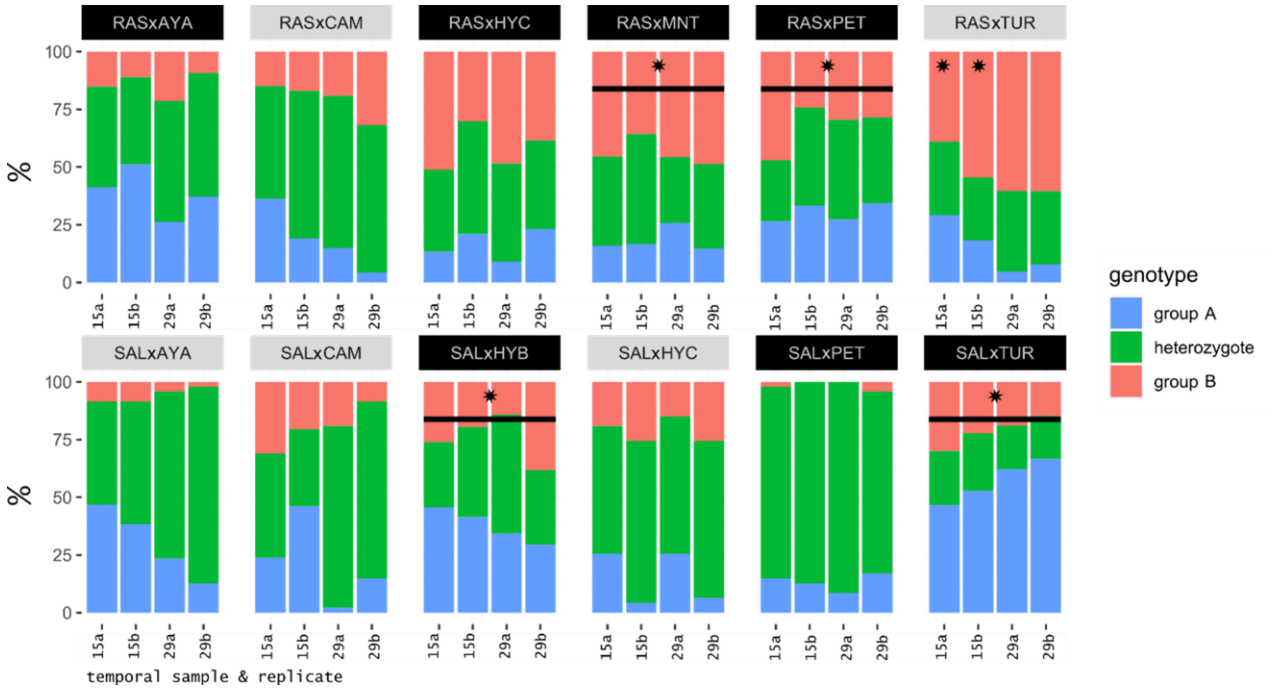

Fig. 4.2 Percentage of diapausing eggs genotypes produced in each population combination for two temporal samples (day 15 and day 29) and two replicates (a and b). Those population combinations with homogeneous samples were pooled for analysis and highlighted in black. Stars indicate significant deficit of heterozygotes, black horizontal lines accounts for significant deficit of heterozygotes in pooled replicates and samples.

The F_{IS} values of the 48 samples ranged from − 0.84 to + 0.54. Correlation of F_{IS} values between the two replicates were highly significant ($P < 0.001$ for both days 15 and 29). Six out of 12 population combinations showed positive average F_{IS} values (Fig. 4.3, average over population combinations: 0.062). A trend of decreasing F_{IS} was observed between day 15 and day 29 in 10 out of 12 population combinations. When comparing the F_{IS} separately for each temporal sample (day 15 vs. day 29), average F_{IS} on day 15 was positive (0.112), while on day 29 was negative (− 0.141). F_{IS} (either per population combination or for each temporal sample) did not show significant correlation with any of the predictors (environmental, geographic or genetic distance) (Suppl. Fig. 4.1).

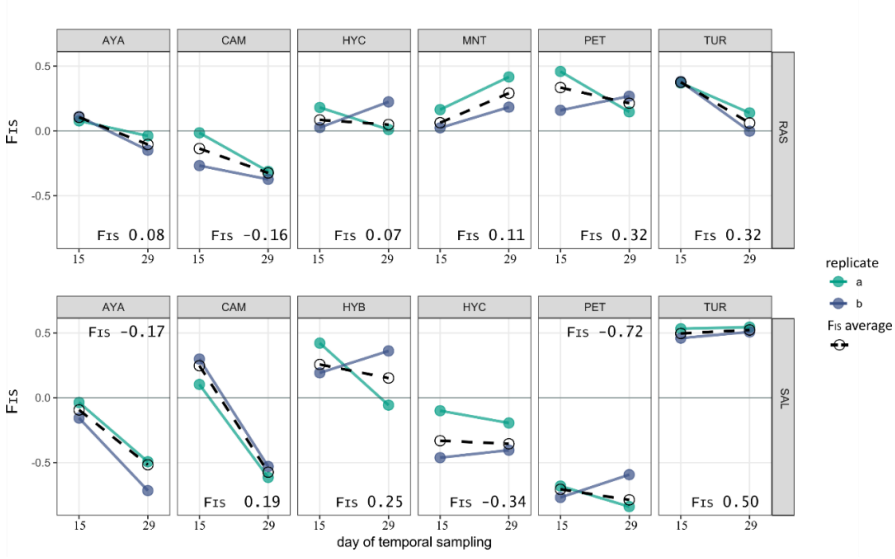

Fig. 4.3 F_{IS} values for each population combination and replicate for the two temporal samples (day 15 and day 29). Black dashed line is averaged over replicates. The number within each panel is F_{IS} averaged over temporal samples and replicates. The area above the line corresponds to within-population mating preferences and below the line to among population mating preferences.

Discussion

In this study we have investigated the evolution of an incipient reproductive isolation among populations of *B. plicatilis* from geographically close ponds that differ in their environmental conditions. We selected nine populations locally adapted to water regime unpredictability (Franch-Gras et al., 2017b). We have produced diapausing eggs from 12 population combinations and found signs of assortative mating (deficit of heterozygotes) in five of them. The emergence of reproductive isolation in the presence of gene flow is not easy to evolve as gene flow tends to blur divergence and because mating recognition systems are under stabilising selection (Lambert et al., 1982; Butlin et al., 1985). Therefore, our observation of deficit of heterozygotes in more than one third of our experimental cultures seems to us biologically significant.

The index of inbreeding (weighted F_{IS}) was positive in eight out of 12 population combinations, providing signatures for outcross avoidance. The overall F_{IS} value was very varying (F_{IS} average 0.062; range − 0.84 to 0.54). The most inbred population combination in this study was SALxHYB, with an average F_{IS} of 0.25. Interestingly, in an independent previous study on mating behaviour, these populations also showed a significant preference for within-population mating (Jezkova et al., 2022a), largest differences in environmental predictability (Franch-Gras et al., 2017a) and each population belongs to a different *mmr-b* haplotype group (Jezkova et al., 2022b).

Regarding those populations that did not show a deficit of heterozygotes (4 out of 12), different explanations could be invoked. Our results could be

due to an actual lack of reproductive barrier between these populations. On the other hand, the experimental setup could have affected the outcome increasing the number of heterozygotes found in several ways. First, the amount of DNA obtained from a single egg is small, and this can affect the genotyping by KASP™ analyses resulting in random loss of one allele. KASP™ was run twice for each egg and every inconsistency (one different homozygote each time) was assigned as heterozygote. By doing this, we may have biased our data against our hypothesis of heterozygote deficit. Second, by admixing populations at high concentrations (150 + 150 females in 6 l of media) we may have increased the probability of encounters and forced random mating, overcoming the effect of the pre-reproductive barrier found in Jezkova et al. (2022a). In the wild, this equifrequent assemblage would not occur. Third, as explained below, asynchronous timing of sexual reproduction may explain events of heterozygote excess.

Whereas average F_{IS}, either globally or in eight out of 12 population combinations, was positive indicating a tendency towards within-population crossing, this index decreased between day 15 (global F_{IS} 0.112) and day 29 (global F_{IS} − 0.141, with low effect due to de decreased egg production). Two different biological factors could contribute to this pattern. One factor may be the environment deterioration between day 15 and day 29 (e.g. accumulation of metabolic compounds or debris). This deterioration might be appreciated by rotifers as the end of the growing season and lead to lower partner discrimination at later stages (better a bad mate than no mate). This would imply F_{IS} to tend to zero. The other

putative factor is related to different timing of induction of sexual reproduction. Asynchrony of reproductive cycles has been reported as one of the important components of reproductive isolation in many species (Wu et al., 2021). In our study system, natural populations are locally adapted to environmental unpredictability by adjusting their density threshold for sex induction, that is, some populations induce sex earlier than others (Franch-Gras et al., 2017b). Accordingly, when the later-inducer population starts producing sexual females, males from the earlier-inducer population are already copulating with them, increasing the number of outbred crosses. Preliminary results from a model simulation (Serra, unpublished) suggest that this coupling might explain a period of heterozygote deficiency followed by a period of heterozygote excess.

Previous studies suggest a tendency towards pre-reproductive isolation related to environmental distance (Jezkova et al., 2022a). In this study, however, despite signatures for within-population crossing, our hypothesis of a stronger deficit of heterozygotes in those populations that are more ecologically distant was not observed. The tendency of populations to avoid interbreeding was not related to environmental distance, nor to geographic or genetic distance. Lack of correlation with genetic distance occurred for neutral markers (microsatellites) and *mmr-b* gene, which is involved in rotifer mate recognition (see Jezkova et al., 2022b). We do not consider this lack of evidence definitive. Firstly, our study was limited to 14 population combinations sampled at a regional scale (240 km2), so lack of correlation could be due to low statistical power in order to detect a small effect. Secondly, as previously pointed, experimental factors and inherent

biological characteristic of our populations may have altered the frequency of heterozygotes in our experiments, and thus, altering the relationship among genetic and environmental distance. The natural populations studied here belong to two aminoacidic haplotype groups of the *mmr-b* gene (Jezkova et al., 2022b). SAL and TUR are in one group and the rest in the other. Correlations of F_{IS} with ϕ_{ST} of *mmr-b* in population combinations involving SAL, but excluding TUR with which there is no genetic distance, resulted in a positive correlation ($P = 0.05$, Suppl. Fig. 4.1) on the edge of the statistical significance. This suggests a role of the mating recognition gene in population divergence but also the high complexity of mating discrimination and behavioural isolation, where a panoply of biotic and abiotic factors might be at work. Indeed, our experimental setup was based on oversimplified populations formed by an equal admixture of differentially adapted individuals in a homogeneous environment. Moreover, the involvement of additional genes controlling successful reproduction (Snell, 2011; Hanson et al., 2013) cannot be discarded.

Zooplankton populations in nature are genetically structured despite their high dispersal capacity (De Meester, 1996; De Meester et al., 2002). This paradox has been explained as the result of persistent founder effects and local adaptation (De Meester et al., 2002; Montero-Pau et al., 2018). There is evidence for local adaptation in cladocerans, rotifers and other zooplanktonic groups (Fernández et al., 2020; Tangwancharoen et al., 2020; White et al., 2022). In this scenario, the evolution of a reproductive barrier among locally adapted populations is advantageous. Our study points in this direction. However, a trade off may exist between preserving

favourable gene combinations that are advantageous in certain habitats and undesirable negative consequences of inbreeding (Tortajada et al., 2009; Cannon, 2021). This trade off might weaken the reproductive barrier, resulting in a gradient of reproductive isolation. In this study we have found signatures of incipient reproductive isolation in some populations of *B. plicatilis* from geographical proximity with putative migration among them. The fact that we worked at an intraspecific level and controlling only a limited set of factors is consistent with not finding clear cuts for isolation. Given that the evolution of reproductive isolation and speciation are rare historical events in nature, finding partial isolation within some of the populations studies seems to us biologically significant. By finding partial within-species reproductive isolation between populations, our results make worthy further investigation on the role of local adaptation in shaping the diversification of small zooplankton populations.

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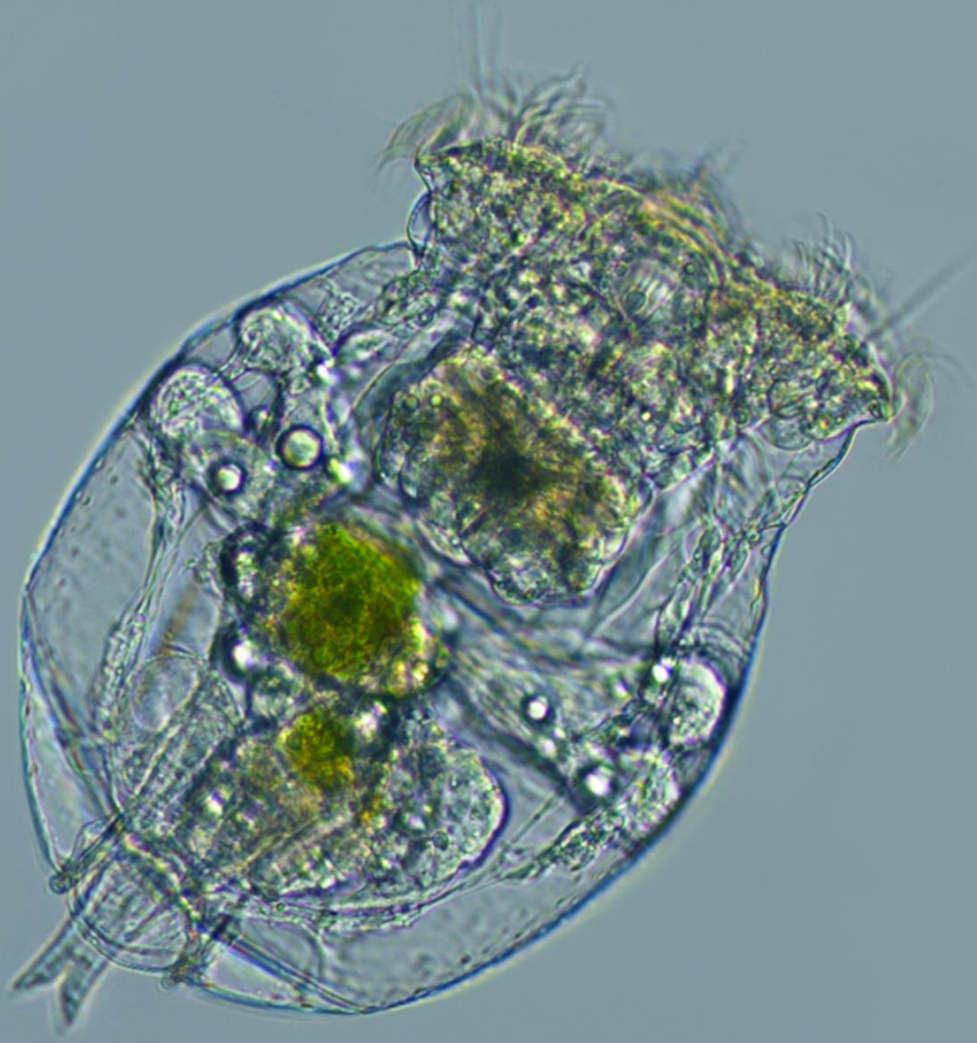

Chapter 5

Genetic Variability of the Mating Recognition Gene in Populations of *Brachionus plicatilis*

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Abstract

The development of reproductive barriers promotes within-species divergence and is a requisite for speciation to occur. Mate recognition in the rotifer *B. plicatilis* is mediated through a surface glycoprotein called Mating Recognition Protein (MRP). Here we investigate the genetic variation of the *mmr-b*, MRP coding, gene in different natural populations of *B. plicatilis* from the Iberian Peninsula, that present different degree of population differentiation, with known adaptive divergence in some cases. The MRP gene consists of several nearly identical tandem repeats. We found a relatively high diversity within and among populations both in the number of repeats, as well as in the nucleotide sequence. Despite that most changes are neutral, variation that can potentially affect the protein function was found in two polymorphic sites within a repeat in some of these populations. Although being mostly subject to stabilizing selection, we have found a noticeable pattern of increasing *mmr-b* gene diversification correlated to increasing differences in environmental factors. The interplay between genetic differentiation, local adaptation and differentiation of the mating recognition system can lead to speciation events in nearly sympatric populations.

Keywords

Behavioral isolation; Signalling; Genetic structure; Reproductive isolation; Speciation

Introduction

Diversification, either within or between species, is an essential process for maintaining the continuity of life on Earth, being more pronounced in sexual species. This is promoted by the emergence of reproductive isolation barriers (pre-mating, prezygotic or postzygotic). Among these, it has been postulated that those acting on pre-mating isolation have clear advantages in the early stages of population differentiation and speciation, as they allow a better allocation of resources in diverging populations, avoiding offspring with reduced fitness (Coyne & Orr, 2004). One of the mechanisms leading to pre-mating isolation is behavioural isolation, involving mate recognition and discrimination. Mate recognition can take different forms, containing visual, auditory or olfactory signalling. In small aquatic metazoans, sexual signalling is usually mediated by non-diffusible molecules attached to the surface of the animal (Snell & Morris, 1993; Rosenthal, 2017).

The mate recognition systems are under strong evolutive pressure to remain stable, assuring that individuals of a particular species are recognized as a potential mating partner (Lambert et al., 1982; Butlin et al., 1985; Brooks et al., 2005). However, when populations start to differentiate, rapid diversification in mating recognition traits may appear allowing a stabilization of this divergence (Swanson & Vacquier, 2002; Wilburn & Swanson, 2016). Rapid diversification in these traits has been detected even in sympatric populations (Porter & Smith, 2020). This may explain high variability of these traits even among closely related species

compared to others (Civetta & Singh, 1995; Martin et al., 2011) either within or between species (Blows & Higgie, 2002).

Population differentiation within species is a ubiquitous phenomenon and can arise through historic and geographic factors or local adaptation to ecological conditions. In small organisms with a high dispersal potential, this population structuring happens even in a limited geographical scale (i.e., "dispersal–gene flow paradox", De Meester et al., 2002). Populations of the rotifer *Brachionus plicatilis* (Müller, 1786) in the Iberian Peninsula are an ideal system to study this population diversification and the incipient stages of reproductive isolation.

The rotifer *B. plicatilis* is a cyclical parthenogen. Clones proliferate asexually and invest in sexual reproduction to produce diapausing eggs. These are a resistant stage needed to survive recurrent periods of adverse conditions. In the Iberian Peninsula, it has been shown that populations of this species present a strong genetic differentiation even among neighbouring populations (Gomez et al., 2002a; Campillo et al., 2011; Montero-Pau et al., 2017). The ponds they inhabit encompass a wide range of environmental conditions including different hydroperiod length, and predictability of flood–desiccation pattern, which results in a gradient of environmental unpredictability (Franch-Gras et al., 2017a). Populations are known to be locally adapted to this unpredictability via diapausing investment patterns; in highly unpredictable environments they will tend to sexually reproduce and, thus, produce diapausing eggs earlier, as a bet-hedging strategy (Franch-Gras et al., 2017b). Additionally, assays of mating behaviour on Iberian populations have shown an incipient behavioural

reproductive isolation associated with isolation by distance and isolation by environment (i.e., ecological divergence) (Jezkova et al., 2022).

The mating recognition mechanism in the *Brachionus* genus occurs through contact chemoreception (Snell et al., 1995). An extracellular glycoprotein, the mate recognition protein (MRP), located on the female body interacts with a mannose receptor expressed by males (Snell, 2011b). Although it may not be the only protein involved in mate recognition (Gribble & Welch, 2012), the experimental removal of MRP was shown to cause elimination of mating behaviour in males, and the artificial addition of MRP on other object triggered initiation of mating behaviour (Snell & Stelzer, 2005). MRP is structurally conserved across *Brachionus* species (Gribble & Welch, 2012). It is encoded by the *mmr-b* gene. Gribble et al. (2011) described the structure of the gene consisting in a 48 bp signalling sequence, followed by several 261-bp repeats (full repeats), plus a 243-bp terminal repeat, the latter differing from the former by an 18-bp deletion on 3' end. The full repeats are nearly identical within the gene, suggesting a concerted evolution (Gribble & Welch, 2012). Differences in the *mmr-b* gene were found among co-generic species and these differences correlated with isolation by mating behaviour (Gribble & Welch, 2012). While differences among species of *Brachionus* genus have been studied, little is known about the intraspecific variability of the *mmr-b* gene and how it may influence reproductive behaviour, despite the interest of knowing its role in population differentiation and incipient speciation.

In this study we investigate variation in the mating recognition gene under a scenario of population divergence. We aim to assess whether local

adaptation is associated with divergence in the mating recognition gene. With this goal we compared neighbouring locally adapted populations of *B. plicatilis* in the Iberian Peninsula with geographically distant populations using distances based on the *mmr-b* gene and on neutral markers.

Materials and Methods

Study Area, Rotifer Collection and Maintenance

A group of nine populations from Eastern Spain were selected based on their known ecological divergence (Table 5.1). These populations differ in the predictability of their environment (related to the duration and uncertainty of the flooding season in the ponds) and are locally adapted to the degree of unpredictability by having different propensity to initiate sexual reproduction (Franch-Gras et al., 2017b). Additionally, two populations geographically distant from this group and belonging to different phylogeographic clades (Gomez et al., 2000) were selected as outgroups. These populations were Salada de Chiprana (CHI) and Torreblanca Norte (TON). Predictability values were obtained from Franch-Gras et al. (2017a) based on constancy and contingency metrics from satellite images (Colwell's predictability index, Colwell, 1974).

Table 5.1 Populations included in the study, location and their estimated degree of predictability of the hydroperiod length in a year based on Franch-Gras et al. (2017b). (nd: data not available).

Population	Acronym	Location [1]	Pond size (m^2)	Predictability [2]
Atalaya de los Ojicos	AYA	38°46'20" N, 1°25'49" W	75,000	0.75
La Campana	CAM	38°51'29" N, 1°29'36" W	29,000	0.11
Hoya Yerba	HYB	38°46'46" N, 1°26'06" W	1060	0.34
Hoya Chica	HYC	38°49'46" N, 1°27'49" W	32,000	0.12
Hoya del Monte	MNT	38°50'44" N, 1°26'38" W	15,800	0.19
Pétrola	PET	38°50'16" N, 1°33'49" W	1,190,000	1
Hoya Rasa	RAS	38°47'06" N, 1°25'37" W	40,000	0.66
Salobralejo	SAL	38°54'52" N, 1°28'06" W	237,000	1
Hoya Turnera	TUR	38°46'36" N, 1°24'37" W	26,000	0.7
Chiprana	CHI	41°14'20" N, 0°11'02" W	230,000	nd
Torreblanca Norte	TON	40°08'54" N, 0°10'07" E	120	nd

[1] Datum WGS84.

[2] Environmental predictability values range from 0 (highly unpredictable) to 1 (highly predictable environmental conditions).

Sediment from the ponds was collected during 2013 and in 2019 (in case of CAM population) and rotifer diapausing eggs were extracted from the sediment in 2017 (or 2019) using a sugar flotation technique (Gomez & Carvalho, 2000). Isolated eggs were individually placed in 96 well-plates with 12 ppt artificial sea water (Instant Ocean® Sea Salt, Aquarium Systems) and clone cultures were established from single hatchlings by parthenogenetic proliferation. *B. plicatilis* belongs to a cryptic species complex (Gomez et al., 2002b; Suatoni et al., 2006). In the Iberian Peninsula only two species belonging to the L (large) morphotype have been described, *B. plicatilis* and *B. manjavacas*. All clones used in this study were visually confirmed as belonging to the L-morphotype, and genetically identified as *B. plicatilis* based on a restriction length polymorphism analysis (RFLP) of a fragment of the mitochondrial gene COI (Campillo et al., 2005). Additionally, the molecular identification was further confirmed by amplification of the microstellite Bp1b, which specifically amplifies in *B. plicatilis* (Tortajada et al., 2010), and by the phylogenetic analysis of a fragment of the *mmr-b* (see below for details). Rotifer clones were maintained under constant conditions (25 °C and illumination of approx. 35 µEm−2 s−1) in 15 mL culture tubes at 12 ppt artificial sea water and fed weekly with the microalgae *Tetraselmis suecica* grown at 12 ppt artificial sea water enriched by f/2 medium (Guillard & Ryther, 1962).

Design of mmr-b *Specific Primers, Amplification and Sequencing*

The available primers to amplify the *mmr-b* gene in the *Brachionus* genus (Gribble & Welch, 2012) showed a very low performance for our focal

populations. Furthermore, in some cases multiple bands appeared after PCR amplification, indicating the possibility that more than one gene was being amplified simultaneously. Thus, we designed a new set of primers (Table 5.2). Available *mmr-b* sequences (Gribble & Welch, 2012) were used to identify homologous regions along three *B. plicatilis* genome reference assemblies (REGN01001155.1, QEOQ01000235.1 and QEOQ01000070.1) using BLAST. Primers were designed using NCBI Primer-BLAST (Ye et al., 2012).

Table 5.2 Primers used for the analysis of *mmr-b* gene.

Code	Forward	Reverse
mmr-b1	CAAGCCGATTCCCATTAAAGCA	AAACCAATAAACAAAAACTAATCCTGG
mmr-b2	GCCTTTTCAGTACCAGTGAAGC	ACAAATAAACAAAAATTTAACCCTGGA

The genetic diversity of the two regions containing *mmr-b* homologs was studied using five to six clones per population. Genomic DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel) from a dense culture of each clone. PCR amplifications were conducted in a final volume of 25 µL containing approx. 50 ng of genomic DNA, 0.4 µM of each primer, 200 mM of each dNTP and 1U TopTaq DNA polymerase (Qiagen, Valencia, CA, USA). PCR condition were set to 4 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 20 s at 62 °C and 40 s at 72 °C, followed by 7 min at 72 °C. PCR products were run in 1% agarose gels, stained with GelRed and visualized under UV illumination. PCR products were cleaned using an Exo-SAP protocol (Bell, 2008), labelled using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) in both directions and run in an ABI3730XL (Applied Biosystems). Sequences were checked and

assembled using the STADEN package (v. 2.0.0) (Bonfield et al., 1995). Sequences were manually phased based on the allelic information found in homozygote individuals. MEGA-X (v. 10.1.7) (Kumar et al., 2018) was then used to align the sequences and extract the repeats. Information about the number of repeats of the *mmr-b* was scored for each clone based on the electrophoretic profiles of each allele.

Microsatellite Genotyping

Variability in microsatellite markers was used to evaluate genetic diversity in neutral markers. In this case, 10 clones per populations were used, including the five clones used in the study of the genetic diversity of *mmr-b* and five additional clones. DNA from the latter was extracted from an individual female using the HotSHOT protocol (Montero-Pau et al., 2008). Six microsatellites (Bp1b, Bp2, Bp3c, Bp4a, Bp5d and Bp6b) (Gomez et al., 1998) were genotyped. The PCRs were performed in 10 µL using 2 µL template DNA, 250 µM of each dNTP, 0.5 µM of each primer (the reverse primer was labelled with VIC, 6-FAM or NED dye) and 0.4 U TopTaq DNA polymerase (Qiagen, Valencia, CA, USA). PCR conditions were set to 5 cycles of 1 min at 94 °C, 1 min at 58 °C and 30 s at 70 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 70 °C, followed by 15 min at 72 °C. PCR products were pooled and run in an ABI PRISM 310 DNA sequencer (Applied Biosystems™) using ROX-500 as internal lane size standard. Scoring of alleles and the genotyping of individuals was performed using OSIRIS software (v. 2.16, https://www.ncbi.nlm.nih.gov/osiris/, accessed on 2 September 2020).

Data Analysis

Genetic distance between populations was computed using Arlequin (v. 3.5.2.2) (Excoffier & Lischer, 2010). For microsatellites, the distance was based on allele frequency (F_{ST}) and for the *mmr-b* on the genetic distance (Φ_{ST}) assuming a Tamura and Nei model, which was the best fitting model assessed in the IQ-TREE Web Server (Trifinopoulos et al., 2016). Analyses considering the sequence of the *mmr-b* gene were performed both using only the terminal repeat or the full repeats. Statistical significance of both genetic distances was obtained by performing 1000 permutations. The median joining network for both the terminal and full repeats were created using PopART software (v. 1.7) (Leigh & Bryant, 2015). Ecological distance between pairs of ponds was calculated using the *dist* function in R software (v. 3.6.2) based on the pond unpredictability (Franch-Gras et al., 2017a). A codon-based test for neutrality (dS/dN ratio) was calculated using the Nei_Gojobori method as implemented in MEGA-X (v. 10.1.7) (Kumar et al., 2018). A maximum likelihood tree was constructed for the terminal repeat. Terminal haplotypes identified in this study and terminal repeat sequences from different species (Gribble & Welch, 2012) retrieved from NCBI (JX239201-JX239258) were used. Duplicated sequences were filtered out, and a multiple sequence alignment was performed using T-Coffee v.13.44 (Notredame et al., 2000). The ML tree was reconstructed using IQ-TREE (version 1.5) (Nguyen et al., 2015) and branch supports were calculated with ultrafast bootstrap based on 1000 replicates (Hoang et al., 2018), aBayes support and SH-aLRT test. Best fitting evolutionary model was determined to be TPM2u+F+I+G4 according to the Bayesian Informative

Criterion using ModelFinder (Kalyaanamoorthy et al., 2017). Correlation between the genetic distance matrix of both microsatellite and terminal repeat of the *mmr-b* gene and geographical distance and environmental predictability was tested using a Mantel test with 1,000,000 permutations as implemented in the *mantel.rtest* function of the package "ade4" (Dray & Dufour, 2007) in R software.

Results

Nucleotide and Amino Acid Diversity of mmr-b *Gene*

After performing a BLAST search against the *B. plicatilis* genome references, two different genomic regions putatively containing an *mmr-b* gene were identified. One of them, amplified using the primer set mmr-b1 was an incomplete copy of the *mmr-b* gene consisting of 1 or 2 repeats alleles with deletions on both extremes (65 bp at the 5' in both lengths, and 108 bp at the 3' ends in 2-repeats length of the locus). Due to the lack of at least one entire repeat, this locus was discarded as a non-functional *mmr-b* gene. The other region, amplified using the primer set mmr-b2, was consistent with the previously described structure of functional *mmr-b* (Gribble & Welch, 2012). It consisted in zero to nine full repeats, always followed by one terminal repeat. Only substitutions, but no insertion or deletions, were observed. Along the 261 or 242 bp length of the full and terminal repeats, 19 and 17 point mutations were found, resulting in 12 and 8 different full repeat and terminal repeat haplotypes respectively (Fig. 5.1). The transition/transversion ratio was 2.4. Only five

mutations leading to three non-synonymous changes in amino acids were detected (amino acid position 47: Asp/Gln, 50: Lys/Thr and 54: Ser/Gln) (Fig. 5.1). All three changes are presumably structurally neutral as all changes involve polar amino acids, however, the changes in the amino acids 50 and 54 could result in the appearance/loss of a phosphorylation or O/N-glycosylation site. The dN/dS ratio test was 2.26 (*p* value = 0.017), which suggests that stabilizing selection has acted on *mmr-b*. Different alleles were found varying both in the number of repeats and in the haplotype composition of the repeats within and among populations (Fig. 5.2). The maximum-likelihood tree of the terminal repeat (Suppl. Fig. 5.1) showed that all the terminal haplotypes found in our study grouped with the two previous groups of sequences of *B. plicatilis* previously described (Gribble & Welch, 2012).

(A)		nucleotide position / amino acid position																				
	nt haplotype	12/4[c]	21/7[c]	36/12[c]	51/17[c]	57/19[c]	96/32[c]	105/35[c]	120/40[c]	139/47[a]	141/47[c]	149/50[b]	156/52[c]	159/53[c]	160/54[a]	161/54[b]	171/57[c]	201/67[c]	222/76[c]	228/76[c]	258/86[c]
full	1	T	T	C	C	C	A	C	C	G	T	A	T	C	T	C	T	T	C	T	C
repeat	2	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
	3	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	C	C	.	T	T	G	T	.	C	A	C	C	T	C	A	C	C	T	.	.
	5	C	C	.	T	T	.	T	.	C	A	C	C	T	C	A	C	C	T	.	.
	6	C	C	.	.	.	.	.	.	C	A	C	C	T	C	A	.	.	T	.	G
	7	.	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	C	.	.	.
	8	C	C	.	.	.	.	T	.	C	A	C	C	T	C	A	.	.	.	C	G
	9	C	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	.	T	.	.
	10	C	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	.	.	C	G
	11	C	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	.	T	.	G
	12	C	C	.	.	.	.	T	.	C	A	C	C	T	C	A	.	.	T	.	G
terminal	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NA
repeat	B	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	NA
	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NA
	D	C	C	.	.	.	.	.	.	C	A	C	C	T	C	A	C	.	.	.	NA
	E	C	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	.	.	.	NA
	F	C	C	.	.	.	.	.	.	C	A	C	C	T	C	A	.	.	T	.	NA
	G	C	C	.	T	T	.	T	.	C	A	C	C	T	C	A	C	.	.	.	NA
	H	C	C	.	T	T	.	.	.	C	A	C	C	T	C	A	C	C	T	.	NA

(B)	nucleotide haplotype				
AA hapl	full	terminal	/47	/50	/54
AA1	1,2,3	A,B,C	D	K	S
AA2	4,5,6,7,8,9,10,11,12	D,E,F,G,H	Q	T	Q

Fig. 5.1 Multiple alignments within full (261 bp) and terminal (243 bp) repeats, respectively. (**A**) Haplotypes based on differences in nucleotide sequences and (**B**) amino acid haplotypes with corresponding AA changes. Non-synonymous substitutions are shown with grey background. Colour symbols are assigned to haplotypes to be used hereafter. [a,b,c] refer to the first, second and third codon positions, respectively.

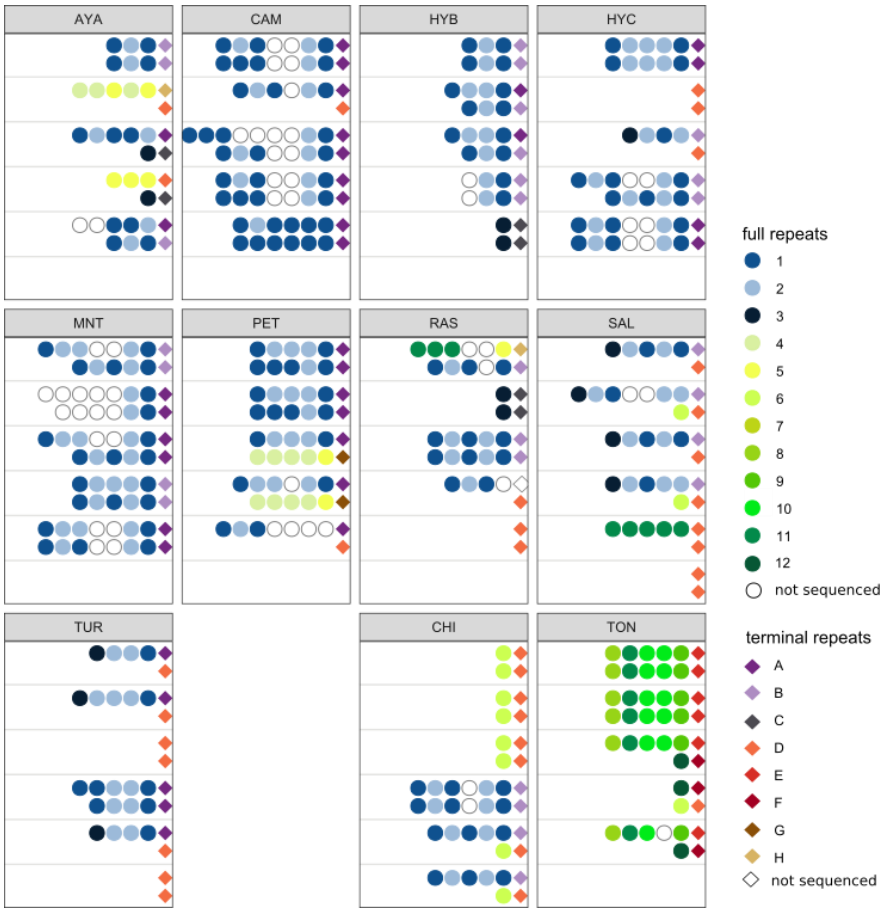

Fig. 5.2 *mmr-b* alleles found in the isolates. Each vertical box represents a population, and each pair of rows with symbols are the two *mmr-b* of a single clone. Each colour represents a different haplotype, either from full repeat (circle) or terminal repeat (diamond). The two detached boxes (CHI and TON) are the outgroup populations.

Network and Geographic Distribution of Nucleotide Haplotypes

Genomic variation in nucleotide sequence and number of repeats was remarkably high, both within and among populations. (Fig. 5.2). Network analysis of the terminal repeat identifies two groups of haplotypes (Fig. 5.3A), separated from each other by two synonymous and five non-synonymous mutations. The same analysis was applied to the full repeats, and then the frequency of the terminal repeat haplotypes was mapped on the full repeat haplotypes (Fig. 5.3B). This shows a high concordance between the structure of variation in the terminal and the full repeats. Due to this concordance, further analysis focused only on the terminal repeat. Regarding the geographical distribution of the different terminal haplotypes, it can be observed, that except for TON population were private haplotypes are found, the rest of the haplotypes are shared among the rest of the populations (Fig. 5.3C). The relative frequency of haplotypes varied from populations, even among relatively geographical proximate populations.

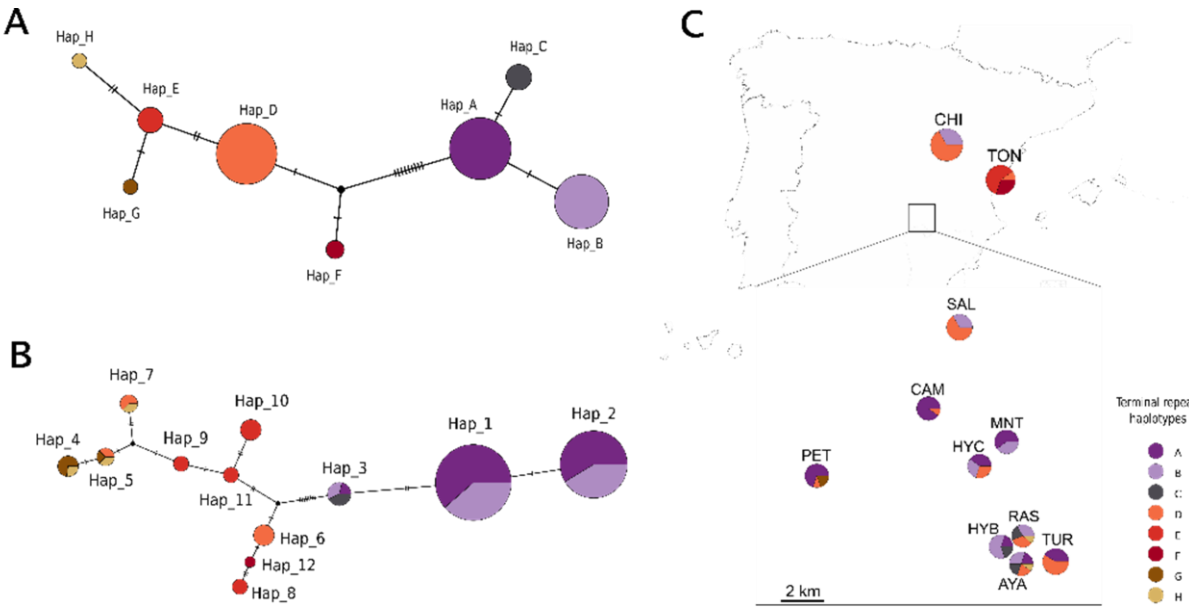

Fig. 5.3 Haplotype network for terminal (**A**) and full (**B**) repeats of *mmr-b*. The colour codes signify different haplotypes of terminal repeat. In the case of a network for full repeats, the colour fractions represent the proportion of terminal repeats appearing in the sequence with the particular full repeat. (**C**) Relative frequency of each terminal repeat in the studied populations with their geographical location on a map of Spain.

Patterns of Population Differentiation in mmr-b

Genetic differentiation between populations, measured as F_{ST}, was computed separately for microsatellites and terminal repeats. Genetic differentiation increased significantly with geographic distance for microsatellites markers, as well as for *mmr-b* terminal repeat. The former relationship is largely dependent on the effect of the long distances involved by the out-group populations, and especially on TON. When the outgroup (distant populations) was removed, a significant correlation of increasing genetic differentiation with geographical distance was observed for microsatellite markers, while this pattern was not found for the *mmr-b* terminal repeats (Fig. 5.4A). Contrasting to the pattern found for the geographic distance, genetic differentiation increased significantly with differences in environmental predictability when based on the *mmr-b* terminal repeat, but not in the case of microsatellite markers (Fig. 5.4B). The phenotypic result of the *mmr-b* gene (i.e., length of the protein which depends on the number of repeats and amino acid sequence of the repeats), showed a certain pattern in relation to environmental unpredictability (Fig. 5.5), with longer sequences of the amino acid haplotype AA1 associated to both extremes of pond predictability, while intermediate predictability tended to possess shorter sequences. At the same time, most AA2 haplotype sequences are found in more predictable environments.

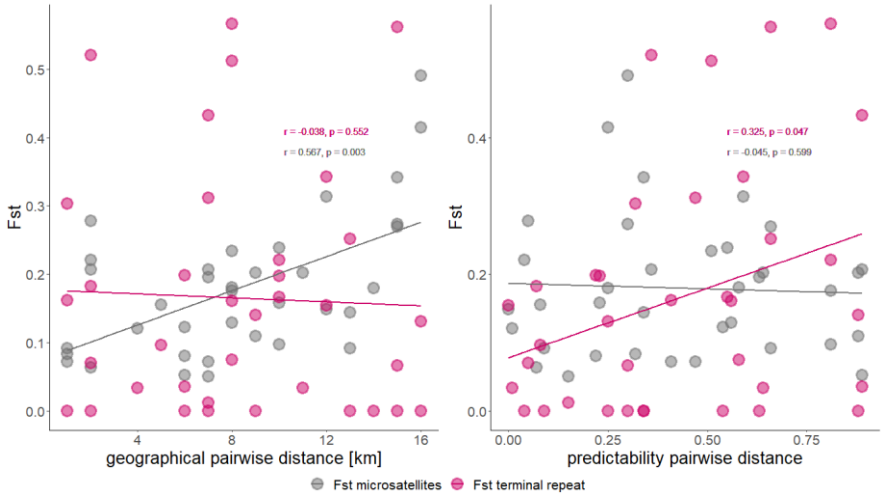

Fig. 5.4 Relationship between F_{ST} for neutral markers (in grey) and terminal repeats of *mmr-b* (in magenta) and geographical distance (**A**) and pond predictability (**B**), respectively. Person's correlation coefficients are shown (*p* values based on a Mantel test with 100,000 permutations).

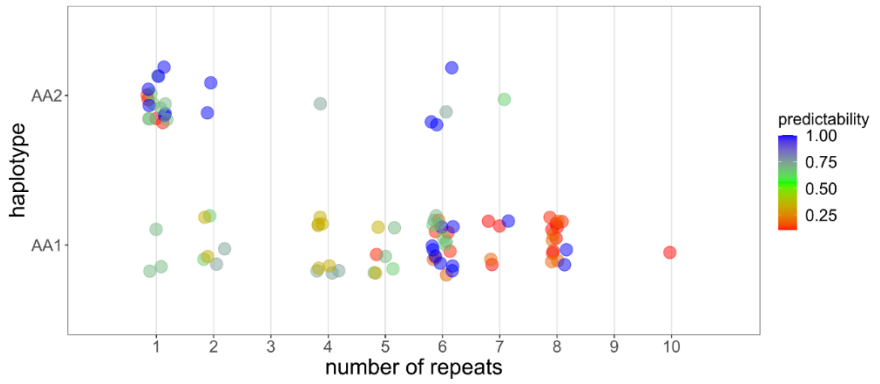

Fig. 5.5 Relationship between the number of repeats in each sequenced allele (full and terminal together), amino acid haplotypes (AA1, AA2) and environmental predictability of the corresponding population.

Discussion

Here we studied the intraspecies variability of the mate recognition protein motif repeat gene (*mmr-b*), whose product, mate recognition protein (MRP), is exposed on the body of *B. plicatilis* females and is implicated in male-mate recognition (Snell et al., 2006). The structure of *mmr-b* is well characterized for species in the *B. plicatilis* species complex and showed a greater level of sequence divergence than housekeeping genes (Gribble & Welch, 2012). However, little is known about within-species variation in *mmr-b*, a variation that could be involved in population differentiation and speciation. Variability in genes for mate recognition is specially intriguing because its emergence must overcome the effect of stabilizing selection usually acting on mate recognition traits (Butlin et al., 1985; Brooks et al., 2005).

In this study, we focused on a single MRP locus (*mmr-b*), in contrast to previous studies where there is uncertainty about the number of loci studied (Gribble & Welch, 2012). Our MRP locus resulted to have high variability in length polymorphism both within and among populations. Alleles varied from one repeat (i.e., zero full and one terminal repeat) to 10 (i.e., nine full and one terminal repeat), suggesting that one terminal repeat might be sufficient for recognition. In addition, relatively large nucleotide variability among repeats was also found. Nevertheless, the nucleotide haplotypes collapsed into only two amino acid haplotypes, as most nucleotide changes corresponded to synonymous substitutions. Two of these amino acid changes may be potential targets for amino acid glycosylation, which might influence the male-female recognition (Gribble

et al., 2011). The dN/dS ratio test on the terminal repeat sequence indicated that stabilizing selection is acting. This is expectable because mate recognition is one of the crucial processes in the life of a sexual individual, so there would be a selective pressure for mating recognition traits to remain stable and ensure that the individual can be recognized as a potential mating partner (Brooks et al., 2005). However, once populations start to diverge, the speed of diversification in these traits is often higher compared to neutral markers (Swanson & Vacquier, 2002). The phylogeographic structure we found showed two highly divergent nucleotide haplotype groups corresponding to the two amino acid haplotypes observed (AA1 and AA2). Haplotypes coding for the AA2 haplotype harbour a higher nucleotide diversity, suggesting a higher divergence time, which is concordant with the geographical distribution and their colonization history (Gomez et al., 2002a).

Overall genetic differentiation in *mmr-b* and neutral markers between populations increased with geographical distance when all the populations were considered (i.e., including the more distant TON and CHI ponds). Focussing on the set of neighbouring locally adapted populations, a remarkable finding is the opposing patterns for *mmr-b* and microsatellites in relation to geographic and environmental distance. On the one hand, the divergence in microsatellites, but not that of *mmr-b*, increases with geographical distance. This difference may be related to the higher mutation rates (followed by drift) for microsatellites, while in *mmr-b* new mutations are likely to be purged. It is also likely that all these neighbour populations share the same historical origin, so that the serial founder

effects causing the isolation by distance pattern (Gomez et al., 2002a) observed on *mmr-b* when the distant populations are considered, would not operate. On the other hand, *mmr-b* divergence, but not that of microsatellites, increased with environmental distance. Non-neutral variation on *mmr-b* might be selected to avoid mating between ecologically diverged genomes (i.e., low fitness of the offspring in the local environment) (Butlin, 1987). Even if admittedly, the evidence is not strong, a non-neutral divergence in *mmr-b* related to local adaptation to predictability is suggested by Fig. 5.5, which focus on phenotypic effects of *mmr-b*. If so, selective divergence in non-synonymous sites could cause correlated divergence in synonymous sites due to physical linkage, then affecting divergence in *mmr-b* as a whole. Regarding the strength of the pattern depicted in Fig. 5.5, it is worth noting that local adaptation is an averaged population feature, thus, variation within population is expectable and therefore, different clones within the populations oscillate around the adaptive optimum (Franch-Gras et al., 2017b). Moreover, it's unlikely that environmental unpredictability would serve as the unique adaptive force, homogenizing whole populations.

Some of the populations included here were studied earlier, focusing on mating behaviour, copulatory preferences and prezygotic reproductive isolation (Jezkova et al., 2022). In this previous study, a positive correlation between behavioural isolation and environmental predictably was observed. Additionally, some of the clones used in that previous work have been sequenced here for the *mmr-b;* thus, we can relate the behavioural reproductive isolation to the genetic sequences of three crosses. Two

clones from CHI pond with different MRP amino acid sequences, as well as different number of repeats were remarkably prone to refuse mating. In addition, a negative mating preference occurred between two clones from HYB pond with the same amino acid repeat haplotype but differing in the number of repeats. On the other hand, clones of TON pond possessing the same amino acid repeat haplotypes and sharing one allele were especially prone to mate. Therefore, the comparison between the two studies is in line with previous evidence for a critical role of *mmr-b* in mate recognition (Snell et al., 2006; Gribble et al., 2011).

Other processes connected to reproduction and subjected to the influence of environmental adaptation can facilitate species differentiation. Diet composition and presence of predators can provoke shifts in pheromone composition related to mating (see Symonds & Elgar, 2008) and references therein). Timing in reproduction has been associated to divergence events in a few groups of organisms (e.g., Aspinwall, 1974; Linn et al., 2004; Hall & Willis, 2006; Papakostas et al., 2012). In cyclical parthenogens, the timing of mixis (i.e., sexual reproduction) has been found to be genetically determined and correlated to the environmental conditions of the ponds (Jankowski & Straile, 2004; Franch-Gras et al., 2017b). This may create another barrier in case of population diversification through local adaptation after a secondary contact. Other zooplankters display a wide array of mechanism of mate choice (e.g., in copepods copulatory dances, mate guarding, stroking) (Titelman et al., 2007) that can also lead to reproductive isolation. Contact mate recognition has been described in other groups besides rotifers (cladorcerans, copepods) and its relevance in

differentiation has been suggested (Snell, 2011a). However, little is known about the genes involved in mate recognition in these taxa and its evolution associated to differentiation.

In this study we have focused in just one selective process along a single environmental gradient. Admittedly, other evolutionary processes, both neutral and adaptive, oppose and may counterbalance local adaptation and divergence of the mate recognition traits. The key counteracting processes include persistent founder effects resulting in high genetic drift (Montero-Pau et al., 2018), selection for multipurpose genotypes and, in relation to mate recognition, the stabilizing selection to remain equal. Despite all of this, we have found clear signatures of non-neutral divergence in *mmr-b* associated to local adaptation to unpredictability. Meaningfully, these signatures were consistent with mating behavioural isolation. Further studies with greater number of populations and ponds will be desirable in order to find stronger patterns and possibly including other gradients. Our study also recalls the need to further investigate other aspects of the function of the mate recognition protein, as a lack of additional functions would allow accumulating genetic variance during the parthenogenetic proliferation phase, thus fuelling selection in the sexual phase. Differences in expression profiles of *mmr-b* genes or the impact of variation in the post-translational modifications could be relevant. In addition, it would be relevant to also study the variation of the MRP mannose receptor expressed by males that could be crucial in the behavioural isolation. All in all, our results have shown the existence of a large amount of genetic variability on the MRP, and that part of this

variation is likely the result of local adaptation to environmental unpredictability. The emergence of this pre-mating barrier can be the first step of an in incipient reproductive isolation among neighbouring populations of the Iberian Peninsula.

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Chapter 6

General discussion, future perspectives and conclusions

Reproductive isolation is essential for the persistence of species and for speciation, especially when talking about animals. The evolution of reproductive isolation involves various factors that act on different stages during reproduction and often interact positively or negatively, promoting or contradicting the results of others (e.g., Coyne & Orr, 2004; Räsänen & Hendry, 2008). It can arise in different ways: (1) neutrally, as a result of divergence by genetic drift on genes involved in reproduction, (2) as a by-product of selection on correlated traits when the loci involved in reproduction are linked to loci under selection (indirect selection) or when local adaptation creates an ecological barrier (e.g., non-overlapping reproductive periods), or (3) as a product of direct selection on reproductive isolation. The immediate consequence of complete reproductive isolation is to maintain the integrity of the isolated lineages, which then become independent evolutionary units. In line with this, to a lesser extent, partial reproductive isolation promotes deeper differentiation and thus nurtures its evolutionary independence. Multiple steps and functional mechanisms can be involved in that isolation. Most relevant is the selection and evolution of premating isolation if some isolation already exists at the final steps of the reproductive cycle (including the fitness of the descendants) (Servedio & Noor, 2003). Premating reproductive isolation has immediate benefits, and selective pressures can facilitate its establishment (Safran et al., 2013). Given its significance, it is not surprising that evolutionary biologists have devoted considerable attention to this topic.

For organisms living in challenging environments, such as zooplankters inhabiting ponds with an unpredictable water regime, fine-tuned adjustment of their life cycles is essential to survive recurrent adverse conditions and to cope with uncertainty. Therefore, development of a series of mechanisms related to local adaptation is expected in these populations (Campillo et al., 2011; Franch-Gras et al., 2017). In the case of populations where migration from other populations can happen, it is crucial to evolve ways to preserve this acquired adaptation and reduce gene flow.

In this thesis three different approaches have been used to study the evolution of reproductive isolation in different populations of *B. plicatilis* from Iberian Peninsula. In Chapter 3, behavioural assays were used to assess the level of premating behavioural isolation among populations as an effect of phylogeographic and adaptive structuring. The setup was based on individual variation (in the form of different clones of a population) in behaviour. The results showed the evolution of low, but not negligible premating reproductive isolation. It was shown that adaptive divergence even with possible gene flow among populations plays a more important role in evolution of premating reproductive isolation, compared with neutral differentiation caused by geographical distances preventing gene flow. However, when considering individual pairs of populations, in some cases no signs of reproductive isolation or preference for between-population reproduction were observed. This may be accounted for a series of factors. It is expected that many factors co-operate to shape the final form of reproduction and avoidance of reproduction between

populations and behavioural isolation accounts for only a part of it (Coyne & Orr, 2004). The lack of behavioural isolation therefore doesn´t have to mean isolating mechanisms do not operate here. On the other hand, an experimental setup may influence the possibility to detect behavioural isolation. Here, for example, high densities of individuals were used, therefore the encounter probability as one of the possible isolating mechanisms was not pondered. The asynchronicity of sexual reproduction was not considered either, as young males and young females susceptible to reproduction were used.

As those populations presenting an adaptive divergence showed stronger effect on reproductive isolation than geographically distant populations (Chapter 3), Chapter 4 concentrated on the relationship between reproductive isolation and local adaptation. The experimental approach was expanded both in terms of the number of populations and the number of clones used. The experimental setup mimicked natural conditions more closely than in Chapter 3 in relation to density of individuals, the possibility of asynchrony in the timing of sex, and mate competition. Also, multiclonal populations were used instead of individual clones. Potential reproductive isolation was assessed at the stage of diapausing egg production, which is the outcome of sexual reproduction. Consistent with the findings from Chapter 3, signs of reproductive isolation were found, in this case in one third of the population combinations. The inbreeding index indicated a preference for within population crosses in two thirds of the cases. No signs of within-population reproductive preferences were found in many pairs of populations. In some cases, even a preference for between-population

reproduction was observed. These contrasting results are not surprising considering that the populations belong to a single species and were sampled in neighbouring ponds. The surprising result would be total isolation, instead of partial one between all, instead some, populations studied. Nevertheless, our laboratory experiments did not account for isolation barriers that might be acting in the wild (Dobzhansky, 1977; Coyne & Orr, 2004). The experimental setup accounts for large densities (although lower that in Chapter 3), so the male-female encounter probabilities were high. The environmental conditions –e.g., temperature or salinity– may, in natural conditions, cause isolation, if immigrants do not perform well in their new locality (Campillo et al., 2011). Here the conditions that were previously shown to be convenient for all studied populations, were used.

To understand the genetic background of behavioural reproductive isolation, the genetic variability of the Mating Recognition Protein was studied in Chapter 5. This protein is expressed on female bodies and is responsible for female-male mate recognition in *B. plicatilis* (Snell et al., 2006). It is coded by the *mmr-b* gene with a structure consisting of a 48 bp signalling sequence, followed by several nearly identical 261 bp "full" repeats and finally one 234 bp "terminal" repeat at the end of the sequence (Gribble et al., 2011). The terminal repeat follows the structure of full repeats with the 18 bp deletion on 3`end of the sequence.

One locus was found in all studied populations, composed of one terminal repeat and between 0 and 9 full repeats. As most of the mutations lead to synonymous changes, the nucleotide haplotypes found merged into two amino acid haplotypes. Stabilizing selection acting on *mmr-b* was found,

which is consistent with the commonly accepted idea of differentiation of traits involved in reproduction (Wojcieszek & Simmons, 2012; Zacchello et al., 2020). It was found that genetic differentiation in *mmr-b* correlated with differences in environmental predictability, while this correlation was not found for neutral markers. This is consistent with the hypothesis that selective divergence acted on *mmr-b*. As expected, for populations of the same species the pattern was relatively weak, and our finding of stabilizing selection likely has implications for this weak effect. Moreover, as the results of *mmr-b* are not entirely consistent with the patterns found at phenotypic level, including mating behaviour, we suggest that other loci may be involved in mate recognition and reproductive isolation.

The results of all three approaches showed major influence of adaptive divergence, rather than geographic distance, on the evolution of reproductive isolation in *B. plicatilis*. The level of reproductive isolation was rather low, which is expectable when taking in account the difficulties in differentiation of traits involved in reproduction (Butlin et al., 1985; Brooks et al., 2005) and the fact that even fully formed *Brachionus* species show some level of among species mating behaviour (Rico-Martínez & Snell, 1995; Gribble & Welch, 2012).

The factors involved in reproductive isolation are complex and influence different phases of reproduction. It is important to emphasise that these studies represent only a reduction from the plethora of different isolation options (Dobzhansky, 1977; Coyne & Orr, 2004). Our experimental approach may lead to artificially strengthened contact between individuals from the studied populations, reducing or even directly eliminating other

barriers that may play role in reproductive isolation, e.g., the effect of timing in rotifer sexual life cycle (Kordbacheh et al., 2023). It was also previously shown that even two fully formed species with no reproduction in nature can breed in captivity and give rise to fully viable and fertile hybrid offspring (Gabryś et al., 2021).

Reproductive isolation embraces a diversity of processes with the effect converging in the same direction, to cessation of gene flow. Although reproductive isolation allows preservation of advantageous allele combinations and thus maintains well adapted genotypes in populations, it does not have to be the only evolutionary trajectory in diverging populations. It can close doors to possible exploration of new adaptive peaks, as the formation of among population genotypes is prevented. In diverging populations of reduced size, impeding of gene flow from outer populations may also lead to the increasing of proportion of inbreeding with possible negative consequences on life cycle (Tortajada et al., 2009).

The origin of reproductive isolation and its causes and evolution remains one of the important topics of evolutionary ecology studies. A panoply of processes with complex interactions, often creating causal loops, are involved and make this topic challenging to study. In this thesis, we shed light on the incipient reproductive isolation in zooplankter *B. plicatilis*.

Future perspectives

The aim of this thesis was to improve our knowledge about the evolution of reproductive isolation using small aquatic invertebrates. Many factors

or reproductive steps have been left aside but might be a target for further investigation. For future research, we advocate by using a population approach as that adopted here. A research strategy might consist in splitting the reproductive sequence, from mate recognition to the production of diapausing eggs and hatchling fitness into smaller steps than investigated here. Practical considerations would impose to reduce the number of populations.

In a contrasting approach, future research could broad the geographic scope to evaluate whether a robust pattern arises when very distant (> 1000 km) populations are investigated. The methodology used in Chapter 4 would be feasible.

Reproductive isolation might be mediated by the differential performance during the parthenogenetic proliferation of the mixed populations. For instance, immigrant genotypes might be incapable of proliferation in their new locality. This putative factor deserves attention and might be investigated by using the methodology of Chapter 4 for specific –a few– population pairs, but in a variety of culture conditions that mimic relevant ones in the wild.

In Chapter 4, we tested the reproductive isolation in form of deficit of heterozygotes on the diapausing eggs. Although using “healthy-looking” eggs with high expectance of hatching, it would be interesting to study the real hatching potential, as well as the life perspectives of the hatchlings, their viability and fertility.

In Chapter 5 we studied the variability of *mmr-b* gene, that codes for a protein driving mate recognition. Although proved to be an indispensable part of mate recognition, it may be not the only one involved in the process of male-female recognition in *B. plicatilis*. Thus, other components from the complex process of mate recognition may be studied. Regarding to *mmr-b* itself, it would be interesting to study the actual effect of individual nucleotide or amino acid haplotypes, as well as the effect of number of repeats, on mate recognition, this may be made by expression of artificially produced and manipulated parts of *mmr-b* gene.

B. plicatilis used in this dissertation belongs to the group of cryptic *B. plicatilis* species. These species are phenotypically very similar and often co-occur at the same habitat. Thus, it would be interesting to compare the evolution of reproductive isolation in other co-occurring *Brachionus* species in order to gain clearer evolutionary perspective.

Conclusions

1. The behavioural assays testing mating reproductive isolation showed signatures of incipient reproductive isolation in the form of a higher propensity for within-population crosses compared to among-population crosses. This pattern was observed in populations selected based on both phylogeographic structure and adaptive divergence.

2. The tendency for within-population mating preferences was stronger in populations chosen because of tested adaptive divergence whose habitats are in geographic proximity.
3. The signs of incipient reproductive isolation are consistent with the hypothesis of reinforcement; i.e., selection for premating reproductive isolation after an establishment of partial postmating isolations.
4. No correlation was found between geographical distance and mating isolation, while a correlation was found between increasing environmental differences and mating isolation, which is consistent with the hypothesis that local adaptation has a stronger effect on the evolution of reproductive isolation than geographic distance.
5. When reproductive isolation was assessed at the level of production of diapausing eggs, incipient reproductive isolation in the form of a deficit of heterozygotes was found in five out of 12 crosses.
6. F_{IS} values in the diapausing eggs were positive on average and in eight out of 12 crosses, indicating a tendency towards within-population crosses.
7. F_{IS} values decreased during the time course of the experiment, which suggests an effect of environmental deterioration or the asynchrony of the induction of reproductive isolation.
8. *mmr-b* gene was amplified, and one locus was found in all studied populations. Its length ranged from 242 bp to 2591 bp, representing 0 – 9 full repeats and one terminal repeat. This

suggests that a single terminal repeat may be sufficient for mate recognition in *B. plicatilis*.

9. Relatively high nucleotide variability was found among *mmr-b* gene repeats, but as most were neutral (i.e., synonymous substitutions), the nucleotide variability grouped into two amino acid haplotypes.
10. The dN/dS ratio indicates stabilizing selection acting on the *mmr-b* gene.
11. When considering neighbouring populations, a positive correlation was found between genetic differences in *mmr-b* and adaptive divergence, as well as between neutral markers and geographic distance. Significant correlations between neutral markers and environmental divergence, and between genetic differences in *mmr-b* and geographic distance were not found.

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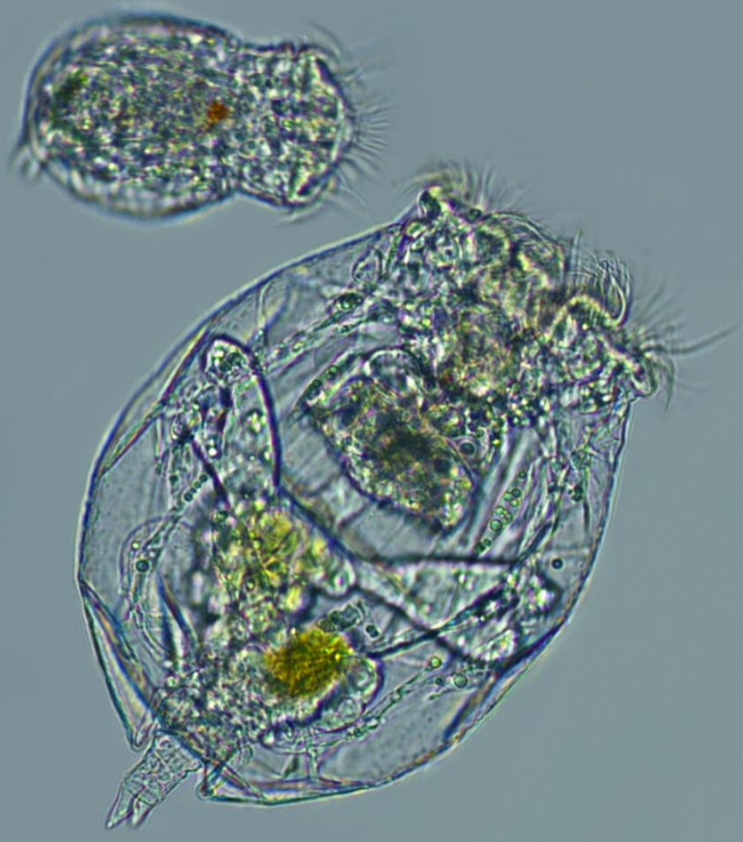
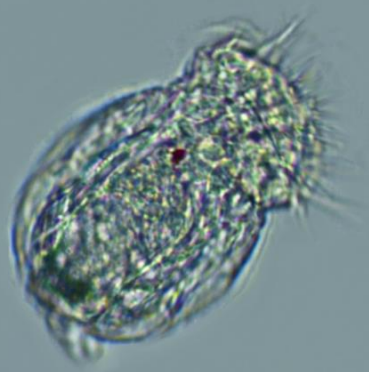


Resumen en extenso



Introducción

Las poblaciones de una especie generalmente habitan una variedad de ambientes y localidades. Con el tiempo, diversos procesos, tanto adaptativos como no adaptativos (neutrales), promueven la diferenciación genética de las poblaciones. A estos procesos, sin embargo, se contraponen otros que mantienen y promueven la homogeneidad dentro de una especie (Dobzhansky, 1977; Holderegger et al., 2006). Dependiendo de qué procesos prevalezcan, la evolución de una población puede tomar diferentes trayectorias. Si prevalecen los primeros, la diferenciación poblacional resultante permite que las poblaciones exploren y utilicen diferentes ambientes con consecuencias positivas para la persistencia de las especies y para la probabilidad de especiación; lo que resulta crucial en el mantenimiento de la biodiversidad (Nosil & Feder, 2012; Wolf & Ellegren, 2016).

La diferenciación neutral, o no adaptativa, se refiere a la variación genética entre poblaciones causada por la deriva genética y que no tiene un efecto directo en la eficacia biológica (Dobzhansky, 1977; Holderegger et al., 2006). Esta diferenciación neutral puede ser promovida por factores extrínsecos, como la distancia geográfica, que protege del efecto homogeneizador de la migración efectiva, o como las fluctuaciones ambientales que afectan al tamaño de las poblaciones. El efecto de los factores neutrales se acentúa especialmente en poblaciones con dinámicas rápidas y tamaños de población fluctuantes, o en aquellas desplazadas de sus sitios originales y que colonizan nuevos nichos (Kawecki & Ebert, 2004). La diferenciación poblacional no neutral, por otro lado, implica selección y

generalmente está asociada con la adaptación divergente a las condiciones ambientales locales. Así, la adaptación local puede ser uno de los resultados evolutivos en caso de poblaciones que se enfrentan a ambientes que difieren entre localidades. Pero incluso cuando las poblaciones se exponen a ambientes selectivos similares, pueden seguir trayectorias evolutivas diferentes, ya que pueden resolver desafíos evolutivos similares a través de diferentes estrategias (Endler, 1986; Arendt & Reznick, 2008). Esto último surge de la interacción entre la deriva genética y la evolución adaptativa. Sin embargo, la divergencia adaptativa no es la única respuesta a la variación ambiental. Los individuos pueden mostrar plasticidad fenotípica o estrategias de apuestas compensadas (*bet hedging*), lo que les proporciona una forma de ajustarse a esa variación, con la consecuencia de que esta flexibilidad puede disminuir la probabilidad de aparición de la divergencia adaptativa (Botero et al., 2015; Perry et al., 2018).

El mantenimiento de la diferenciación poblacional se ve favorecido por la evolución de aislamiento reproductivo (Mayr, 1963; Coyne & Orr, 2004). Las barreras reproductivas se pueden dividir en intrínsecas y extrínsecas. Las intrínsecas son aquellas que resultan de características del sistema reproductivo en sí mismo. Por el contrario, las barreras extrínsecas son la debidas a factores externos de tipo histórico-geográfico o ecológico o adaptaciones que no operan directamente sobre el sistema reproductivo; es decir, se trata de aislamiento geográfico o ecológico (Coyne & Orr, 2004; Matute et al., 2009). Estas barreras pueden actuar en diferentes fases de la reproducción, que en animales con sexos separados irán desde el encuentro macho-hembra, el reconocimiento de pareja y el cortejo (si es

aplicable) hasta la interacción de las células reproductivas, el desarrollo del cigoto, y la viabilidad y fertilidad de la descendencia entre otros (Mayr, 1963; Coyne & Orr, 2004). Una distinción evolutivamente relevante es la que existe entre aislamiento pre-apareamiento (*premating*) y aislamiento post-apareamiento (*postmating*).

Las barreras reproductivas pueden involucrar una amplia gama de factores, como los factores espacio-temporales para poblaciones que habitan diferentes localidades o la misma localidad en diferentes momentos; los factores ecológicos para poblaciones adaptadas que habitan diferentes nichos ecológicos; los factores etológicos durante el apareamiento; los factores fisiológicos en forma de diferencias estructurales en los órganos reproductivos, o incompatibilidades genéticas generales, entre otros (Coyne & Orr, 2004). Estos factores tienden a impedir el intercambio de genes entre especies o poblaciones y, por lo tanto, la recombinación entre genomas (por ejemplo, Orr, 2005; Matute et al., 2009; Matute, 2010). En consecuencia, se mantiene la divergencia de poblaciones, la integridad genética y las combinaciones de alelos ventajosas que surgen a través de la divergencia adaptativa y la adaptación local. El aislamiento reproductivo puede evitar apareamientos poco productivos (Coyne & Orr, 2004). Por otro lado, puede influir negativamente en las poblaciones al prevenir la introgresión adaptativa intraespecífica (Edelaar et al., 2012; Tigano & Friesen, 2016).

Objetivos

El objetivo de esta tesis es estudiar el aislamiento reproductivo entre diferentes poblaciones del microinvertebrado acuático *Brachionus plicatilis* (Müller 1786) (Ciros-Pérez et al., 2001). El propósito es encontrar patrones y relaciones que puedan proporcionar información sobre la evolución del aislamiento reproductivo. Existen estudios previos de *B. plicatilis* en la península ibérica que han caracterizado estas poblaciones desde el punto de vista de la divergencia filogenética (Gomez et al., 2002b) y la respuesta adaptativa a la impredecibilidad ambiental en sus hábitats (Franch-Gras et al., 2017b). Por ello, este sistema proporciona una oportunidad para investigar el aislamiento reproductivo entre las poblaciones y evaluar el potencial de preferencias reproductivas dentro de las poblaciones.

Se proponen las siguientes hipótesis:

- Existe aislamiento reproductivo parcial entre las diferentes poblaciones de *B. plicatilis* que habitan lagunas en la península ibérica.
- El aislamiento reproductivo parcial, en caso de estar presente, se correlaciona con la distancia filogenética entre las poblaciones.
- El aislamiento reproductivo parcial, en caso de estar presente, se correlaciona con la adaptación de las poblaciones de *B. plicatilis* al nivel de predecibilidad ambiental.
- El aislamiento reproductivo parcial, en caso de estar presente, se correlaciona con la divergencia en el locus cuyo producto, *Mating Recognition Protein* (la proteína de reconocimiento de pareja,

MRP), es responsable del reconocimiento entre machos y hembras en *B. plicatilis*.

Metodología

El rotífero B. plicatilis

Las especies de microinvertebrados acuáticos son adecuadas para abordar la interacción evolutiva entre la diferenciación poblacional y el sistema reproductivo. En esta tesis, hemos utilizado el rotífero monogonontе *B. plicatilis* como organismo modelo. Los rotíferos son uno de los grupos de metazoos más abundantes en el zooplancton continental. Se pueden encontrar en todo tipo de hábitats acuáticos continentales de diversos tamaños o condiciones ambientales y forman parte de una variedad de comunidades. Algunos de ellos habitan hábitats extremos, como aguas termales, gotas de agua en musgos y líquenes o localidades con alta radiación (Wallace et al., 2005; Iakovenko et al., 2015; Rebecchi et al., 2019). La capacidad de sobrevivir en una amplia gama de ambientes está mediada por varios factores, como la producción de formas resistentes (Schröder, 2005), su alta capacidad de dispersión (Bohonak & Jenkins, 2003) y su capacidad de colonizar rápidamente nuevos ambientes y localidades (Gomez et al., 2002a; Schröder, 2005).

Las poblaciones de monogonontes suelen estar activas solo estacionalmente en la columna de agua. A este período se le denomina periodo de crecimiento. El ciclo de vida típico de los monogonontes comienza con la aparición de hembras diploides de rotíferos por eclosión

de los huevos de diapausa que existen en el sedimento al comienzo del periodo de crecimiento. A partir de estas hembras, en la columna de agua, tiene lugar una fase de proliferación clonal por un número indeterminado de generaciones, mediante la producción partenogenética de huevos subitáneos que se desarrollan en hembras asexuales (partenogenéticas) (Serra et al., 2018). Esta reproducción asexual permite a las poblaciones alcanzar altas densidades en un tiempo relativamente corto. La reproducción sexual es inducida por la densidad poblacional después de un período obligatorio de reproducción asexual (Hagiwara & Hino, 1989; Stelzer & Snell, 2003). El umbral de densidad para inducir el sexo depende de una serie de factores y varía de una población a otra (Snell & Boyer, 1988; Franch-Gras et al., 2017b). Después de alcanzar el umbral de densidad, las hembras asexuales empiezan a producir partenogenéticamente una fracción de hijas (diploides) que se denominan sexuales. Estas producen huevos haploides que, si no son fertilizados, dan origen a machos haploides. Los machos son de menor tamaño y están anatómica y funcionalmente reducidos en comparación con las hembras. Cuando las hembras sexuales son inseminadas en las primeras horas de su vida (Snell & Childress, 1987; Snell & Hoff, 1987), producen huevos de diapausa diploides. A pesar de ser llamados "huevos", en realidad se trata de embriones. Los huevos de diapausa, después de ser transportados por la madre durante algún tiempo, caen al sedimento y finalmente eclosionan en uno de los próximos periodos de crecimiento (Wallace et al., 2015).

El apareamiento entre machos y hembras sigue una secuencia de tres pasos: primero se produce el encuentro entre la hembra y el macho. Una

vez se produce el encuentro, el macho desarrolla un comportamiento consistente en dar vueltas alrededor de la hembra (*circling*) y, finalmente, se produce la cópula. Dado que los rotíferos no secretan feromonas en el medio, la primera fase de encuentro es aleatoria. El reconocimiento de la pareja, en cambio, sí que se lleva a cabo a través de quimiorrecepción de contacto, en la cual la proteína de reconocimiento de pareja (MRP) es un componente importante (Snell & Morris, 1993; Snell et al., 1995; Gribble et al., 2011).

Las poblaciones de *B. plicatilis* ofrecen una oportunidad única para la realización de estudios de ecología evolutiva debido a diversas características de su biología (ver en Serra et al., 2019). Como resultado de estudios previos, se conoce que las poblaciones albergan una gran diversidad genética (Gomez & Carvalho, 2000; Ortells et al., 2006; Montero-Pau et al., 2017), los eventos históricos han moldeado su estructura filogeográfica (Gomez & Carvalho, 2000; Campillo et al., 2011b), las poblaciones muestran divergencia en rasgos ecológicos (Campillo et al., 2011a), y también en la adaptación local derivada de la impredecibilidad ambiental (Franch-Gras et al., 2017b).

La península ibérica como laboratorio de campo

La península ibérica es una región de alto interés evolutivo, ya que es uno de los refugios glaciares más importantes del Pleistoceno en Europa (Hewitt, 2004; Abellán & Svenning, 2014). Estos refugios permitieron la supervivencia y diferenciación de las poblaciones y sirvieron como

reservorio de diversidad genética y de especies para los períodos posteriores a la glaciación, lo que ha influido en los patrones filogeográficos en Europa (Gomez & Lunt, 2007; Millette et al., 2011).

Los rotíferos no han sido una excepción, y en las poblaciones de *B. plicatilis* de la península ibérica se han descrito mediante marcadores neutrales (gen mitocondrial COI) tres clados filogeográficos resultado de este proceso (Gomez et al., 2002a). Así pues, encontramos poblaciones con una alta divergencia genética debida a procesos neutrales. Además, dentro de uno de los clados, se ha estudiado detalladamente un grupo de poblaciones que ocupan una pequeña área geográfica de aproximadamente 240 km^2. Las lagunas del área donde habitan estas poblaciones de rotíferos varían en cuanto a la fluctuación y predecibilidad del régimen hídrico (Franch-Gras et al., 2017a). Se conoce que la propensión a la reproducción sexual (i.e., la densidad a la que una hembra empieza a producir descendencia sexual, uno de los rasgos del ciclo de vida de *B. plicatilis*) esta correlacionada con el nivel de impredecibilidad ambiental de estas localidades (Franch-Gras et al., 2017b). En entornos poco predecibles, los rotíferos tienden a iniciar la reproducción sexual antes, asegurando así la producción de al menos algunos huevos de diapausa en caso de que el periodo de crecimiento (de proliferación clonal) fuera inesperadamente corto.

Por lo tanto, debido a (1) la estructura filogeográfica que afecta a áreas geográficamente distantes y (2) la diferenciación ecológica relacionada con la predecibilidad ambiental en una pequeña área, las poblaciones ibéricas de B. plicatilis son un buen sistema para probar la influencia de estos factores en la evolución del aislamiento reproductivo intrínseco.

Enfoque experimental

El núcleo experimental de esta tesis se desarrolla en sus Capítulos 3, 4 y 5, el conjunto de los cuales combina varios enfoques para caracterizar el aislamiento reproductivo, la divergencia ecológica y la variación genética en las poblaciones de *B. plicatilis*.

En el **Capítulo 3** se estudian las preferencias de apareamiento entre poblaciones de *B. plicatilis* con diferentes niveles de distancia genética o ecológica. El enfoque experimental consistió en seleccionar dos grupos de poblaciones. El primer grupo reunió poblaciones pertenecientes a diferentes clados filogeográficos, mientras que en el segundo grupo se incluyeron poblaciones que mostraban diferenciación en la adaptación a las condiciones locales, específicamente en la predecibilidad del régimen hídrico. Se realizaron ensayos de apareamiento entre individuos jóvenes de clones de la misma o de distinta población. Se realizaron pruebas bidireccionales, de manera que para cada par se ensayaros dos situaciones según qué población proporcionaba los machos y cuál las hembras. se. El protocolo de observación siguió el de Gomez & Serra (1995) con modificaciones menores y se registraron encuentros, *circlings* y cópulas en un periodo de 10 minutos por ensayo. El encuentro se definió como cualquier contacto físico entre la corona del macho y el cuerpo de la hembra. El *circling* (que implica un encuentro previo) se definió como el comportamiento de natación del macho alrededor de la hembra cuando se completa al menos un círculo. La cópula se definió como la inserción del pene del macho en la hembra, lo que ocurre en las partes blandas del esta (corona o abertura del pie). Siempre se observó *circling* antes de las

cópulas. Ningún clon fue utilizado en más de un ensayo dentro de un grupo. El objetivo fue evaluar la presencia de aislamiento pre apareamiento y determinar si se correlacionaba con la divergencia ecológica o la distancia geográfica.

Para cada combinación de poblaciones, se calculó el porcentaje de *circlings* tras los encuentros, el porcentaje de cópulas tras *circlings* y el porcentaje de cópulas tras los encuentros. Este último es una composición de los otros dos. Se utilizó un modelo lineal generalizado de efectos mixtos (GLMM por sus siglas en inglés) para analizar la significación de los efectos en el diseño experimental sobre las cópulas tras los encuentros. De manera semejante, se utilizó una descomposición lineal de los efectos considerados en el diseño experimental para cuantificar la propensión en los cruces de poblaciones. Uno de los factores del GLMM fue la interacción entre la población de machos y la población de hembras, que da cuenta de la existencia de preferencias dependientes de las poblaciones que se cruzan. Esta interacción muestra una propensión al comportamiento desviada del azar, por lo que, para encontrar signos significativos para los cruces entre poblaciones, este factor debe ser mayor para los cruces entre poblaciones, en comparación con los cruces entre poblaciones. Además, para cuantificar la propensión entre cruces de población, se estimaron coeficientes asociados al efecto de interacción.

Con el fin de comparar este ensayo con otros estudios, también se calculó el déficit-exceso relativo de cópulas dentro y entre poblaciones utilizando un índice de aislamiento reproductivo (RI, Sobel & Chen, 2014). También, se usó el coeficiente de correlación de Spearman para evaluar la asociación

entre las métricas de comportamiento de apareamiento y diferentes variables vinculadas a las características de las poblaciones (diferenciación genética en rasgos neutros, distancia geográfica y diferenciación en predecibilidad ambiental).

En el **Capítulo 4** se adoptó un enfoque más integrado para evaluar el aislamiento reproductivo en la fase de los huevos de diapausa. El objetivo fue evaluar en conjunto varias barreras de la reproducción, como el apareamiento preferencial, el éxito de la fertilización y el aborto temprano de los embriones en poblaciones de una escala geográfica pequeña (distancia máxima entre sus localidades: 16 km; área: 240 km^2) que mostraban diferenciación en la adaptación a la predecibilidad del régimen hídrico (regularidad en los periodos en los que la laguna posee agua). Se eligieron nueve poblaciones que abarcan una gama de adaptación a la predicibilidad ambiental (Franch-Gras et al., 2017b) y se establecieron cultivos de laboratorio mezclando clones de dos poblaciones diferentes a densidades de población bajas, más cercanas a las naturales que las necesarias para realizar las observaciones etológicas (Capítulo 3). De cada combinación de poblaciones se realizaron dos replicados. Los cultivos se dejaron proliferar durante varias semanas, de manera que dieran lugar a la formación de huevos diapáusicos. Estos huevos se recogieron periódicamente. Se aisló ADN de una fracción de huevos (de cada huevo de diapausa por separado) y se genotipó mediante el uso de SNPs privados de población con el fin de determinar si eran resultado de cruces intra o interpoblacionales.

Se calculó el porcentaje de los tres genotipos posibles (los dos parentales y el híbrido interpoblacional) en los huevos de diapausa para cada cruce de poblaciones, distinguiendo entre los dos momentos de recolección y los dos replicados). También se calculó el índice de fijación (F_{IS}) y su significación para el déficit de heterocigotos con respecto a las proporciones de Hardy-Weinberg (pruebas unidireccionales). Se calculó la correlación entre los valores de F_{IS} para las dos réplicas dentro de las combinaciones poblacionales, así como la correlación entre F_{IS} y los distintos predictores (distancia geográfica, distancia ambiental, distancia genética en marcadores neutrales y distancia genética en un gen que ha sido relacionado con el reconocimiento de pareja entre poblaciones). La distancia genética neutral se estimó como el valor de F_{ST} para microsatélites. En el reconocimiento de pareja interviene el gen *mmr-b* (capítulo 5). La distancia genética para este gen se estimó como el valor ϕ_{ST} para el motivo terminal del gen. La hipótesis de interés fue que los genotipos son más propensos a la reproducción intrapoblacional que a la interpoblacional. Además, interesó evaluar si esta tendencia es más marcada en las poblaciones que están más distantes en términos de predecibilidad ambiental.

El objetivo del **Capítulo 5** fue estudiar la variación genética en el locus de MRP (del gen *mmr-b*) y su relación con la adaptación local. Se muestrearon las once poblaciones previamente estudiadas en los Capítulos 3 y 4. Como los cebadores disponibles para amplificar el gen *mmr-b* en el género *Brachionus* (Gribble et al, 2011) no mostraron un rendimiento suficiente para nuestras poblaciones focales, se diseñaron nuevos cebadores a partir

de las secuencias de referencia del genoma de *B. plicatilis*. Se extrajo ADN genómico de cultivos monoclonales de alta densidad y se realizó una amplificación por PCR en cada clon por separado. El producto se secuenció de ambas direcciones. Además, se evaluó la variabilidad en microsatélites para estimar la diversidad genética en marcadores neutrales.

Se calculó la distancia genética entre poblaciones. Para los microsatélites, la distancia se evaluó en función de la frecuencia alélica (F_{ST}) y para el gen *mmr-b* en función de la distancia genética (ϕ_{ST}). Los análisis de la secuencia del gen *mmr-b* se realizaron (a) utilizando únicamente el motivo repetitivo completo y (b) el motivo terminal. También se estimó la correlación de las distancias genéticas basadas en microsatélites y basadas en el motivo terminal del gen mmr-b con la distancia geográfica y las diferencias en predecibilidad ambiental (cuatro correlaciones).

Resultados y discusión

Aislamiento reproductivo y conducta

Los porcentajes de cópulas tras los encuentros alcanzaron una media del 48% en los cruces dentro de poblaciones y del 42% en los cruces entre poblaciones. Al analizar los cruces dentro de población y entre poblaciones restringiendo la comparación de manera que se comparta una población, se encontró un mayor porcentaje de cópulas en los cruces intrapoblacionales en 15 de las 24 comparaciones. El resultado del GLMM para la cópula tras el encuentro mostró efectos significativos para todos los factores fijos: población que proporciona los machos, población que

proporciona las hembras y el del efecto de interés, la interacción entre la población de machos y la población de hembras, para cada grupo de estudio (poblaciones diferenciadas ecológicamente; poblaciones diferenciadas filogeográficamente).

La descomposición lineal de la respuesta mostró que los coeficientes medios del efecto de interacción fueron mayores para los cruces dentro de población que para los cruces entre poblaciones en ambos grupos de estudio, lo que indica una mayor proclividad a los cruces dentro de población que a los cruces entre poblaciones. Esta tendencia fue mayor en el grupo de poblaciones que mostraba diferenciación en la adaptación local a la predecibilidad del régimen hídrico que en el grupo de estudio que incluyó diferentes clados filogeográficos.

El índice de aislamiento precigótico, RI, fue en general ligeramente positivo para todas las combinaciones de poblaciones, lo que puede indicar a un aislamiento reproductivo de comportamiento relativamente débil.

La correlación de Spearman entre los coeficientes de interacción de los dos grupos de estudio y las características poblacionales (diferencia en la predecibilidad ambiental, distancia geográfica y valores de F_{ST} en los marcadores neutros) fueron, excepto para la distancia geográfica en el grupo de estudio de poblaciones pertenecientes a diferentes clados filogeográficos, significativamente negativas, como era de esperar para una preferencia por los cruces entre poblacionales. Al mismo tiempo, las correlaciones entre RI y las características de la población fueron positivas, como cabía esperar de una preferencia por los cruces intrapoblacionales.

Sin embargo, estas últimas correlaciones no fueron estadísticamente significativas.

Los ensayos de preferencia de apareamiento mostraron aislamiento reproductivo parcial en el comportamiento de apareamiento. Aun siendo bajo el nivel de aislamiento, parece ser biológicamente significativo, y es más notable en el caso de poblaciones próximas (con probable migración ente ellas) escogidas por su divergencia adaptativa que en poblaciones escogidas por sus diferencias filogeográficas. Como era de esperar entre poblaciones de la misma especie, en algunos pares de poblaciones no se observaron signos de apareamiento preferencial intrapoblacional. En estos casos no se puede excluir que realmente exista cierto aislamiento reproductivo bien debido a otros factores no estudiados aquí o bien debido a que las condiciones experimentales pueden haber afectado al comportamiento de apareamiento. En relación con lo primero, al enfocarse en la conducta de apareamiento y utilizar machos ya producidos, no entran en consideración aspectos como la asincronía ente poblaciones en sus fases de reproducción sexual, ya que se utilizaron machos y hembras jóvenes susceptibles a la reproducción. En relación con lo segundo, las altas densidades de individuos en los ensayos pueden propiciar alta probabilidad de encuentro, que sería poco probable de alcanzar en condiciones naturales, donde se espera que los migrantes representen una baja proporción de los rotíferos presentes en la columna de agua.

Producción de huevos sexuales en cultivos mixtos de poblaciones

El total de los 28 cultivos experimentales en los ensayos del capítulo 4 produjeron huevos de diapausa. Las densidades de huevos producidos variaron entre réplicas y sobre todo a lo largo del curso temporal experimental, ya que la tasa de producción mostró una disminución notable. Se observó deficiencia de heterocigotos en 26 de las 48 muestras. Los valores F_{IS} de las muestras oscilaron entre - 0,84 y + 0,54 (con una media de 0,062). Ocho de las doce combinaciones de poblaciones mostraron valores F_{IS} positivos, lo que indica la propensión a favor de los cruces dentro de las poblaciones. Los valores de F_{IS} (ya sea por combinación de poblaciones o por cada muestra temporal) no mostraron una correlación significativa con ninguno de los predictores (distancia en predecibilidad ambiental, distancia geográfica o genética).

Consistentemente con los resultados del capítulo anterior, en este estudio también se encontraron señales de aislamiento reproductivo. En 5 de las 12 combinaciones de poblaciones se encontró déficit de heterozigotos y el índice de endogamia indicó una preferencia por los cruces dentro de la población en dos tercios de los casos. Por otro lado, no se encontraron signos de preferencias reproductivas dentro de la población en muchos cruces experimentales. En algunos casos, incluso se observó la preferencia por la reproducción entre poblaciones. Estos resultados heterogéneros no son sorprendentes. Si se tiene en cuenta que las poblaciones pertenecen a una sola especie y se muestrearon en lagunas cercanas, el resultado sorprendente sería un aislamiento total en lugar de parcial y entre todas las poblaciones estudiadas. De nuevo, en los casos en los que las

observaciones se apartan de las esperadas no se puede descartar que en la naturaleza no opere aislamiento. En primer lugar, las densidades poblacionales experimentales, aunque menores que en los ensayos de comportamiento, son más altas que las naturales y podrían propiciar mayor fertilización cruzada. Las condiciones ambientales, como la temperatura o la salinidad, pueden causar aislamiento en condiciones naturales si los inmigrantes tienen un desempeño deficiente en su nueva localidad (Campillo et al., 2011a).

Por otra parte, la estrategia de evaluación del déficit de heterocigotos también puede afectar al resultado. La cantidad de ADN obtenida de un solo huevo es baja, y esto podría afectar críticamentea los análisis de genotipado dando lugar a la pérdida aleatoria de uno de los dos alelos. Los ensayos de genotipado se realizaron dos veces para cada huevo y cada inconsistencia se asignó como heterocigoto. Al hacer esto, también podemos haber sesgado nuestros datos en contra de nuestra hipótesis de déficit de heterocigotos.

Diferenciación poblacional en el gen mmr-b

Se identificaron dos regiones genómicas diferentes que putativamente contenían el gen de *mmr-b*. Debido a la falta de al menos un motivo repetitivo completo o un motivo terminal, se descartó un locus como gen mmr-b no funcional. La otra región era consistente con la estructura descrita anteriormente del gen *mmr-b* funcional (Gribble et al, 2011).

Se encontró un locus en todas las poblaciones estudiadas. Este estaba compuesto por entre 0 y 9 motivos repetitivos completos y un motivo terminal. Sólo se observaron sustituciones en el locus, pero no inserciones ni deleciones. La variación genómica en la secuencia de nucleótidos y el número de repeticiones fue relativamente alta, tanto dentro de las poblaciones como entre ellas. Dado que la mayoría de las mutaciones condujeron a cambios sinónimos, los haplotipos detectados a nivel de nucleótidos se correspondieron a dos haplotipos aminoacídicos. El análisis del número de cambios sinónimos y no sinónimos mostró selección estabilizadora actuando sobre *mmr-b* (dN/dS *ratio test* = 2.26, valor p = 0.017), lo cual concuerda con la idea comúnmente aceptada de diferenciación de rasgos involucrados en el reconocimiento de pareja (Wojcieszek & Simmons, 2012; Zacchello et al., 2020). Dado que se observó una gran concordancia entre la estructura de la variación en el motivo repetitivo completo y en el motivo terminal, los análisis posteriores se centraron únicamente en el motivo terminal. Los haplotipos fueron comunes en todas las poblaciones, salvo en un caso en el que se encontraron haplotipos privados. La frecuencia relativa de los haplotipos variaba de una población a otra, incluso entre poblaciones relativamente próximas geográficamente.

La diferenciación genética entre poblaciones, medida como F_{ST}, se calculó por separado para los microsatélites y los motivos terminales. Se encontró que la diferenciación genética en mmr-b se correlacionó con diferencias en la predecibilidad ambiental, mientras que no se observó correlación de *mmr-b* s con marcadores neutrales. Esto es consistente con la hipótesis de

que la divergencia selectiva actúa sobre *mmr-b*. Por otro lado, la divergencia de microsatélites, pero no la de *mmr-b*, se incrementa con la distancia geográfica. Esta diferencia puede estar relacionada con las mayores tasas de mutación (seguidas de deriva genética) de los microsatélites, mientras que en el gen *mmr-b* las nuevas mutaciones pudieron ser purgadas. También es muy probable que todas estas poblaciones vecinas compartan el mismo origen histórico, de modo que no operarían los efectos fundadores en serie que causan el patrón de aislamiento por distancia (Gómez et al., 2002a) y que fueron observados en mmr-b cuando se consideran las poblaciones distantes.

Como era de esperar para poblaciones de la misma especie, la diferenciación en *mmr-b* fue relativamente débil. El efecto de la selección estabilizadora debe de tener implicaciones para que esta diferenciación sea débil. Por otro lado, una vez que las poblaciones empiezan a divergir, la velocidad de diversificación en estos rasgos suele ser mayor en comparación con los marcadores neutros (Swanson & Vacquier, 2002). Además, dado que los resultados de mmr-b no son totalmente consistentes con los patrones que encontramos a nivel fenotípico, incluido el comportamiento de apareamiento, sugerimos que otros loci pueden estar involucrados en el reconocimiento de pareja. En los machos, el receptor de manosa sirve de receptor para el MRP de las hembras, sin embargo, no se estudió su variación entre poblaciones. Al mismo tiempo, es poco probable que la predecibilidad ambiental del régimen hídrico sirva como única fuerza adaptativa, homogeneizando poblaciones enteras. Otros procesos relacionados con la reproducción y sometidos a la influencia de la

adaptación ambiental pueden facilitar la diferenciación genética. Por ejemplo, la composición de la dieta y la presencia de depredadores pueden provocar cambios en la composición de feromonas relacionadas con el apareamiento (ver en Symonds & Elgar, 2008). Al mismo tiempo, otros factores, como el inicio de la mixis (es decir, la reproducción sexual), pueden crear otra barrera en caso de diversificación de la población mediante la adaptación local.

Comentarios finales y conclusiones

Los resultados de los tres enfoques han mostrado indicios de aislamiento reproductivo en poblaciones naturales de *B. plicatilis*. Se ha observado una mayor influencia de la divergencia adaptativa, en comparación con la de la distancia geográfica, sobre la evolución del aislamiento reproductivo. El nivel de este aislamiento ha sido bajo, lo cual es de esperar dado que son poblaciones de la misma especie y que la diferenciación de rasgos involucrados en la reproducción presenta dificultades (Butlin et al., 1985; Brooks et al., 2005), así como por el hecho de que incluso las especies completamente formadas de *Brachionus* muestran algún nivel de comportamiento de apareamiento entre especies (Rico-Martínez & Snell, 1995; Gribble & Welch, 2012).

Los factores involucrados en el aislamiento reproductivo presentan una alta complejidad, y pueden influir en diferentes fases de la reproducción. Es importante destacar que estos estudios representan solo una reducción de la multitud de opciones de aislamiento (Dobzhansky, 1977; Coyne & Orr,

2004). Nuestro enfoque experimental puede conducir a un contacto artificialmente alto entre individuos de las poblaciones estudiadas, reduciendo o incluso eliminando directamente otras barreras que pueden actuar en el aislamiento reproductivo (Kordbacheh et al., 2023). También se ha demostrado previamente que incluso dos especies completamente formadas sin reproducción en la naturaleza pueden reproducirse en cautividad y dar lugar a descendencia híbrida completamente viable y fértil (Gabryś et al., 2021).

El aislamiento reproductivo abarca muchos procesos con el efecto de converger en la misma dirección, el cese del flujo genético. Aunque permite preservar combinaciones favorables de alelos y, por lo tanto, ayuda a mantener genotipos bien adaptados, la evolución del aislamiento reproductivo no tiene que ser la única trayectoria evolutiva en poblaciones divergentes. Puede cerrar las puertas a la exploración de nuevos picos adaptativos, ya que se impide la formación de genotipos entre poblaciones. En poblaciones divergentes de tamaño reducido, evitar el flujo genético desde otras poblaciones puede llevar al aumento de la proporción de endogamia con posibles consecuencias negativas sobre la eficacia biológica (Tortajada et al., 2009).

El origen del aislamiento reproductivo, sus causas y su evolución siguen siendo uno de los temas centrales en ecología y evolución. Una serie de procesos con interacciones complejas, frecuentemente creando bucles causales, están involucrados y hacen que este tema represente un desafío de investigación. En esta tesis, el objetivo fue incrementar el conocimiento sobre la evolución del aislamiento reproductivo incipiente en el

invertebrado *B. plicatilis*. Se han dejado de lado muchos factores o pasos reproductivos que podrían ser objeto de investigaciones futuras. Una estrategia de investigación podría consistir en descomponer la secuencia reproductiva, desde el reconocimiento de pareja hasta la producción de huevos de diapausa y la aptitud de la descendencia, en pasos más pequeños que los investigados en esta tesis, incorporando posibles factores extrínsecos o ampliando el estudio para investigar el aislamiento postzigótico en forma de potencial de eclosión, viabilidad y fertilidad de la descendencia. En cuanto al reconocimiento de pareja, sería interesante estudiar otros componentes además del gen *mmr-b*, ya que seguramente están presentes e influyen en ese reconocimiento. B. plicatilis pertenece a un grupo de especies crípticas de *B. plicatilis* que a menudo coexisten en el mismo hábitat. Por lo tanto, sería interesante comparar la evolución del aislamiento reproductivo en estas otras especies.

Conclusiones

1. Los ensayos de conducta para evaluar el aislamiento reproductivo en la fase de apareamiento mostraron indicios de un aislamiento reproductivo incipiente en forma de una mayor propensión para los apareamientos dentro de la misma población en comparación con los apareamientos entre poblaciones. Este patrón se observó en poblaciones seleccionadas tanto por su estructura filogeográfica como por su divergencia adaptativa.

2. La tendencia hacia el apareamiento dentro de la misma población fue más fuerte en las poblaciones seleccionadas por su divergencia adaptativa, cuyos hábitats están en proximidad geográfica.
3. Los indicios de un aislamiento reproductivo incipiente son consistentes con la hipótesis del refuerzo; es decir, con la selección del aislamiento reproductivo por apareamiento preferencial cuando ya existe cierto grado de asilamiento pos-apareamiento.
4. No se encontró correlación entre la distancia geográfica y el aislamiento por apareamiento preferencial, mientras que sí se encontró una correlación entre el aumento de las diferencias ambientales y ese tipo de aislamiento, lo cual es consistente con la hipótesis de que la adaptación local tiene un efecto más fuerte en la evolución del aislamiento reproductivo que la distancia geográfica.
5. Al evaluar el aislamiento reproductivo a nivel de la producción de huevos de diapausa, se encontró un aislamiento reproductivo incipiente en forma de un déficit de heterocigotos en cinco de los doce cruzamientos.
6. Los valores de F_{IS} en los huevos de diapausa fueron positivos en su media y en ocho de los doce cruzamientos, lo que indica una tendencia hacia los apareamientos dentro de la misma población.
7. Los valores de F_{IS} disminuyeron durante el transcurso del experimento, lo que sugiere un efecto del deterioro ambiental o de la asincronía de las fases reproductivas.
8. El gen *mmr-b* fue amplificado y se encontró un locus común en todas las poblaciones estudiadas. Su longitud osciló entre 242 pb y

2591 pb, lo que representa de 0 a 9 motivos repetitivos completos y un motivo terminal. Esto sugiere que un solo motivo terminal puede ser suficiente para el reconocimiento de pareja en *B. plicatilis*.

9. Se encontró una variabilidad nucleotídica relativamente alta entre los motivos repetitivos completos, como los motivos terminales, del gen *mmr-b*, pero la mayoría de ellas eran neutrales (es decir, sustituciones sinónimas), lo que resulta en una variabilidad nucleotídica que se agrupa en dos haplotipos de aminoácidos.
10. La relación dN/dS indica una selección estabilizadora actuando sobre *mmr-b*.
11. Aun usando poblaciones cercanas, se encontró una correlación positiva entre las diferencias genéticas en *mmr-b* y la divergencia adaptativa, así como entre marcadores neutrales y la distancia geográfica. No se encontraron correlaciones significativas entre marcadores neutrales y la divergencia adaptativa, ni entre las diferencias genéticas en *mmr-b* y la distancia geográfica.

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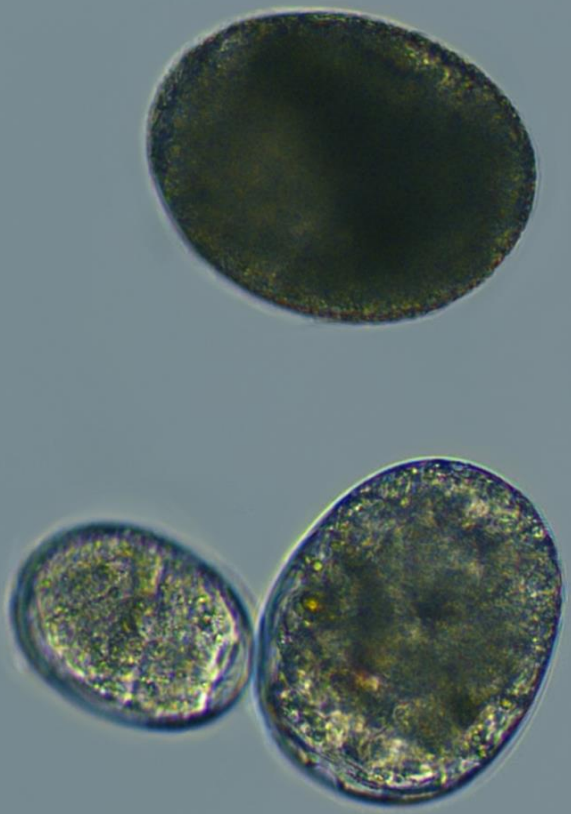


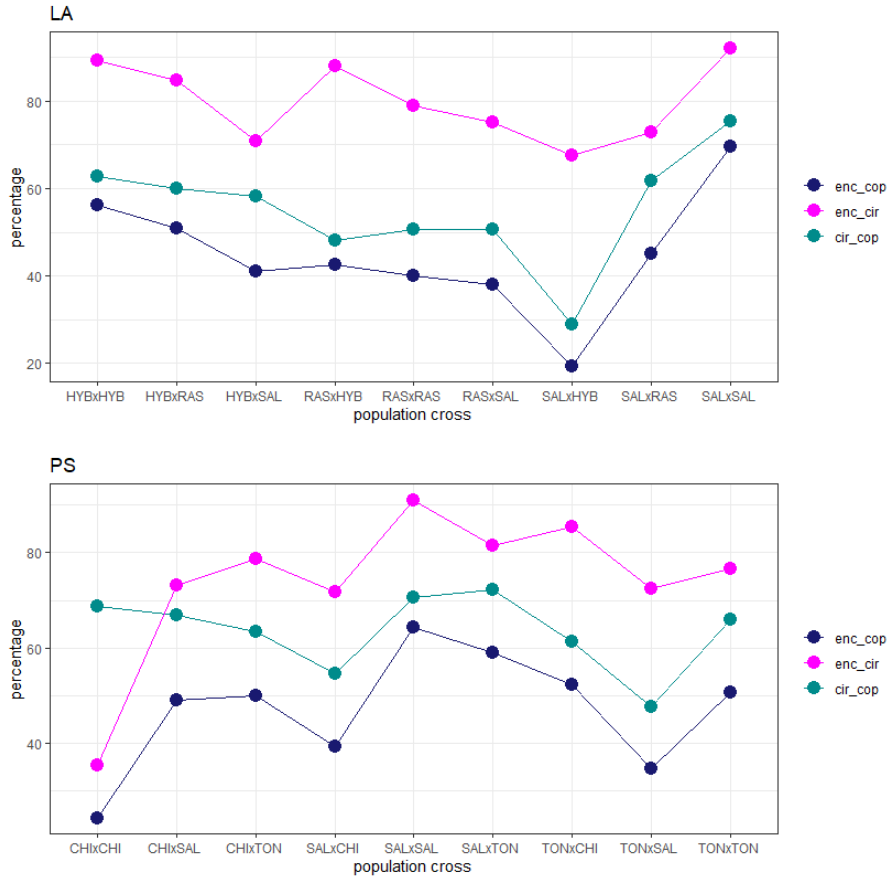
Appendix

Supplementary Table 3.1 Raw data from the mating experiments. Total counts of encounters, circlings and copulations for the five replicates in each population cross and the two study groups, locally adapted (LA) and phylogeographically structured (PS). Each replicate consisted in crosses of different clones, with 25 females and 6 males, the latter added one by one and observed for 10 minutes.

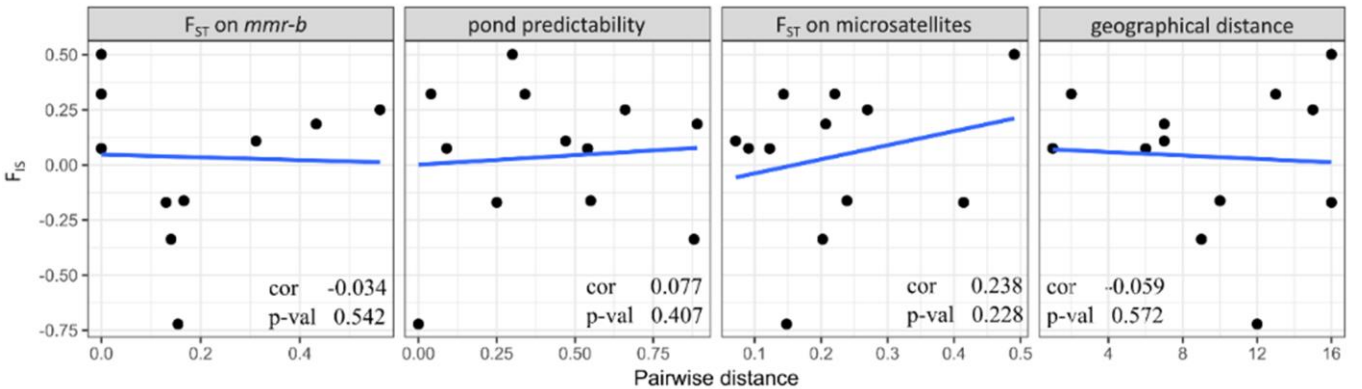
Study group	Female population	Male population	Replicate No.	Mating response		
				encounter	circling	copulation
LA	SAL	SAL	1	22	20	19
LA	SAL	SAL	2	27	22	17
LA	SAL	SAL	3	31	30	19
LA	SAL	SAL	4	30	29	22
LA	SAL	SAL	5	31	29	21
LA	RAS	RAS	1	58	32	23
LA	RAS	RAS	2	25	23	16
LA	RAS	RAS	3	66	52	22
LA	RAS	RAS	4	78	70	22
LA	RAS	RAS	5	33	28	21
LA	HYB	HYB	1	35	30	23
LA	HYB	HYB	2	60	55	19
LA	HYB	HYB	3	30	27	24
LA	HYB	HYB	4	25	24	17
LA	HYB	HYB	5	28	23	17
LA	SAL	RAS	1	25	17	13
LA	SAL	RAS	2	37	35	28
LA	SAL	RAS	3	28	23	17
LA	SAL	RAS	4	46	39	18
LA	SAL	RAS	5	75	40	19
LA	SAL	HYB	1	105	78	15
LA	SAL	HYB	2	65	23	14
LA	SAL	HYB	3	72	66	13
LA	SAL	HYB	4	85	51	11
LA	SAL	HYB	5	33	25	17
LA	RAS	SAL	1	37	33	21

LA	RAS	SAL	2	34	30	17
LA	RAS	SAL	3	69	57	20
LA	RAS	SAL	4	40	36	16
LA	RAS	SAL	5	54	20	15
LA	RAS	HYB	1	27	23	20
LA	RAS	HYB	2	36	30	24
LA	RAS	HYB	3	47	47	22
LA	RAS	HYB	4	43	37	22
LA	RAS	HYB	5	92	79	16
LA	HYB	SAL	1	69	25	13
LA	HYB	SAL	2	42	34	20
LA	HYB	SAL	3	38	33	20
LA	HYB	SAL	4	52	43	27
LA	HYB	SAL	5	42	37	20
LA	HYB	RAS	1	31	27	13
LA	HYB	RAS	2	33	30	22
LA	HYB	RAS	3	22	18	17
LA	HYB	RAS	4	38	29	14
LA	HYB	RAS	5	59	51	27
PS	TON	TON	1	46	39	28
PS	TON	TON	2	47	34	12
PS	TON	TON	3	36	34	29
PS	TON	TON	4	34	27	22
PS	TON	TON	5	52	31	18
PS	SAL	SAL	1	48	41	31
PS	SAL	SAL	2	39	34	26
PS	SAL	SAL	3	50	47	24
PS	SAL	SAL	4	18	18	16
PS	SAL	SAL	5	25	24	19
PS	CHI	CHI	1	39	29	22
PS	CHI	CHI	2	36	33	22
PS	CHI	CHI	3	132	37	24
PS	CHI	CHI	4	209	31	17
PS	CHI	CHI	5	35	30	25

PS	TON	SAL	1	30	25	17
PS	TON	SAL	2	70	48	27
PS	TON	SAL	3	70	61	24
PS	TON	SAL	4	39	36	18
PS	TON	SAL	5	68	31	10
PS	TON	CHI	1	53	42	18
PS	TON	CHI	2	44	41	28
PS	TON	CHI	3	60	52	31
PS	TON	CHI	4	50	43	30
PS	TON	CHI	5	26	21	15
PS	SAL	TON	1	46	40	31
PS	SAL	TON	2	40	32	26
PS	SAL	TON	3	41	33	21
PS	SAL	TON	4	37	36	28
PS	SAL	TON	5	53	36	22
PS	SAL	CHI	1	57	42	21
PS	SAL	CHI	2	51	33	15
PS	SAL	CHI	3	33	29	20
PS	SAL	CHI	4	68	37	23
PS	SAL	CHI	5	43	40	20
PS	CHI	TON	1	40	36	31
PS	CHI	TON	2	45	37	19
PS	CHI	TON	3	87	60	25
PS	CHI	TON	4	38	28	21
PS	CHI	TON	5	34	31	26
PS	CHI	SAL	1	43	19	15
PS	CHI	SAL	2	35	28	24
PS	CHI	SAL	3	39	32	16
PS	CHI	SAL	4	34	25	16
PS	CHI	SAL	5	43	38	24



Supplementary Fig. 3.1 Percentage of circlings after encounters (enc_cir), copulations after circlings (cir_cop) and copulations after encounters (enc_cop) for all population combinations in both study group (LA and PS).



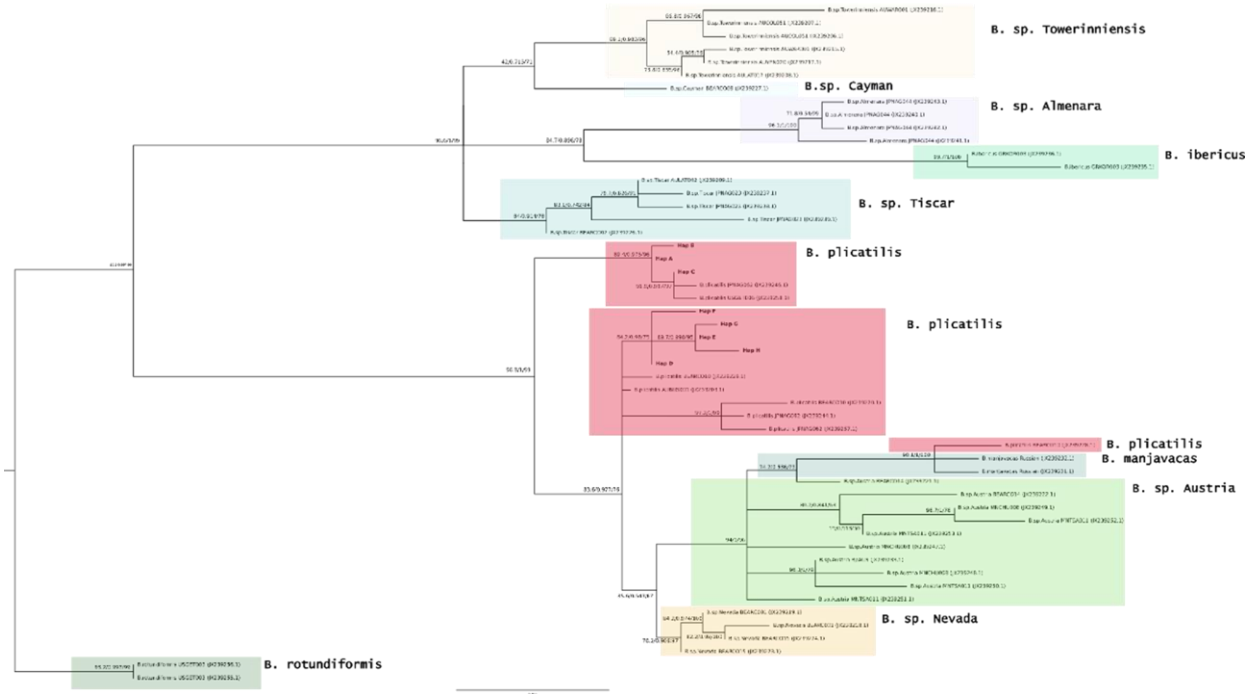
Supplementary Fig. 4.1 One sided Pearson correlation between averaged F_{IS} values for each population combination and different predictors (environmental predictability distance, geographic distance and genetic distance - ϕ_{ST} of *mmr-b* gene or F_{ST} of microsatellites). Correlation coefficient values with p values are indicated at the bottom of each panel. When TUR was dropped, the one sided Pearson correlation between averaged F_{IS} values for population combinations involving SAL were: ϕ_{ST} of *mmr-b* gene: cor 0.794, p-val 0.05; FST of microsatellites: cor 0.368, p-val 0.271; environmental predictability distance: cor 0.713, p-val 0.088; geographic distance: cor 0.020, p-val 0.487.

Supplementary Table 4.1 SNPs used in KASP analysis with the accession number (Franch-Gras et al., 2018) with their position in the scaffold, and allele frequencies for both populations in each population combination.

			Population combination		Alleles (frequency)	
GenBank	Scaffold name	Position	Population A	Population B	Population A	Population B
REGN01000603.1	scaffold2159_len11196_cov69	951	HYC	RAS	C (0.09); G (0.91)	C (1.00); G (0.00)
REGN01001801.1	scaffold6500_len58321_cov77	21914	HYB	SAL	A (0.00); G (1.00)	A (0.97); G (0.03)
REGN01005106.1	scaffold22581_len26821_cov83	21806	TUR	RAS	T (0.02); G (0.98)	T (0.96); G (0.04)
REGN01005285.1	scaffold23526_len68915_cov84	12418	TUR	SAL	G (0.00); T (1.00)	G (0.05); T (0.95)
REGN01006240.1	scaffold29685_len4788_cov57	2342	MNT	RAS	A (0.00); G (1.00)	A (1.00); G (0.00)
REGN01006597.1	scaffold32254_len2672_cov45	2180	AYA	SAL	A (0.05); G (0.95)	A (1.00); G (0.00)
			CAM	SAL	A (0.02); G (0.98)	A (1.00); G (0.00)
			HYC	SAL	A (0.05); G (0.95)	A (1.00); G (0.00)
REGN01013605.1	scaffold131895_len5770_cov90_single	4528	PET	SAL	G (1.00); A (0.00)	G (0.09); A (0.91)
REGN01013691.1	scaffold134414_len14929_cov83_single	12842	AYA	RAS	A (0.05); C (0.95)	A (0.96); C (0.04)
			CAM	RAS	A (0.96); C (0.04)	A (0.00); C (1.00)
			PET	RAS	A (0.00); C (1.00)	A (0.96); C (0.04)

Supplementary Table 4.2 Egg density on day 15 and day 29 for both replicates of all population combinations. Note that on the first temporal sample, eggs accumulated over 15 days and on the second temporal sample they accumulated over 7 days, as there was another egg extraction on day 22 (see methods).

		eggs.mL^{-1}	
Population combination	day	replicate a	replicate b
RASxAYA	15	3.91	18.09
	29	1.51	2.22
RASxCAM	15	7.61	7.03
	29	1.95	0.47
RASxHYC	15	2.38	3.89
	29	2.28	0.50
RASxMNT	15	1.56	3.95
	29	0.63	0.73
RASxPET	15	5.03	3.55
	29	0.51	0.62
RASxTUR	15	11.04	11.32
	29	2.08	2.55
SALxAYA	15	3.88	3.55
	29	1.46	0.17
SALxCAM	15	7.71	21.60
	29	1.23	1.12
SALxHYB	15	6.30	16.08
	29	0.69	0.69
SALxHYC	15	4.95	8.77
	29	0.98	3.20
SALxPET	15	7.62	3.10
	29	2.00	0.53
SALxTUR	15	4.24	4.32
	29	1.00	1.41



Supplementary Fig. 5.1 Unrooted Maximum-Likelihood phylogenetic tree of the terminal repeat sequence of *mmr-b* based on the substitution model TPM2u+F+I+G4. Terminal repeats identified in this study (Hap A – Hap H in bold in the tree) and available sequences for other species of the *B. plicatilis* species complex were used. Node values represent SH-aLRT support (%)/aBayes support/ultrafast bootstrap support (%). Nodes with an ultrafast bootstrap support lower than 50 % have been collapsed.