Genetic survey of polymorphic populations of Podarcis muralis (Sauria: Lacertidae) using molecular markers

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Color polymorphisms are widespread in nature and may show a high variability not only within the same population, but also among populations of the same species., Color polymorphism is a common feature among reptiles and is often linked to evolution of alternative strategies. Alternative and discrete color morphs have been reported in the common wall lizard, *Podarcis muralis*, which show six different ventral colorations in both sexes: three pure phenotypes (i.e., *white*, *yellow* and *red*) and three intermediate phenotypes (i.e., *white-yellow*, *white-red* and *yellow-red*). The relative frequencies of color morphs show micro-geographic variations at local scale, which could be related to gene flow reduction among populations. Therefore, in this work I studied genetic polymorphism by using molecular markers in order to assess gene flow and dispersal among populations of common wall lizard, with the aim to describe the geographic structure of polymorphic populations at local scale. Moreover, nuclear markers were used to investigate the presence of genetic divergence among sympatric color morphs of common wall lizard.

1 Introduction

1.1 Color polymorphism: evolution and maintenance

Polymorphism is defined as the coexistence within the same population and in the same habitat of two or more distinct and genetically determined phenotypes (named *morphs*), the rarest of which being too frequent to be maintained only by recurrent mutations (i.e., true polymorphism; [1]). Color polymorphism occurs in many vertebrates taxa and is widespread among reptiles. In lizards, true color morphs often occur in well-discrete color phenotypes, which are genetically determined [2] and remain stable through the individual life [3,4]. The co-occurrence of discrete color morphs within the same population implies a balance between different strategies reaching alternative equilibria under the same environmental and social conditions. Inter-population variability in morph frequency have been observed in many discrete-polymorphic lizards, such as *Uta stansburiana* and *Urosaurus ornatus* [3,5,6], in several *Sceloporus* species [7,8] and in *Lacerta vivipara* [9], even in the absence of geographical barriers or genetic segregation among individuals.

Polymorphisms maintenance may rely on the degree of "population connectivity", a concept based on the movement of individuals among discrete populations. Gene flow and dispersal among populations may therefore contribute significantly to polymorphism persistence [10]. For instance, in metapopulations discrete subpopulations exchange genes with one another through dispersal and favour deterministic processes of density- and frequency-dependent selection, generating more linkage disequilibria than expected in a panmictic population [11]. Local subdivision increases inbreeding, lowers effective recombination rate and increases levels of linkage disequilibria present in each deme [11, 12], enhancing the probability of chromosomal rearrangements that drive the evolution of super-genes responsible for morph-specific behavioural and mating strategies. Neutral processes such as drift and mutation may further contribute to the maintenance of spatial variation in morph-frequencies.

Another interesting observation concerns the evolution of sympatric alternative morphs, which often derive from multivariate disruptive natural selection favoured by non-random mating system within the population. Polymorphic system which show positive correlation between color and morph-specific suites of characters are expected to experience higher rates of genetic divergence either in response to direct selection for assortative mating or as a consequence of phenotype spatial sorting at the time of mating or allochrony. Therefore, looking for deviations in the distribution of neutral genetic diversity would be of particular interest for testing hypotheses on mating system and life-history evolution of polymorphic species that invoke selection due to metapopulation dynamics [13,14,15,16].

1.2   Color polymorphism in the common wall lizard

The common wall lizard (*Podarcis muralis*, Laurenti, 1768) is a small-sized lizard (snout-vent length, SVL, 45–75 mm) widespread in southern and central Europe. In northern Italy, populations exhibit a clear and remarkable polymorphism in throat and belly colorations, both sexes showing three pure phenotypes or morphs (i.e., *white*, *yellow* and *red*) and three apparently intermediate phenotypes (i.e., *white-yellow*, *white-red* and *yellow-red*; Fig. 1). Throat and belly colorations start to develop from the second year of life, when individuals attain sexual maturity [17]; then the same color is maintained throughout the individual life [18,19], suggesting a strong genetic determination of this traits [19,20].

Recent image analyses of color variation has shown that only one color is expressed in pure morphs, and no continuous variation exists among these three alternative color phenotypes. Moreover, the two intermediate-red phenotypes appear clearly dichromatic (the two pure colors being both expressed on different scales of throat and belly or on the same scale at a time), while *white-yellow* does not represent a real morph, but most likely an intermediate phenotypic phase to the *yellow* morph.

Fig. 1 Ventral CP in the common wall lizard (top row: females, bottom row: males). From left to right: *white*, *yellow*, *red*, *white-red*, *yellow-red*, *white-yellow*.

Although these chromatic differences are remarkable, the adaptive significance of color polymorphism in common wall lizard is unclear. As already observed for several polymorphic lizards, colorations may underlie alternative behavioral strategies, which can be also implicated in shaping species mating system [19]. At present, several morphological [20], immunological [21] and physiological [22] differences among color morphs have been identified thanks to both field and laboratory works.

Morph frequencies seem similar in neighbouring populations, although a clear geographic cline is hardly recognizable [20]. Since the habitats between the investigated sites are likely unfavourable to individual dispersal, populations may represent local demes of a metapopulation. Connectivity among populations may therefore be lower than expected if populations are fully connected, leading to a local shift in morph frequency..

In the present study, I analysed the pattern of gene flow among northern Italian populations of common wall lizard, as a mechanism to maintain polymorphism, testing for the occurrence of limited gene flow among them, as expected in a metapopulation scenario. In addition, I also investigated the presence of genetic divergence among sympatric color morphs of common wall lizard with neutral molecular markers to test if population structuring parallels colorations, as expected if disruptive selection and non-random mating system occurs among morphs.

2   Materials and methods

2.1   Population survey and genetic sampling

To describe geographic variation in frequencies of common wall lizard color morphs, during spring and summer 2009-2010 (i.e., along the reproductive season) 8 polymorphic populations of *P. muralis* were sampled in Lombardy (northern Italy). At small spatial scale, 5 populations were sampled within the city of Pavia (45°11'N–9°30'E, PV1-PV2) and in neighbouring farms (PV3, PV4, PV5), in the typical farmland landscape of the Po Plain. Average distance among these populations was 6 km (from 1.6 km between PV1 and PV2 to 12 Km between the easternmost farm PV3 and the westernmost farm PV5).

To assess large-scale geographic variation in morph frequencies, 2 populations located in the surroundings of Milano (45°27'N–9°11'E) were also sampled within two natural protected areas (urban parks), 50 km apart from our main sampling area (MI1, MI2), as well as 1 mountain population up to 580 m a.s.l. (LE) located 100 km apart from the city of Pavia, in the village of Lemna, near Como (45°51'N–9°9'E). At each site, 36 lizards were sampled on average. The ventral morph was assessed univocally by visual inspection (see Fig. 1 for the definition of color phenotypes).

All individuals were measured for SVL to the nearest 0.1 mm, and a small (< 3 mm) piece of tail tissue was sampled for further gene flow and genetic population structure analyses. Tail tissues were preserved in 95% ethanol in the field and then storage in the laboratory at -20°C until DNA analyses. After sampling, all lizards were immediately released at capture point.

2.2   DNA extraction and sequencing

Total genomic DNA was extracted for 288 adult lizards (156 males, 132 females) using the ArchivePure DNA Cell/Tissue kit (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. The whole cytochrome *b* (cyt *b*) gene (1143 bp length) was amplified using previously described primer pair [23,24]. Polymerase chain reactions (PCRs) profile comprised an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation (45 s at 94°C), annealing (45 s at 51°C) and elongation (90 s at 72°C), and a final elongation step at 72°C for 10 min. All lizards were also genotyped for 7 microsatellite loci available for the species [25]. Forward microsatellite primers were fluorescently labeled and amplifications were performed in a 10 µl reaction volume containing 1 µl of template DNA. PCR conditions involved an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 45 s denaturation at 95°C, 30 s at locus-specific annealing temperatures [25], and 30 s extension at 72°C. After last cycle, a final extension was done at 72°C for 10 min. For fluorescent detection on an ABI 3130 sequencer, PCR products were mixed with formamide and an internal size standard (500(-250)LIZ, Applied Biosystem).

2.3   Statistical analysis

Mitochondrial DNA sequences were corrected by eye, then alignments were made using Clustal W [26] implemented in BioEdit 7.0 [27]. The software TCS 1.21 [28] was used to generate haplotype networks for populations as well as for color morphs. Concerning microsatellite data, observed and expected heterozygosities (H_O and H_E), as well as the number of private allele (A_P), was calculated with GenAlex 6.4 [29]. Allelic richness (A_R), averaged over all loci and estimated for the minimum number of individual *per* morph class was calculated with FSTAT 2.9.3 [30]. Population differentiation was assessed by calculating pairwise multilocus estimates of F_{ST} [31] using Arlequin 3.11 [32]. For the sequence data, pairwise distance model was selected. The same software was used to test for correlations between genetic and geographic distance matrices with Mantel test.

In three populations for which the largest number of samples were available (PV2, *N*=36; PV3, *N*=45; PV4 *N*=33) I also tested whether individuals were randomly sampled from a panmictic unit, checking for the presence of more than one putative reproductive group in each population according to color morph. A factorial correspondence analysis (FCA) was conducted with the software Genetix 4.05 to determine the similarity of allelic composition among the color morph classes within the same population. Differences in the FCA factors were then tested by a MANOVA using R 1.2.1. statistic package.

3   Results

3.1   Phylogeographic structure and gene flow among polymorphic populations

Overall, 24 unique haplotypes were found in our dataset (Fig. 2). Haplotype PM1 was found in all the populations and in all the three morph types (Fig. 2). The 75% of the haplotypes identified were private of a single population, while 25% occurred in a single morph class. On average, two private haplotypes were found in all the surveyed populations (Fig. 2). Concerning individual morph, the majority of samples sharing the widespread PM1 haplotype was *white* (41%), while the *yellow* morph (31%) and the *red* (i.e., pure *red*) one (27%) were less represented.

Reconstruction of the haplotype networks led to the identification of a single cluster at the 95% parsimony threshold. Networks showed a classical *star-like* phylogeographic structure, with one wide-ranging ancestral haplotype and many weakly differentiated locally restricted haplotypes. Overall, the *white* morph seem to be less structured than the *yellow* and the *red* ones, as fewer derivates haplotypes has been found in *white* individuals of all the surveyed populations.
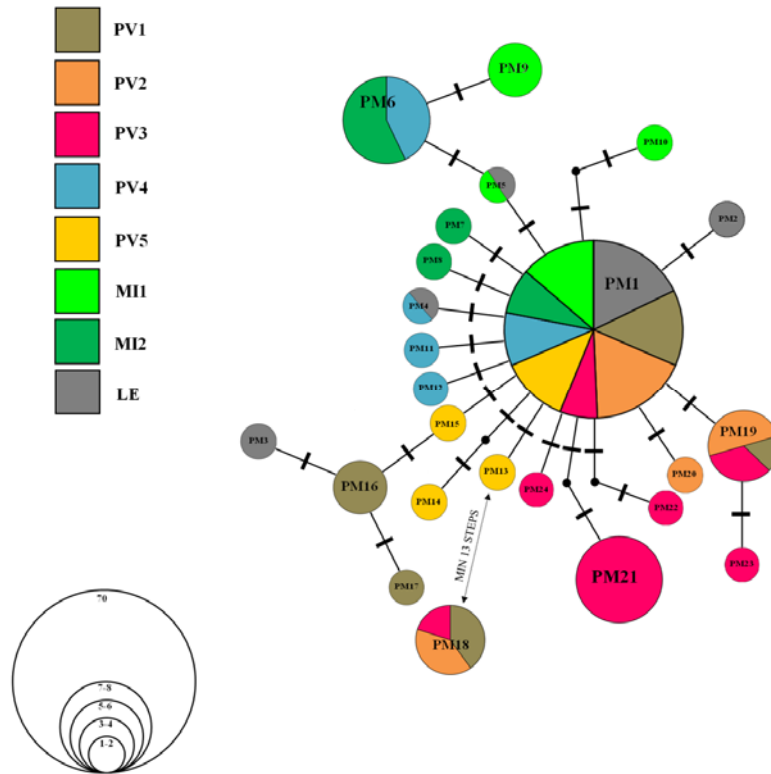
Fig. 2 Haplotype network reconstruction for northern Italian population of common wall lizard based on the whole cyt *b* sequences (1143 bp). The relative frequency of population sharing each haplotype is indicated with different color.

All microsatellite loci were polymorphic across the 8 populations. Across all populations and sites 122 alleles were found, ranging from 10 to 21 (17.42 ± 0.39 on average). Allelic richness was similar among all the studied populations (ranging from 0.847 in PV5 to 0.873 in PV2). Private alleles were found in all the populations but PV1, the highest number (4) being found in the mountain population LE.

Estimates of population differentiation with microsatellite markers were positive and significantly different from zero, with an overall F_{ST}-value of 0.0250, indicating reduced gene flow among populations. All population comparisons were significant, ranging from 0.0159 to 0.0352 (Table 1). Genetic differentiation among populations based on the mitochondrial cyt *b* data ranged from -0.0213 to 0.1529 (Tab. 1). Interesting enough, linear regression of $F_{ST}/(1 - F_{ST})$ on the logarithm of geographical distances showed a significant positive correlation between population differentiation and geographical distances ($P < 0.001$).

Table 1 Differentiation between populations of common wall lizard from northern Italy by pairwise F_{ST} for microsatellite (lower diagonal) and cyt b sequences (upper diagonal). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	PV1	PV2	PV3	PV4	PV5	MI1	MI2	LE
PV1	-	0.0219	0.1099**	0.1412	0.0873	0.1529***	0.1568**	0.1223**
PV2	0.0194***	-	0.0431	0.0624	0.0137	0.0777	0.0927	0.0433
PV3	0.0242***	0.0163***	-	0.1066**	0.0688*	0.1200***	0.1311**	0.1001**
PV4	0.0220***	0.0238***	0.0301***	-	0.0644***	-0.0069	-0.0213	0.0537
PV5	0.0253***	0.0284***	0.0287***	0.0313***	-	0.0878	0.1486	-0.0057
MI1	0.0217**	0.0230***	0.0254***	0.0352***	0.0302***	-	-0.0118	0.0835*
MI2	0.0160**	0.0159**	0.0189**	0.0227***	0.0240***	0.0210**	-	0.1404**
LE	0.0166**	0.0273***	0.0264***	0.0315***	0.0312***	0.0271***	0.0209**	-

3.2   Genetic divergence among color morph classes

Factors generated by the FCA differed significantly among morphs only in the lowland Populations PV2 ($P = 0.017$) and PV3 ($P = 0.028$). In both these populations, significant differences were found between all the three morph classes ($P < 0.05$). The non-significant values observed for the site PV4 may be due to smaller sample sizes of this population (N=33) compared to the other populations considered in the analysis. Although morph classes substantial overlapped one another, three well-distinguishable groups were graphically identified by the first two axis in all the sites considered (Fig. 3).
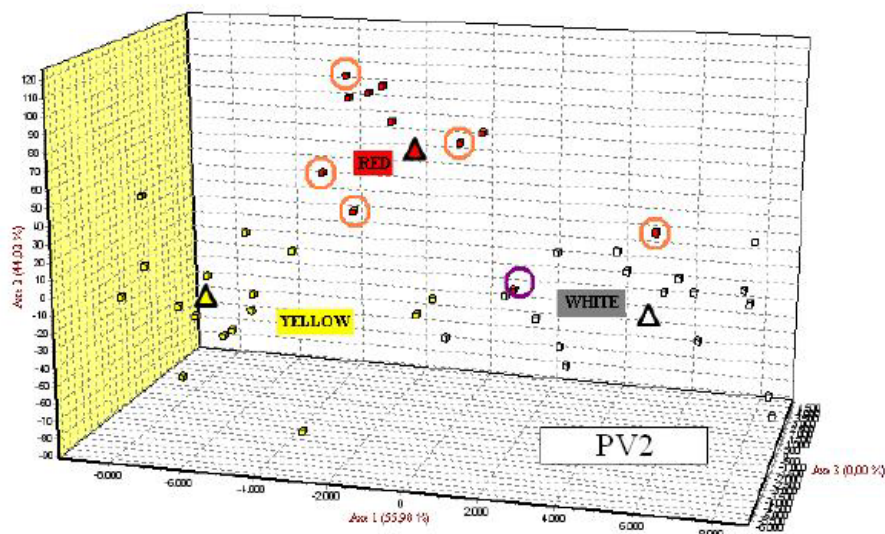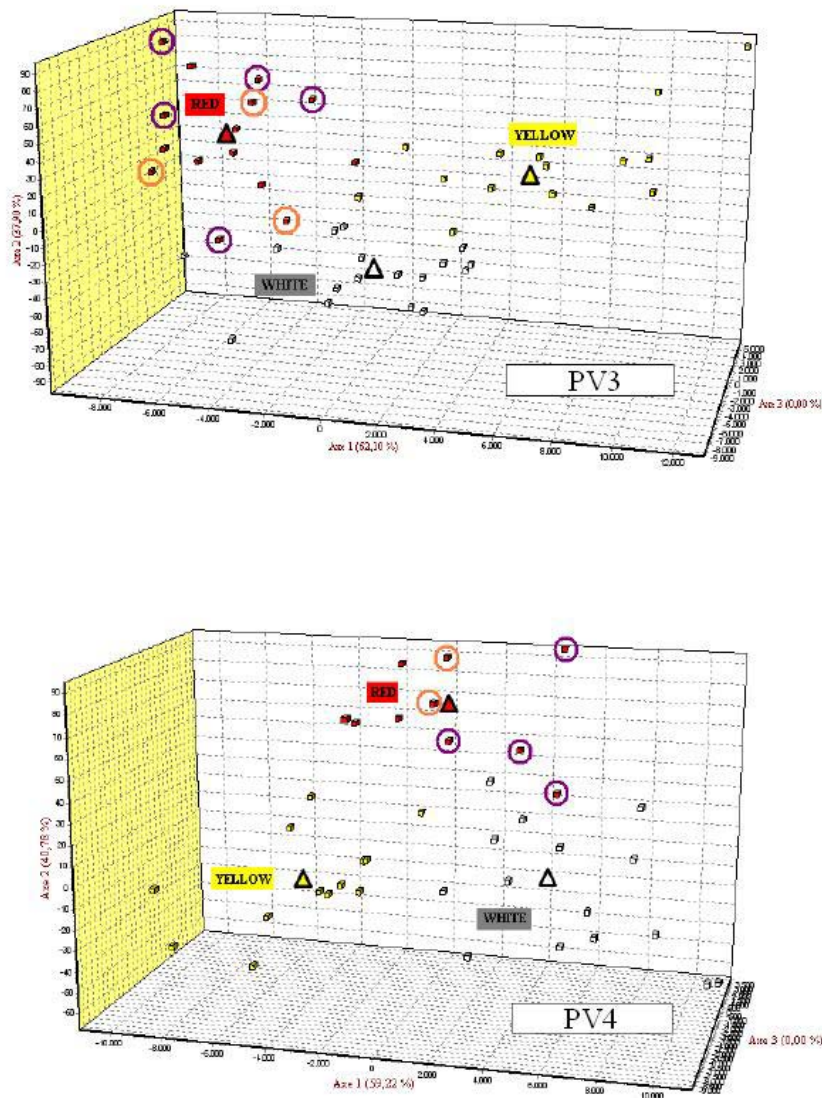
Fig. 3 Tridimensional graphics showing spatial relationships among individuals of alternative color morph classes for populations PV2 PV3 and PV4. Axis Triangles represent the barycentre of each samples (i.e., each of the three color morph classes considered). Finally, purple rings indicate *white-red* individuals, while orange rings signal *yellow-red* lizards.

4   Conclusions

The general aim of this study was to understand the possible mechanisms that maintain intra-specific color variation in natural populations of common wall lizard by using molecular markers. In particular, I focused my research on the analysis of gene flow and genetic structure of polymorphic populations, testing also for the presence of genetic differentiation among color morphs in sympatry. I found variation

in morph frequencies among populations and gene flow reduction among geographic populations of common wall lizard according to spatial distance. Both mitochondrial and microsatellite markers suggested that local variation in morph frequencies may be strongly related to founder effect and asymmetrical gene flow among populations.

Analysis with nuclear microsatellite markers confirmed the presence of significance differentiation even among nearby populations located less than 2 km apart from each other (see Tab. 1). The observed general increment of genetic divergence according to geographic distance (IBD) suggested limited gene flow among populations as expected in a classical metapopulation scenario, where individuals show low dispersal ability [33]. In fact, farms of Po Plain are highly suitable for the occurrence of very dense common wall lizard populations, offering many shelters (old and desert buildings), food resources and protection from predators, but habitats among farms, mainly consisting of intensive cereal crops (maize, wheat and rice), do not represent optimal corridors for lizard dispersal and settlement. [20]

I also investigated if reduced gene flow and genetic divergence among color morphs of common wall lizard occur in sympatry by using highly polymorphic microsatellite loci. When alternative color morph coexist in a population, correlational selection favouring certain trait value combinations at the expense of others may result in modification of genomic architecture and may lead to divergence among color morphs in many morphological, physiological and behavioural (e.g., reproductive) traits [34]. In the three populations examined, significance genetic divergence at neutral loci was found to match ventral color polymorphism in sympatry. These positive correlations can arise from a variety of mate choice processes, including a preference for phenotypically similar mates (as assumed in many sympatric speciation models), but also a spatial sorting of phenotypes at the time of mating and allochrony [35]. In polymorphic systems such as those observed in the common wall lizard, where morphs may represent co-adapted complexes trait, disruptive selection can then be enhanced by concomitant assortative mating among morphs [36].

In conclusion, our results emphasizes the benefit of multilocus approaches in a population genetics framework for study not only polymorphisms in the wild, but also to assess the role of gene flow, mating system and natural selection in shaping genetic structure of polymorphic populations.

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