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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; signaling; genetic structure; reproductive isolation; speciation

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1. 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 [1]. 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 [2,3].

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 [4–6]. However, when populations start to differentiate, rapid diversification in mating recognition traits may appear allowing a stabilization of this divergence [7,8]. Rapid diversification in these traits has been detected even in sympatric populations [9]. This may explain high variability of these traits even among closely related species compared to others [10,11] either within or between species [12].

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" [13]). 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 [14–16]. 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 [17]. 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 [18]. 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) [19].

The mating recognition mechanism in the *Brachionus* genus occurs through contact chemoreception [20]. An extracellular glycoprotein, the mate recognition protein (MRP), located on the female body interacts with a mannose receptor expressed by males [21]. Although it may not be the only protein involved in mate recognition [22], 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 [23]. MRP is structurally conserved across *Brachionus* species [22]. It is encoded by the *mmr-b* gene. Gribble et al. [24] 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 [22]. Differences in the *mmr-b* gene were found among co-generic species and these differences correlated with isolation by mating behaviour [22]. 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.

2. Materials and Methods

2.1. Study Area, Rotifer Collection and Maintenance

A group of nine populations from Eastern Spain were selected based on their known ecological divergence (Table 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 [18]. Additionally, two populations geographically distant from this group and belonging to different phylogeographic clades [25] were selected as outgroups. These populations were Salada de Chiprana (CHI) and Torreblanca Norte (TON). Predictability values were obtained from [17] based on constancy and contingency metrics from satellite images (Colwell's predictability index, [26]).

Table 1. Populations included in the study, location and their estimated degree of predictability of the hydroperiod length in a year based on [18]. (nd: data not available).

Population	Acronym	Location [1]	Pond Size (m^2)	Predictabillity [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	1.00
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	1.00
Hoya Turnera	TUR	38°46′36″ N, 1°24′37″ W	26,000	0.70
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 [27]. 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 [28,29]. 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 [30]. Additionally, the molecular identification was further confirmed by amplification of the microstellite Bp1b, which specifically amplifies in *B. plicatilis* [31], 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 [32].

2.2. Design of mmr-b Specific Primers, Amplification and Sequencing

The available primers to amplify the *mmr-b* gene in the *Brachionus* genus [22] 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 2). Available *mmr-b* sequences [22] 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 [33].

Table 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 [34], 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) [35]. Sequences were manually phased based on the allelic information found in homozygote individuals. MEGA-X (v. 10.1.7) [36] 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.

2.3. 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 [37]. Six microsatellites (Bp1b, Bp2, Bp3c, Bp4a, Bp5d and Bp6b) [38] 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).

2.4. Data Analysis

Genetic distance between populations was computed using Arlequin (v. 3.5.2.2) [39]. 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 [40]. 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) [41]. Ecological distance between pairs of ponds was calculated using the *dist* function in R software (v. 3.6.2) based on the pond unpredictability [17]. 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) [36]. A maximum likelihood tree was constructed for the terminal repeat. Terminal haplotypes identified in this study and terminal repeat sequences from different species [22] 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 [42]. The ML tree was reconstructed using IQ-TREE (version 1.5) [43] and branch supports were calculated with ultrafast bootstrap based on 1000 replicates [44], 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 [45]. 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" [46] in R software.

3. Results

3.1. 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* [22]. 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 (Figure 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) (Figure 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 (Figure 2). The maximum-likelihood tree of the terminal repeat (Figure S1) showed that all the terminal haplotypes found in our study grouped with the two previous groups of sequences of *B. plicatilis* previously described [22].

(A)

	nt haplotype	nucleotide position / amino acid position																			
		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)

AA hapl	nucleotide haplotype 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

Figure 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.

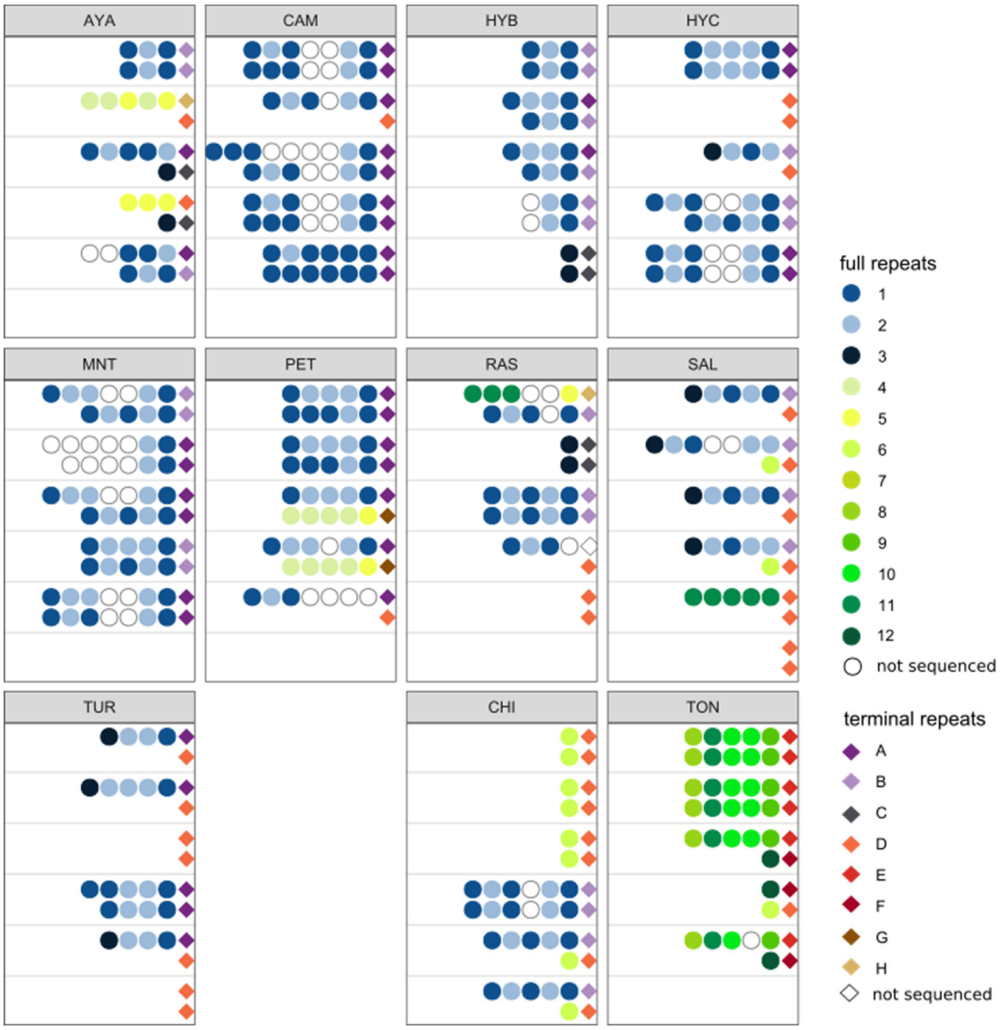

Figure 2. *mmr-b* alleles found in the isolates. Each vertical box represents a population, and each pair of rows with symbols are the two *mmr-b* of a single clone. Each colour represents a different haplotype, either from full repeat (circle) or terminal repeat (diamond). The two detached boxes (CHI and TON) are the outgroup populations.

3.2. Network and Geographic Distribution of Nucleotide Haplotypes

Genomic variation in nucleotide sequence and number of repeats was remarkably high, both within and among populations. (Figure 2). Network analysis of the terminal repeat identifies two groups of haplotypes (Figure 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 (Figure 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 (Figure 3C). The relative frequency of haplotypes varied from populations, even among relatively geographical proximate populations.

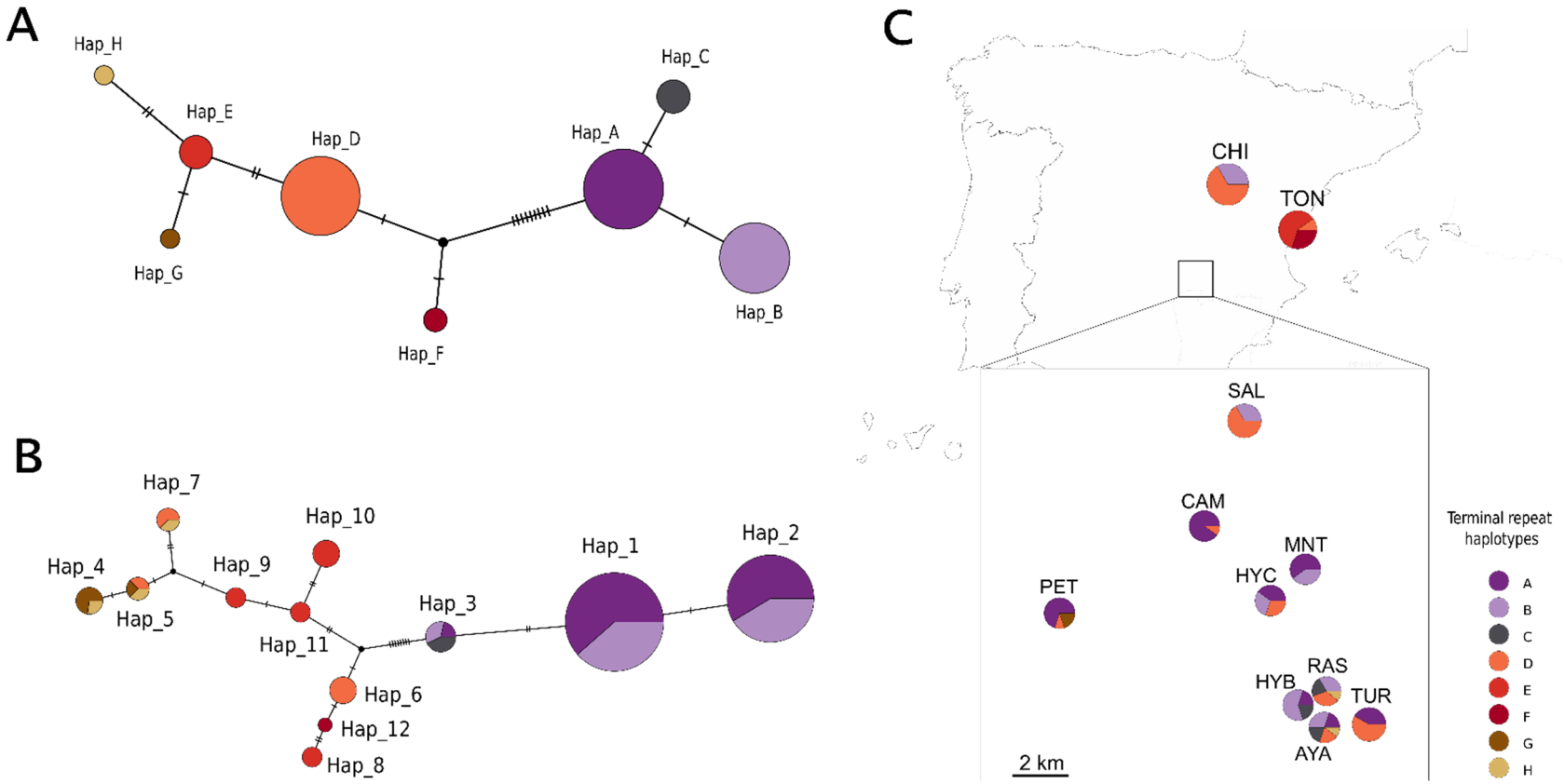

Figure 3. Haplotype network for terminal (**A**) and full (**B**) repeats of *mmr-b*. The colour codes signify different haplotypes of terminal repeat. In the case of a network for full repeats, the colour fractions represent the proportion of terminal repeats appearing in the sequence with the particular full repeat. (**C**) Relative frequency of each terminal repeat in the studied populations with their geographical location on a map of Spain.

3.3. 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 (Figure 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 (Figure 4B).

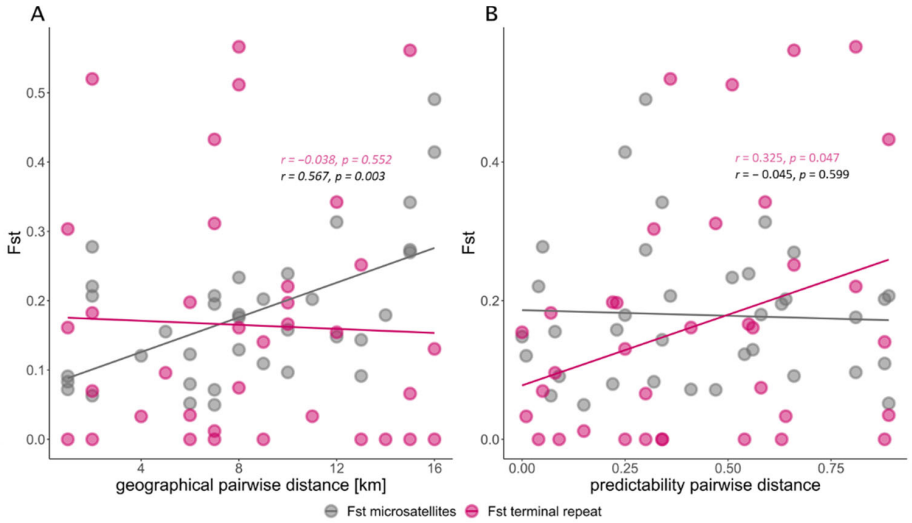

Figure 4. Relationship between F_{ST} for neutral markers (in grey) and terminal repeats of *mmr-b* (in magenta) and geographical distance (**A**) and pond predictability (**B**), respectively. Person's correlation coefficients are shown (*p* values based on a Mantel test with 100,000 permutations).

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 (Figure 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.

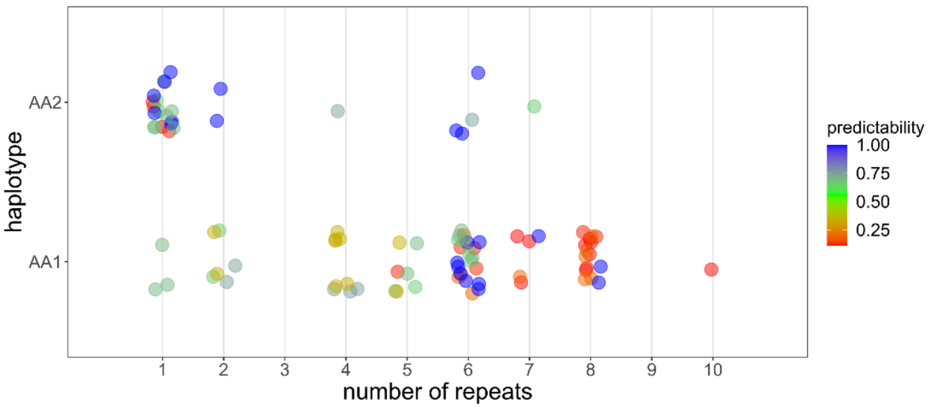

Figure 5. Relationship between the number of repeats in each sequenced allele (full and terminal together), amino acid haplotypes (AA1, AA2) and environmental predictability of the corresponding population.

4. 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 [47]. 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 [22]. 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 [5,6].

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 [22]. 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 [24]. 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 [5]. However, once populations start to diverge, the speed of diversification in these traits is often higher compared to neutral markers [7]. 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 [15].

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 [15] 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) [48]. Even if admittedly, the evidence is not strong, a non-neutral divergence in *mmr-b* related to local adaptation to predictability is suggested by Figure 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 Figure 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 [18]. 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 [19]. 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 [24,47].

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 [49] and references therein). Timing in reproduction has been associated to divergence events in a few groups of organisms (e.g., [50–53]). 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 [18,54]. 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) [55] 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 [56]. 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 [57], 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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14030155/s1, Figure S1: 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.

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References

1. Coyne, J.A.; Orr, H.A. *Speciation*; Sinauer Associates: Sunderland, MA, USA, 2004; pp. 55–82. ISBN 9780878930913.
2. Snell, T.W.; Morris, P.D. Sexual communication in copepods and rotifers. *Hydrobiologia* **1993**, *255–256*, 109–116. [CrossRef]
3. Rosenthal, G.G. *Mate Choice: The Evolution of Sexual Decision Making from Microbes to Humans*; Princeton University Press: Princeton, NJ, USA, 2017; pp. 1–648. ISBN 9780691150673.
4. Lambert, D.M.; Kingett, P.D.; Slooten, E. Intersexual selection: The problem and a discussion of the evidence. *Evol. Theory* **1982**, *6*, 67–78.
5. Brooks, R.; Hunt, J.; Blows, M.W.; Smith, M.J.; Bussière, L.F.; Jennions, M.D. Experimental evidence for multivariate stabilizing sexual selection. *Evolution* **2005**, *59*, 871–880. [CrossRef] [PubMed]
6. Butlin, R.K.; Hewitt, G.M.; Webb, S.F. Sexual selection for intermediate optimum in *Chorthippus brunneus* (Orthoptera: Acrididae). *Anim. Behav.* **1985**, *33*, 1281. [CrossRef]
7. Swanson, W.J.; Vacquier, V.D. The rapid evolution of reproductive proteins. *Nat. Rev. Genet.* **2002**, *3*, 137–144. [CrossRef] [PubMed]
8. Wilburn, D.B.; Swanson, W.J. From molecules to mating: Rapid evolution and biochemical studies of reproductive proteins. *J. Proteomics* **2016**, *135*, 12–25. [CrossRef] [PubMed]
9. Porter, C.K.; Smith, J.W. Diversification in trophic morphology and a mating signal are coupled in the early stages of sympatric divergence in crossbills. *Biol. J. Linn. Soc.* **2020**, *129*, 74–87. [CrossRef]
10. Civetta, A.; Singh, R.S. High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in *Drosophila melanogaster* and *Drosophila virilis* group species. *J. Mol. Evol.* **1995**, *41*, 1085–1095. [CrossRef]
11. Martin, S.H.; Wingfield, B.D.; Wingfield, M.J.; Steenkamp, E.T. Causes and consequences of variability in peptide mating pheromones of ascomycete fungi. *Mol. Biol. Evol.* **2011**, *28*, 1987–2003. [CrossRef] [PubMed]
12. Blows, M.W.; Higgie, M. Evolutionary Experiments on Mate Recognition in the *Drosophila Serrata* Species Complex. *Genetica* **2002**, *116*, 239–250. [CrossRef] [PubMed]
13. De Meester, L.; Gomez, A.; Okamura, B.; Schwenk, K. The Monopolization Hypothesis and the dispersal-gene flow paradox in aquatic organisms. *Acta Oecologica* **2002**, *23*, 121–135. [CrossRef]
14. Campillo, S.; Serra, M.; Carmona, M.J.; Gomez, A. Widespread secondary contact and new glacial refugia in the halophilic rotifer *Brachionus plicatilis* in the Iberian Peninsula. *PLoS ONE* **2011**, *6*, e20986. [CrossRef]
15. Gomez, A.; Adcock, G.J.; Lunt, D.H.; Carvalho, G.R. The interplay between colonization history and gene flow in passively dispersing zooplankton: Microsatellite analysis of rotifer resting egg banks. *J. Evol. Biol.* **2002**, *15*, 158–171. [CrossRef]
16. Montero-Pau, J.; Serra, M.; Gomez, A. Diapausing egg banks, lake size, and genetic diversity in the rotifer *Brachionus plicatilis* Müller (Rotifera, Monogononta). *Hydrobiologia* **2017**, *796*, 77–91. [CrossRef]
17. Franch-Gras, L.; García-Roger, E.M.; Franch, B.; Carmona, M.J.; Serra, M. Quantifying unpredictability: A multiple-model approach based on satellite imagery data from Mediterranean ponds. *PLoS ONE* **2017**, *12*, e0187958. [CrossRef] [PubMed]
18. Franch-Gras, L.; García-Roger, E.M.; Serra, M.; José Carmona, M. Adaptation in response to environmental unpredictability. *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20170427. [CrossRef] [PubMed]
19. Jezkova, I.; Ortells, R.; Montero-Pau, J.; Serra, M. Insight into incipient reproductive isolation in diverging populations of a rotifer *Brachionus plicatilis*. *Hydrobiologia*, 2022; *submitted*.
20. Snell, T.W.; Rico-Martinez, R.; Kelly, L.N.; Battle, T.E. Identification of a sex pheromone from a rotifer. *Mar. Biol.* **1995**, *123*, 347–353. [CrossRef]
21. Snell, T.W. A review of the molecular mechanisms of monogonont rotifer reproduction. *Hydrobiologia* **2011**, *662*, 89–97. [CrossRef]

22. Gribble, K.E.; Welch, D.B.M. The mate recognition protein gene mediates reproductive isolation and speciation in the *Brachionus plicatilis* cryptic species complex. *BMC Evol. Biol.* **2012**, *12*, 134–151. [CrossRef]
23. Snell, T.W.; Stelzer, C.P. Removal of surface glycoproteins and transfer among *Brachionus* species. *Hydrobiologia* **2005**, *546*, 267–274. [CrossRef]
24. Gribble, K.E.; Snell, T.W.; Welch, D.B.M. Gene and protein structure of the mate recognition protein gene family in *Brachionus manjavacas* (Rotifera). *Hydrobiologia* **2011**, *662*, 35–42. [CrossRef]
25. Gomez, A.; Carvalho, G.R.; Lunt, D.H. Phylogeography and regional endemism of a passively dispersing zooplankter: Mitochondrial DNA variation in rotifer resting egg banks. *Proc. R. Soc. B Biol. Sci.* **2000**, *267*, 2189–2197. [CrossRef] [PubMed]
26. Colwell, R.K. Predictability, Constancy, and Contingency of Periodic Phenomena. *Ecology* **1974**, *55*, 1148–1153. [CrossRef]
27. Gomez, A.; Carvalho, G.R. Sex, parthenogenesis and genetic structure of rotifers: Microsatellite analysis of contemporary and resting egg bank populations. *Mol. Ecol.* **2000**, *9*, 203–214. [CrossRef] [PubMed]
28. Gomez, A.; Serra, M.; Carvalho, G.R.; Lunt, D.H. Speciation in ancient cryptic species complexes: Evidence from the molecular phylogeny of *Brachionus plicatilis* (rotifera). *Evolution.* **2002**, *56*, 1431–1444. [CrossRef]
29. Suatoni, E.; Vicario, S.; Rice, S.; Snell, T.; Caccone, A. An analysis of species boundaries and biogeographic patterns in a cryptic species complex: The rotifer-*Brachionus plicatilis*. *Mol. Phylogenet. Evol.* **2006**, *41*, 86–98. [CrossRef] [PubMed]
30. Campillo, S.; García-Roger, E.M.; Martínez-Torres, D.; Serra, M. Morphological stasis of two species belonging to the L-morphotype in the *Brachionus plicatilis* species complex. *Hydrobiologia* **2005**, *546*, 181–187. [CrossRef]
31. Tortajada, A.M.; Carmona, M.J.; Serra, M. Effects of population outcrossing on rotifer fitness. *BMC Evol. Biol.* **2010**, *10*, 312. [CrossRef] [PubMed]
32. Guillard, R.R.; Ryther, J.H. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. *Can. J. Microbiol.* **1962**, *8*, 229–239. [CrossRef] [PubMed]
33. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 1–11. [CrossRef] [PubMed]
34. Bell, J.R. A simple way to treat PCR products prior to sequencing using ExoSAP-IT®. *Biotechniques* **2008**, *44*, 834. [CrossRef]
35. Bonfield, J.K.; Smith, K.F.; Staden, R. A new DNA sequence assembly program. *Nucleic Acids Res.* **1995**, *23*, 4992. [CrossRef]
36. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
37. Montero-Pau, J.; Gomez, A.; Muñoz, J. Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnol. Oceanogr. Methods* **2008**, *6*, 218–222. [CrossRef]
38. Gomez, A.; Clabby, C.; Carvalho, G. Isolation and characterization of microsatellite loci in a cyclically parthenogenetic rotifer. *Brachionus Plicatilis Mol.* **1998**, *7*, 1613–1621.
39. Excoffier, L.; Lischer, H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef] [PubMed]
40. Trifinopoulos, J.; Nguyen, L.T.; von Haeseler, A.; Minh, B.Q. W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* **2016**, *44*, W232–W235. [CrossRef] [PubMed]
41. Leigh, J.W.; Bryant, D. POPART: Full-feature software for haplotype network construction. *Methods Ecol. Evol.* **2015**, *6*, 1110–1116. [CrossRef]
42. Notredame, C.; Higgins, D.G.; Heringa, J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **2000**, *302*, 205–217. [CrossRef]
43. Nguyen, L.T.; Schmidt, H.A.; Von Haeseler, A.; Minh, B.Q. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* **2015**, *32*, 268–274. [CrossRef] [PubMed]
44. Hoang, D.T.; Chernomor, O.; Von Haeseler, A.; Minh, B.Q.; Vinh, L.S. UFBoot2: Improving the Ultrafast Bootstrap Approximation. *Mol. Biol. Evol.* **2018**, *35*, 518–522. [CrossRef] [PubMed]
45. Kalyaanamoorthy, S.; Minh, B.Q.; Wong, T.K.F.; Von Haeseler, A.; Jermiin, L.S. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* **2017**, *14*, 587–589. [CrossRef] [PubMed]
46. Dray, S.; Dufour, A.B. The ade4 Package: Implementing the Duality Diagram for Ecologists. *J. Stat. Softw.* **2007**, *22*, 1–20. [CrossRef]
47. Snell, T.W.; Kubanek, J.; Carter, W.; Payne, A.B.; Kim, J.; Hicks, M.K.; Stelzer, C.P. A protein signal triggers sexual reproduction in *Brachionus plicatilis* (Rotifera). *Mar. Biol.* **2006**, *149*, 763–773. [CrossRef]
48. Butlin, R. Speciation by reinforcement. *Trends Ecol. Evol.* **1987**, *2*, 8–13. [CrossRef]
49. Symonds, M.R.E.; Elgar, M.A. The evolution of pheromone diversity. *Trends Ecol. Evol.* **2008**, *23*, 220–228. [CrossRef]
50. Hall, M.C.; Willis, J.H. Divergent selection on flowering time contributes to local adaptation in *Mimulus guttatus* populations. *Evolution* **2006**, *60*, 2466–2477. [CrossRef]
51. Aspinwall, N. Genetic analysis of north american populations of the pink salmon, *Oncorhynchus gorbuscha*, possible evidence for the neutral mutation-random drift hypothesis. *Evolution* **1974**, *28*, 295–305. [CrossRef] [PubMed]
52. Papakostas, S.; Michaloudi, E.; Triantafyllidis, A.; Kappas, I.; Abatzopoulos, T.J. Allochronic divergence and clonal succession: Two microevolutionary processes sculpturing population structure of *Brachionus rotifers*. *Hydrobiologia* **2012**, *700*, 33–45. [CrossRef]

53. Linn, C.E.; Dambroski, H.R.; Feder, J.L.; Berlocher, S.H.; Nojima, S.; Roelofs, W.L. Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: Reduced response of hybrids to parental host-fruit odors. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17753–17758. [CrossRef]
54. Jankowski, T.; Straile, D. Allochronic differentiation among *Daphnia* species, hybrids and backcrosses: The importance of sexual reproduction for population dynamics and genetic architecture. *J. Evol. Biol.* **2004**, *17*, 312–321. [CrossRef] [PubMed]
55. Titelman, J.; Varpe, Ø.; Eliassen, S.; Fiksen, Ø. Copepod mating: Chance or choice? *J. Plankton Res.* **2007**, *29*, 1023–1030. [CrossRef]
56. Snell, T. Contact chemoreception and its role in zooplankton mate recognition. In *Chemical Communication in Crustaceans*; Springer: New York, NY, USA, 2011; pp. 451–466. ISBN 9780387771014.
57. Montero-Pau, J.; Gomez, A.; Serra, M. Founder effects drive the genetic structure of passively dispersed aquatic invertebrates. *PeerJ* **2018**, *2018*, e6094. [CrossRef] [PubMed]