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Comparative pattern of genetic structure in two Mediterranean killifishes (*Aphanius fasciatus* and *Aphanius iberus*) inferred from both mitochondrial and nuclear data.

A. M. PAPPALARDO[1], E. G. GONZALEZ[2], C. TIGANO[1], I. DOADRIO[2] AND V. FERRITO[1*]

[1] *Department of Biological, Geological and Environmental Sciences, University of Catania, Italy*

[2]*Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, MNCN-CSIC, Madrid, Spain*

Running headline: Pattern of genetic structure in *Aphanius* sp.

*** Correspondence: A.M. Pappalardo, Department of Biological, Geological and Environmental Sciences, University of Catania, Via Androne 81, I-95124 Catania, Italy.**

Email: pappalam@unict.it

ABSTRACT

The genetic structure of contemporary populations is the outcome of their connectivity, of historical events and current ecological condition. In this work, genetic diversity and population structure were assessed in two species of *Aphanius* (*A. fasciatus* and *A. iberus*) characterized by similar ecological traits but having a very different distribution range in the Mediterranean area. Five Iberian populations of *A. iberus* and five Mediterranean populations of *A. fasciatus* were analyzed using five polymorphic microsatellite loci and partial mitochondrial control region sequences. Congruent results were found with both nuclear and mitochondrial molecular markers. Our results showed that similar levels of genetic divergence, based on mitochondrial control region sequences, are present among populations of *A. iberus* and among populations of *A. fasciatus* despite of the very different geographic distance existing among the examined populations of the two species (low geographic distance in *A. iberus* and high in *A. fasciatus*). This allows us to infer that the populations of *A. iberus* were isolated for a longer time than the populations of *A. fasciatus* supporting the hypothesis that the split of the lineage leading to *A. iberus* is older than the split of the lineage leading to *A. fasciatus*. However, we cannot rule out that the wide circum-Mediterranean distribution of *A. fasciatus* could ensure a high connectivity of its populations, preventing, in some case, local differentiation.

Key words: Teleosts, killifish species, genetic structure, mtDNA control region, microsatellites.

INTRODUCTION

The population structure of brackish water species has attracted considerable interest, because they often exhibit narrow habitat specificity, which in combination with geographic isolation of the different populations may lead to higher chances of evolutionary divergence among populations and speciation (e.g. Carvalho, 1993; Cognetti & Maltagliati, 2000; Bilton *et al*., 2002). Among teleost fish, killifish (order Cyprinodontiformes) usually inhabit estuarine and salt marshes along the coastlines of the New and Old World, and some species have been used as model for understanding local and regional adaptation to variable environments (e.g. Bernardi & Talley, 2000; Adams *et al*., 2006; Whitehead, 2010; Teimori *et al.*, 2012) as well as past palaeogeographic and palaeoclimatic events (April & Turgeon, 2006; Triantaphyllidis *et al*., 2007; Haney *et al*., 2009; Reichenbacher & Kowalke, 2009; Ferrito *et al*., 2013). *Aphanius* killifishes living in freshwater as well as in brackish water bodies along the Mediterranean coast have been considered a model system to study speciation phenomena (e.g. Hrbek & Meyer, 2003; Villwock, 2004; Fuller *et al*., 2007). For instance the reconstruction of phylogenetic relationships among the species of the killifish of the genus *Aphanius* in the Mediterranean basin using mitochondrial (mt) DNA supported a predominantly vicariant-based speciation hypothesis with a major role played by the Messinian Salinity Crisis (Hrbek & Meyer, 2003).

Here, we focus on the population structure of *A. fasciatus* (Valenciennes, 1821) and *A. iberus* (Cuvier & Valenciennes, 1846). The two species belong to the western clade formed when the final closing of the Tehys Sea (approximately 20 MYA) separated the ancestral *Aphanius* species inhabiting coastal areas of the Tethys, into an eastern and a western group (Hrbek & Meyer, 2003). *A. fasciatus* is currently distributed in the saline coastal waters and occasionally in inland freshwater bodies of the central and eastern Mediterranean

(Wildekamp, 1993). The life-history traits of this species (that produces large benthic eggs, lacks planktonic larval stages and has a relatively sedentary adult stage with limited migrating capabilities) promote a certain degree of isolation of the populations; although these can be subjected to occasional events of gene flow that potentially balance the effects of isolation (Maltagliati, 1998).

The disappearance of several populations of *A. fasciatus* in some parts of distribution area has been already documented (Ferrito & Tigano, 1995; 1996), although the species is not yet facing problems of extinction due to its wide distribution (Kottelatt & Freyhof, 2007).

A. iberus is endemic of the Iberian Peninsula and characterized by fast growth, early maturity, high reproductive effort and reduced life span (Garcia-Berthou & Moreno-Amich, 1992; Doadrio *et al*., 2011; Oltra & Todolì, 2000). Its distribution is mostly restricted to coastal habitats, such as salt marshes, lagoons and some river mouths along the Mediterranean coast of the Iberian Peninsula. The life-history traits of *A. iberus* are similar to those observed in *A. fasciatus* (e.g. benthic eggs and absence of larval stages) (Maltagliati & Camilli, 2000), and should also prevent gene flow between *A. iberus* populations. The species is considered endangered (Doadrio *et al*., 2011), its populations suffer a strong regression, and many surviving only in captivity (Doadrio, 2001a,b).

Surveys of genetic variation in Teleosts have been frequently carried out comparing nuclear and mitochondrial data to test for population structure (Barluenga & Meyer, 2005; Mesquita *et al.*, 2005; McGlashan *et al*., 2001; Carlsson *et al*., 2004; Gonzalez & Zardoya, 2007). Studies on *A. fasciatus* focusing on partial sequencing of the control region (Tigano *et al*., 2004, 2006; Rocco *et al*., 2007; Pappalardo *et al.*, 2008, Annabi *et al*., 2013; Ferrito *et al*., 2013) and on allozymes (Maltagliati 1998, 1999; Maltagliati *et al*., 2003) identified relatively high levels of genetic divergence among populations. Nuclear (allozyme) and mitochondrial

(cyt b) molecular markers analyzed in three populations of *A. iberus* (Doadrio *et al*., 1996; Perdices *et al.*, 2001) showed congruent results differentiating the Mediterranean and Atlantic populations into two independent lineages, *A. iberus* and *A. baeticus* respectively. To date, there are no published microsatellite studies for these killifish species, with the exception of the eight microsatellite loci that had been identified in *A. fasciatus* (Babbucci *et al.*, 2007). The specific objectives of this study are: i) to examine differences in genetic diversity and population structure in two killifish species (*A. fasciatus* and *A. iberus*) characterized by similar ecological traits but having a very different distribution range in the Mediterranean area; ii) to test for the cross-amplification of microsatellite between the two species and to perform a pilot study based on microsatellites and mitochondrial control region sequence data to assess the congruence between these independent genetic markers in defining the differentiation patterns of the two species; iii) to pinpoint the advantages of using data from two genetic and life history descriptors (5'D-loop, and microsatellites) with different evolutionary characteristics (ploidy, inheritance and mutation rate) to assess a preliminary picture of geographic population structure of these two endangered killifishes as a tool for the genetic management of their populations.

MATERIALS AND METHODS

Sample collection

A total of 293 specimens from five *A. fasciatus* and five *A. iberus* populations were analyzed (Fig. 1 and Table I). Microsatellites were PCR amplified and genotyped in all tissue samples (N=146 *for A. fasciatus* and N=147 for *A. iberus*). The 5'D-loop of *A. iberus* was PCR amplified and the sequences obtained in 73 samples were deposited in GenBank (accession

nos. JX431288 and KC710025 - KC710043). Additional 5'D-loop sequences of *A. fasciatus* (N= 118) were deposited in GenBank under the following accession nos. KC741410-KC741414 ; AM183201,AM183202, AM183178-AM183182; AM884569, AM88456970, AM184197; GU947110; GU969235, GU969236, GU980872-GU980883.

Microsatellite amplification and genotyping

Total genomic DNA was extracted from muscle tissue sample (25-30 mg) of specimens preserved in 95% ethanol, using the Dneasy tissue kit (Qiagen) following manufacturer's instructions. DNA extracts were screened for variation at five microsatellite loci [Af7, Af8, Af9, and Af20 from Babbucci *et al.* (2007) and one additional locus (Af11) developed for this study]. Amplifications were carried out in a total volume of 20 µl following the conditions described in Babbucci *et al.* (2007), and individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using one of the primer pairs end-labeled with a fluorescent tag. Allele scoring was carried out using GENEMAPPER software version 3.7 (Applied Biosystems).

D-loop amplification and sequencing

PCR amplification of the 5' hypervariable portion of the *A. iberus* mt control region (5'D-loop) was accomplished with the primers LPRO1 and HDL1 (Ostellari *et al*., 1996). Reactions were carried out in 50 µl. Each reaction contained 0.5 µM of each e primer, 0.2 µM dNTP, 1.5 µM $MgCl_2$, 1X PCR buffer, 1 U of *Taq* Polymerase (Invitrogen) and 50-100 ng of genomic DNA. The PCR conditions for amplification of *A. iberus* 5'D-loop as follows: initial denaturation at 94°C for 2 min, followed by denaturation at 94°C (30s), annealing at 50°C (30s) and the extension at 72°C (40s) repeated for 35 cycles and by a final extension at 72°C

for 7 min. Amplified DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen). Sequencing was performed for both strands using an ABI Prism 3100 automated sequencer (Applied Biosystem).

Microsatellite data analyses

Genetic polymorphism for each population, estimated as the number of alleles per locus (N_A), and the expected (H_e) and observed heterozygosities (H_o), (Nei 1987) were computed using GENETIX ver. 4.04 (Belkir, *et al.* 2004). Linkage disequilibrium between pairs of loci, and deviations from Hardy–Weinberg expectations (HWE) were tested using GENEPOP ver. 3.3 (Raymond & Rousset, 1995). Genetic population structure was tested with a hierarchical analysis of molecular variance (AMOVA, ARLEQUIN ver.3.0) (Excoffier *et al.*, 2005). Population differentiation was analyzed using Wright's F_{ST} (Wright, 1969). In all cases correction for multiple tests of overall significance values was carried out using the sequential Bonferroni procedure of Rice (1989).

To sort populations based on allelic frequencies, a principal component analysis (PCA) was performed using the computer package PCA-GEN written by J. Goudet (downloadable at http://www.unil.ch/izea/softwares/pcagen.html). This program tests the significance of both the total and the individual PCA axis inertias by using a randomization procedure (Manly, 1997). We performed 1000 randomizations of genotypes to test for significance of individual axis inertia.

The assignment of individuals to populations was carried out using a model based clustering method as implemented in the program STRUCTURE version 2.1(Pritchard *et al.*, 2000). This method assumes linkage equilibrium, and that loci are at HWE within populations. The program applies a Bayesian clustering algorithm to infer the number of clusters (*K*) with highest probability given each particular *K*, without using prior information

of the individual sampling locations and estimates the posterior probability of the data given each particular *K* (Pritchard *et al*., 2000). Both the *A. fasciatus* and the combination of the two species allele dataset were analyzed. We assumed a prior value for *K* that ranged from 1 to 11, and 10^6 MCMC iterations were used after a "burn-in" period of 10^5 iterations. All simulations were conducted using an admixture model and correlated allele frequencies between populations. An additional ad hoc statistic (ΔK) was also estimated because it was shown to provide a better predictor of the number of groups (i.e. *K*) at the uppermost hierarchical level (Evanno *et al*., 2005).

Tests based on summary statistics were applied to infer population demography history in *A. fasciatus* and *A. iberus*, using BOTTLENECK ver. 1.2.02 (Cornuet & Luikart, 1996; Luikart *et al.*, 1998). The method assumes that recently bottlenecked populations should exhibit a significant excess of heterozygosity (H_e) compared to expected at mutation-drift equilibrium (H_{eq}), The analysis were carried out assumingan infinite allele (IAM), a stepwise-mutation (SMM) a the two-phase (TPM) models, and applying the Wilcoxon test for statistical detection of H_e excess. The distribution of allele frequency classes was examined for a distortion from the normal L-shaped distribution (Luikart *et al*., 1998).

Differences between each pair of populations were summarized using Cavalli-Sforza & Edwards (1967) chord distance (D_{CE}). Clustering relationship among populations was displayed using an unrooted Neighbor-Joining (NJ) tree, which was estimated based on pairwise D_{CE} using the program POPULATIONS 1.2.14 (Langella, 2001).

Mitochondrial DNA data analyses

Sequences were aligned using CLUSTAL X (Thompson *et al.*,1997). Ambiguous regions of the alignment were systematically identified and removed using the program GBlocks v.091b

193 (Castresana, 2000) with default settings. The number of haplotypes and polymorphic sites was
194 estimated using the program Collapse (version 1.2) and verified with DnaSp (version 4.10.3;
195 Rozas *et al*., 2003). Genetic variability was estimated using two parameters: nucleotide
196 diversity (π) described as the average number of nucleotide differences per site between two
197 sequences, and haplotype diversity (*h*), using DnaSp.
198 An analysis of molecular variance (AMOVA), implemented in the program
199 ARLEQUIN ver. 3.0 (Excoffier *et al*., 2005) was applied to the distance matrix to estimate
200 variance components and population pairwise distance measures. Analyses of genetic
201 variance were performed grouping all populations into two distinct species in a single
202 analysis, as well as considering populations of each species separately in two different
203 analyses. *Φst* values were estimated both for population pairs and for all populations, using a
204 "weighted" analysis of variance (Weir & Cockerham, 1984).
205 Pairwise divergence between populations was calculated using Kimura 2 parameter
206 (K2P) distances (Kimura, 1980) in MEGA 5 (Tamura *et al*., 2011). Evolutionary relationships
207 among populations of the two species were inferred using NJ (Saitou & Nei, 1987) and K2P
208 distances (Kimura, 1980) with MEGA 5 (Tamura *et al*., 2011). Robustness of internal
209 branches was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicates. A
210 hierarchical series of likelihood ratio tests (Huelsenbeck & Rannala, 1997), implemented by
211 the 0.1.1 jModelTest (Posada, 2008) and using the Akaike Information Criterion, was applied
212 in order to identify the appropriate nucleotide substitution model, with which to perform the
213 maximum likelihood (ML) tree in MEGA 5 (Tamura *et al*., 2011). The K2P+I model best
214 fitted the 5'D-loop dataset. To maximize the power of inference concerning the relationship
215 between haplotypes and their different frequencies/locality, a haplotype network was built
216 using the median joining algorithm (Bandelt *et al*.,1999) in the program NETWORK v.

4.5.1.0 (www.fluxus-engineering.com).

Tajima's D statistic (Tajima, 1989) and Fu's Fs test (Fu, 1997) for selective neutrality were calculated using ARLEQUIN ver. 3.0 (Excoffier *et al*., 2005) with the aim of detecting changes in population size. Results with significant negative D and Fs values can be interpreted as signatures of population expansion.

RESULTS

Microsatellite data

Genetic variability per loci and population site and measures of genetic differentiation

All examined loci were polymorphic in *A. fasciatus* except locus Af11, which was fixed in this species. Moreover, Af20 locus did not amplify in the population of Tourlida (TR). The genotyping of five polymorphic microsatellite loci revealed a relatively low number of alleles per locus. Overall allele number ranged from four to nine for Af7; from three to five for Af8; from two to three for Af11, and from two to four for Af20. Overall, H_o ranged from 0.31 (Trapani, TRA) to one (S'Ena Arrubia, SR); H_E ranged from 0.35 (TRA) to 0.81 (TRA). Three *Fis* values (obtained at S'Ena Arrubia, SR and Lesina, LE populations for Af7 locus and at SR population for Af20 locus) remained significant after Bonferroni correction, which indicates their departure from HW equilibrium due to a strong heterozygote deficit (Table II, Fig. SI).

The Wilcoxon test under the IAM detected recent bottleneck ($P < 0.05$) in two populations (SR, TRA) and for SR also under the TPM model (Table SI). The test performed under the SMM model rendered non-significant results for any population.

A shift in the distribution of allele frequency classes in two populations was detected (Table SI). Consistently, only SR exhibited significant bottleneck effects under IAM and

241 SMM models. No trace of genetic bottleneck was detected for TR population under any
242 specific mutation model, however, the low number of polymorphic loci in these populations
243 resulted in a low power of the Wilcoxon test.
244 The PCA analysis performed on allelic frequencies (Fig. 2) allowed for a clear distinction of
245 the five populations of *A. fasciatus*. The first component explained 44.76% of the total genetic
246 variance, corresponding to a global F_{ST} estimate of 0.067, and provided a straightforward
247 discrimination between Vendicari (VE) and the rest of populations, whereas the second
248 component explained 35.95% of the variance corresponding to a global F_{ST} estimate of 0.054,
249 discriminating Lesina (LE) and S'Ena Arrubia (SR) populations from the rest.
250 The *A. fasciatus* unrooted NJ tree based on D_{CE} separated Vendicari (VE) and Tourlida (TR)
251 populations from those of Trapani (TRA) Lesina (LE) and S'Ena Arrubia (SR). Within the
252 latter, LE and SR clustered together (Fig. SII). However, all groupings received low bootstrap
253 support. The topology of the NJ tree agrees with the results of the PCA based on allelic
254 frequencies (Fig. 2).
255 The number of alleles obtained for Af7 and Af8 loci in *A. iberus* was two; for Af11 and Af9 loci
256 ranged from two to three, whereas locus Af20 did not amplify for this species (Table II). Overall,
257 the H_o varied between 0.27 (Vinalopo,VIN) to 1.0 (Villena,VIL), and the H_E ranged from 0.23
258 (VIL) to 0.57 (Albuixech, ALB). The Wilcoxon test failed to detect recent bottlenecks under any
259 kind of mutation model (IAM, TPM and SMM). However, the low number of polymorphic loci in
260 these populations resulted in a low power of the Wilcoxon test.
261 The PCA analysis performed on allelic frequencies revealed that the five populations of *A.*
262 *iberus* are well differentiated on the first and second principal components; the PC1 explained
263 64.53% of the total genetic variance corresponding to a global F_{ST} estimate of 0.024, and
264 separated the populations of VIN and SAX from the others, whereas the PC2 explained

30.50% of the total genetic variance corresponding to a global F_{ST} estimate of 0.011, and provided a straightforward discrimination between SPO and the remaining populations (Fig. 2). The *A. iberus* unrooted NJ tree based on D_{CE} recovered two clusters with a high bootstrap support: one including SAX and VIN populations, and the other including the remaining populations (Fig. SII). The topology of the NJ tree agrees well with the results of the PCA based on allelic frequencies (Fig. 2).

Estimation of the number of possible populations per species

The Bayesian clustering analysis of all samples together showed a modal value of ΔK at $K = 2$ (Fig. SIII), separating the two species into different clusters. If only individuals of *A. fasciatus* were included in the analyses, the highest likelihood was found at K=5, whereas the modal value of ΔK was shown at $K = 2$ (corresponding to the Ionian and the Mediterranean samples respectively). The analysis of only individuals of *A. iberus* did not resolved distinct genetic clusters (i.e. the highest posterior probability value was found at K=1).

Mitochondrial DNA analyses

A 365-378 bp portion of the D-loop was sequenced in 73 individuals of *A. iberus* from five Iberian populations (Table I). The analyzed dataset included also the orthologous sequences of five populations of *A. fasciatus* previously studied (see accession number in Tigano *et al.*, 2006; Pappalardo *et al*., 2008; Ferrito *et al*., 2013).

Base composition of analyzed sequences showed an A-T bias (A+T content = 0.67) as usual for the D-loop. Saturation analysis demonstrated that D-loop data set bears little substitution saturation and should be used for phylogenetic reconstruction (the observed values of the substitution saturation index Iss, were significantly lower than the critical value,

289 Iss.c).

290

291 *Mitochondrial genetic differentiation*

292 Sequence comparisons of the *A. fasciatus* D-loop yielded 28 haplotypes from 118 individuals.
293 The alignment had 380 positions, and 33 polymorphic sites, of which 30 were parsimoniously
294 informative. The population of VE showed the lower values of haplotype and nucleotide
295 diversity (h= 0.541, π= 0.0022) (Fig. SIV). The AMOVA revealed overall significant genetic
296 structuring of the analyzed samples (Φst value = 0.83; P<0.00) (Table SII). The majority of
297 the variance was distributed among (83.13%) and not within (16.87%) populations. The
298 percentage of divergence among populations based on the genetic distance matrix (K2P)
299 ranged between 0.5% (in the TRA/SR comparison) and 3.8% (in the VE/SR comparison)
300 (Table SIII). The NJ tree based on K2P distances (Fig. SII) and ML tree (Fig.3) revealed the
301 presence of two distinct clades. The first one included samples from the Sicilian population of
302 Vendicari (VE) and the second cluster included the remaining populations (LE, TRA, SR,
303 TR). Within this second cluster, the Greek population of Tourlida form a well defined
304 subcluster while the Western Sicilian population of Trapani (TRA) clustered together with the
305 Sardinian population of SR. The topology of the mt-based NJ tree agrees well with the
306 topology of the microsatellite-based tree (Fig. SII) and the results of the PCA based on
307 microsatellite data (Fig. 2). In agreement with the pattern recovered in the corresponding NJ
308 tree (Fig. SII), the median joining network analysis of *A. fasciatus* 5'D-loop sequences
309 produced three haplogroups (Fig. 4). The first haplogroup (southeastern) includes the
310 haplotypes of the greek population from Tourlida (TR) and those of the Sicilian population
311 from Vendicari (VE). The second haplogroup (western) includes the population from Trapani
312 (TRA) and S'Ena Arrubia (SR). The third haplogroup including the Adriatic Lesina (LE)

haplotypes seems to connect the first and the second haplogroups. The main part of the haplotypes is private but H_9 haplotype which is shared among S'Ena Arrubia (SR) and Trapani (TRA) populations.

The alignment of the 73 *A. iberus* sequences had 378 positions. A total of 25 sites were variable, of which 24 were parsimoniously informative. These polymorphisms defined 20 distinct mtDNA haplotypes. The populations of Santa Pola (SPO) and Albuixech (ALB) showed the lower values of haplotype and nucleotide diversity, respectively (h= 0.54, π= 0.0074; h= 0.69, π= 0.0039)(Fig. SIV). The AMOVA revealed overall significant genetic structuring of the analyzed samples (Φst value =0.73; P<0.00) (Table SII). The majority of the molecular variance (72,60 %) was due to the variation among populations. The percentage of divergence among populations based on the genetic distance matrix (K2P) ranged between 1.3% (in the VIL/ALB and SAX/VIN comparisons) and 3,3% (in the SPO/SAX comparison) (Table SIII). The unrooted NJ tree (K2P) showed the separation of Sax and Vinalopò (VIN) from the remaining populations (Fig. SII). The tree topology agrees well with the microsatellite-based NJ tree and PCA results (Fig. 2). The median-joining network of 20 mtDNA haplotypes was characterized by two main haplogroups: the first including Sax and Vinalopò haplotypes and the second included the remaining haplotypes (Fig. 4).

DISCUSSION

The main outcomes of this work concern i) the presence of similar levels of population structure in *A. iberus* and *A. fasciatus*; ii) the congruence of the 5'D-loop and microsatellite data in defining the pattern of genetic structuring of the two species. Previous studies aimed to determine levels of genetic variation within and among populations in the Mediterranean killifish *A. fasciatus* were based on allozymes (Maltagliati 1998, 1999, 2002); 5'D-loop

337 (Tigano *et al*., 2004, 2006; Pappalardo *et al.*, 2008, Ferrito *et al.*, 2013), RAPDs and 5'D-loop
338 (Rocco *et al*., 2007). Similar studies in *A. iberus* were based on allozymes and RFLP analysis
339 of 5'D-loop (Doadrio *et al.*, 1996; Fernandez-Pedrosa *et al.*, 1995; Perdices *et al.*, 2001). The
340 present study is the first characterizing population genetic structure in both species using
341 microsatellite variation and comparing it to mtDNA variation. The percentage of divergence
342 among the two studied species based on the genetic distance matrix (K2P) ranged between
343 16.1% and 18.9%. Comparing these divergence values with those of the 5'D-loop sequences
344 obtained from pairwise comparisons among *Aphanius* species studied by Parker & Kornfield
345 (1997) (*A. chantrei= A. danfordii*; *A. dispar* and *A. mento*) reveals the lower value in the
346 comparison *A. fasciatus vs A. danfordii* (12,5%), *A. iberus vs A. danfordii* (17,0%) and *A.*
347 *fasciatus vs A. iberus* (17,4%). The pairwise comparisons among the remaining species
348 yielded divergence values ranging between 21,4% (*A. fasciatus vs A. dispar*) and 24,4% *(A.*
349 *mento vs A. fasciatus, A. mento vs A. danfordii* and *A. iberus vs A. dispar*). The relationships
350 shown in our ML tree confirm the greater affinity between *A. fasciatus* and *A. danfordii*
351 already reported by Hrbek & Meyer (2003). Performing the microsatellite and 5'D-loop
352 analyses separately on the two species the *Φst* and F_{ST} average values were relatively lower
353 in *A. iberus* (*Φst* = 0.72 and F_{ST} =0.07) rather than in *A. fasciatus* (*Φst*= 0.83 and F_{ST} = 0.18).
354 The percentage of molecular variance was higher among populations using the 5'D-loop
355 sequences and within populations using microsatellites. This difference is not unexpected
356 since the two molecular markers show different evolutionary characteristics (ploidy,
357 inheritance and mutation rate) reflecting pattern of geographical structure on different
358 timescales. Moreover, it is also known that the high mutation rate of microsatellites could lead
359 to an underestimation of differentiation among populations (Hedrick, 1999).
360 The topologies of the unrooted NJ trees, based on D_{CE} using microsatellite loci, the

results of the PCA as well as the topologies of NJ and ML trees inferred from 5'D-loop sequences well agreed in each species. In particular, all results showed the separation of the population of *A. fasciatus* from Vendicari (VE), which differed from other *A. fasciatus* samples by a 3.5% mean sequence divergence (3.1-3.8%). This population lives in a coastal lagoon in an area of southeastern Sicily known as the Hyblean region, which is clearly differentiated from the rest of Sicily because of its peculiar geological history (La Greca, 1989; Pedley *et al*., 2007). Thus, all the populations of *A. fasciatus* of the Hyblean region were always described as a different lineage with respect to other Mediterranean populations of this species based on both morphological and molecular evidence (Tigano *et al*., 2006; Ferrito *et al*., 2007; Pappalardo *et al.*, 2008, Ferrito *et al.*, 2013). Our microsatellite data confirms the singularity of the Hyblean population of Vendicari (VE).

In *A. iberus*, microsatellites and mtDNA sequence analyses revealed a clear separation of Vinalopò (VIN) and Sax populations from the others. At our best knowledge this is the first population genetic study that includes these two populations while we confirm the results obtained by Perdices *et al.* (2001) based on allozymes and cytochrome *b* sequences for Albuixech, Villena and Santa Pola populations. Testing for signatures of population expansion yielded a recent past demographic expansion for the population of *A. fasciatus* from Lesina (LE) characterized by a star-like median-joining network, low haplotype diversity value and significantly negative neutrality tests (Table III). Negative but not significant values of Fu's *Fs* and Tajima *D* were revealed for the populations of Trapani (TRA) and Tourlida (TR). The parameters rejected the null hypothesis of population expansion for *A. iberus* populations. Bottleneck analysis showed signature for a recent bottleneck under IAM and TPM model only for the population of *A. fasciatus* from S'Ena Arrubia (SR) confirmed by the shift in the distribution of allele frequencies classes. All these

385 results taken together outline a peculiar picture in which two species having similar ecological
386 traits, share similar genetic traits but show a very different distribution range that would for
387 example promote a higher level population structure in the widely distributed *A. fasciatus*
388 rather than in *A. iberus* distributed in a more restricted area. Therefore, the detection of the
389 same level of intraspecific divergence in *A. iberus* and in *A. fasciatus* leads us to assume a
390 different scenario for the evolution of the two species on a historical timescale. Studies by
391 Reichenbacher & Kowalke (2009) on the zoogeography of killifishes in the Mediterranean
392 area focused on fossil distribution and palaecology of the *Aphanius* and *Aphanolebias* species
393 in the Miocene. The results showed that in the Late Miocene two fossil species endemic for
394 Spain (*A. illunensis* and *A. jeani*) were clearly isolated from *A. crassicaudus*, the widespread
395 fossil species in the Central and Eastern Mediterranean. Based on otoliths morphology was
396 hypothesized that *A. jeani* could be the ancestor of the present day *A. iberus* and that the
397 divergence of the lineage leading to *A. iberus* is older than the split of the lineage leading to
398 *A. fasciatus*. This statement is supported by our results and by Hrbek & Meyer (2003) trees
399 topology which clearly indicate that the divergence of *A. iberus* happened earlier than that of
400 *A. fasciatus*. However, looking at the high divergence of the Sicilian population of Vendicari
401 (VE) from all the other populations (including the neighboring Sicilian population from
402 Trapani, TRA) we can infer that at local scale very different gene pools of this species could
403 coexist in a same area, that would need to be preserved to keep the genetic diversity of the
404 species. If many conservation actions have been undertaken for *A. iberus* recognized as
405 endangered species (Doadrio, 2001a,b; Doadrio *et al.*, 2011), the same cannot be said for *A.*
406 *fasciatus*. In fact, the wide distribution of this killifish within the Mediterranean area prevents
407 considering it as a threatened species, although many populations disappeared at a local scale
408 (Ferrito & Tigano, 1996; Rondinini *et al.*, 2013).

In conclusion, in recent years, the widespread recognition of a decline in cyprinodontiform populations in the Iberian waters has led to an increasing need to assess genetic variability in wild populations for conservation management purposes (Perdices *et al*., 2001); our results suggest that similar initiatives would be important for *A. fasciatus*. Maintenance of genetic diversity and population distinctiveness must be two major goals in the conservation management of these killifish and in this regard, studies advancing in the knowledge of their population genetic structuring are rather helpful when taking conservation decisions.

In this context, the congruent patterns derived from microsatellite and 5'D-loop data in the two killifish suggest that both markers could be further used to infer the relative role of life-history traits and historical factors in shaping the genetic population structure of the two species.

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Table

Table I - List of the populations of *A. fasciatus* and *A. iberus* examined in this work; sampling areas, population identification code and number of specimens examined in microsatellite and mtDNA analysis.

Species	Sampling areas	Code	microsatellite analysi	mtDNA analysis
A. fasciatus	S'Ena Arrubia (Sardinia)	SR	N = 29	N = 24
	Lesina (Adriatic coast)	LE	N = 28	N = 23
	Vendicari (eastern Sicily)	VE	N = 30	N = 23
	Trapani (western Sicily)	TRA	N = 29	N = 25
	Tourlida (Greece)	TR	N = 30	N = 23
A. iberus	Villena	VIL	N = 30	N = 14
	Albuixech	ALB	N = 30	N = 16
	Vinalopò	VIN	N = 27	N = 15
	Sax	SAX	N = 30	N = 15
	Santa Pola	SPO	N = 30	N = 13

Table

Table II- Summary of genetic variation detected at five microsatellite DNA

	Aphanius fasciatus				
LOCUS	SR (29)	LE (28)	VE (30)	TRA (29)	TR (30)
Af7 (13)					
N_A	4	4	5	9	5
H_o	1.0000	0.8929	0.7333	0.9310	0.6000
H_e	0.7556	0.6279	0.7181	0.8082	0.5136
F_{is}	**−0.331**	**−0.433**	−0.022	−0.155	−0.172
Af8 (6)					
N_A	3	5	5	3	1
H_o	0.8966	0.6786	0.3667	0.3448	/
H_e	0.5777	0.5890	0.3802	0.4301	/
F_{is}	−0.567	−0.155	0.036	0.201	/
Af9 (7)					
N_A	4	5	2	6	6
H_o	0.6897	0.6071	0.6000	0.8276	0.7548
H_e	0.5802	0.5468	0.5062	0.6776	0.7000
F_{is}	−0.193	−0.113	−0.189	−0.226	0.074
Af11 (3)					
N_A	1	1	1	1	1
H_o	/	/	/	/	/
H_e	/	/	/	/	/
F_{is}	/	/	/	/	/
Af20 (4)					
N_A	4	3	2	2	/
H_o	0.6997	0.3929	0.3333	0.3103	/
H_e	0.6745	0.5422	0.4520	0.3539	/
F_{is}	**−0.023**	0.279	0.266	0.125	/

Sample size is indicated in parentheses beneath each population. The total number of alle
locus name. N_A, number of alleles; H_o, observed heterozigosity; H_e, expected heterozi
probability estimates after Bonferroni correction (P <0.005).

loci within five populations of A. fasciatus and five populations of A. iberus.

Aphanius iberus				
VIL (30)	ALB (30)	VIN (27)	SAX (30)	SPO (30)
2	2	2	2	2
0.2667	0.5000	1.0000	0.9000	0.5333
0.2350	0.3814	0.5094	0.5034	0.3977
−0.137	**−0.318**	**−1**	−0.813	−0.349
2	2	2	2	2
0.2667	0.7000	0.6667	0.4667	0.4000
0.3254	0.4627	0.5031	0.5062	0.3254
0.183	−0.526	−0.333	0.079	−0.234
/	/	/	/	/
/	/	/	/	/
/	/	/	/	/
/	/	/	/	/
2	3	1	1	2
0.3667	0.6000	/	/	0.5333
0.4804	0.5695	/	/	0.3977
0.240	−0.055	/	/	−0.349
/	/	/	/	/
/	/	/	/	/
/	/	/	/	/
/	/	/	/	/

les scored for each locus is indicated in parentheses next to the
gosity; F_{is}, inbreeding coefficient. Bold F_{is} values are significant

Table

Table III - Neutrality test results displayed for *A. fasciatus* and *A. iberus* samples. Significant neutrality tests ($p < 0.05$) are indicated in bold.

	Population	Fu's *F* s		Tajma's *D*	
		Fs	*p*	D	*p*
A. fasciatus	SR	-0.437	0.423	0.764	0.787
	LE	-3.922	**0.001*** **	-1.764	**0.021***
	VE	0.768	0.471	-0.864	0.213
	TRA	-1.967	0.067	-1.457	0.058
	TR	-1.381	0.299	-0.014	**0.021***
A. iberus	VIL	0.836	0.677	1.191	0.893
	ALB	0.835	0.838	0.840	0.810
	VIN	0.595	0.641	0.771	0.801
	SAX	1.177	0.737	0.045	0.558
	SPO	5.649	0.988	2.392	0.998

* *p* -value <0.05

** *p* -value <0.01

*** *p* -value <0.001

FIGURE CAPTIONS

Fig. 1 – Geographical map depicting sample collection of *A. fasciatus* and *A. iberus* at the central Mediterranean Sea and in the Iberian Peninsula respectively.

○ *A. fasciatus,* ● *A. iberus.* SR, S'Ena Arrubia; TRA, Salina Chiusa Trapani; VE, Vendicari; LE, Lesina lagoon; TR, Tourlida; ALB, Albuixech; VIL, Villena; VIN, Vinalopò; SAX, Sax; SPO, Santa Pola.

Fig. 2 – PCA performed on allele frequencies at five microsatellite loci of 5 *A. fasciatus* populations (A) and 5 *A. iberus* populations (B). Shown are the first two principal component axes (PC1 and PC2) with the corresponding inertia percentage.

Fig. 3 – Phylogenetic relationships of *A. fasciatus* and *A. iberus* based on D-loop haplotype data using Maximum Likelihood (ML). The best fit model of nucleotide substitutions identified using the Akaike Information Criterion (AIC) implemented in jModeltest 0.1.1 (Posada 2008), K2P+ I, was used by the programme MEGA 5 (Tamura et al. 2011). Bootstrap values greater than 50% are shown. ◇ Shared haplotype between SR and TRA.

Fig. 4 – Median-Joining Network showing the relationships among 5'D-loop haplotypes of *A. fasciatus* and *A. iberus* used in the present study and included sequences published by Pappalardo *et al*., 2008 and Tigano *et al*., 2006; Ferrito *et al*., 2013. Numbers of mutations (greater than one) between haplotypes are indicated near branches and circle sizes are proportional to the number of similar haplotypes (n) observed in the data set.

SUPPORTING INFORMATION

Table SI – Bottleneck analysis. Heterozygote excess deviated allele frequency classes.

Table SII - Analysis of Molecular Variance (AMOVA) calculated for the populations examined in this work with Arlequin ver. 3.0.

Table SIII - Per cent sequence divergence (Kimura 2 parameter distances) based on the 5'D-loop.

Fig. SI - Allelic distribution at five microsatellite loci in populations of the killifishes *A. fasciatus* and *A. iberus*. Locus Af9 and locus Af20 do not amplify in *A. iberus*.

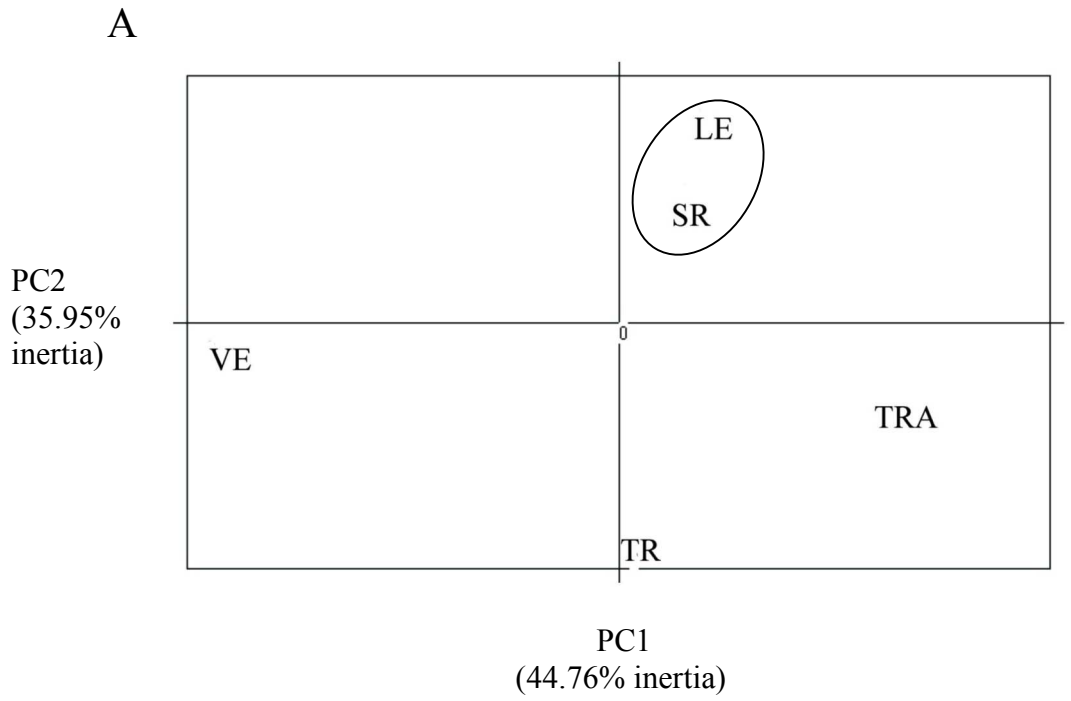
Fig. SII - A) Unrooted Neighbour-Joining trees of *A. fasciatus* populations based on Kimura 2-parameters distance from CR (on the left) and based on the Cavalli-Sforza and Edwards's chord distance Dc from microsatellites (on the right). B) Unrooted Neighbour-Joining trees of *A. iberus* populations based on Kimura 2-parameters distance from CR (on the left) and based on the Cavalli-Sforza and Edwards's chord distance Dc from microsatellites (on the right).

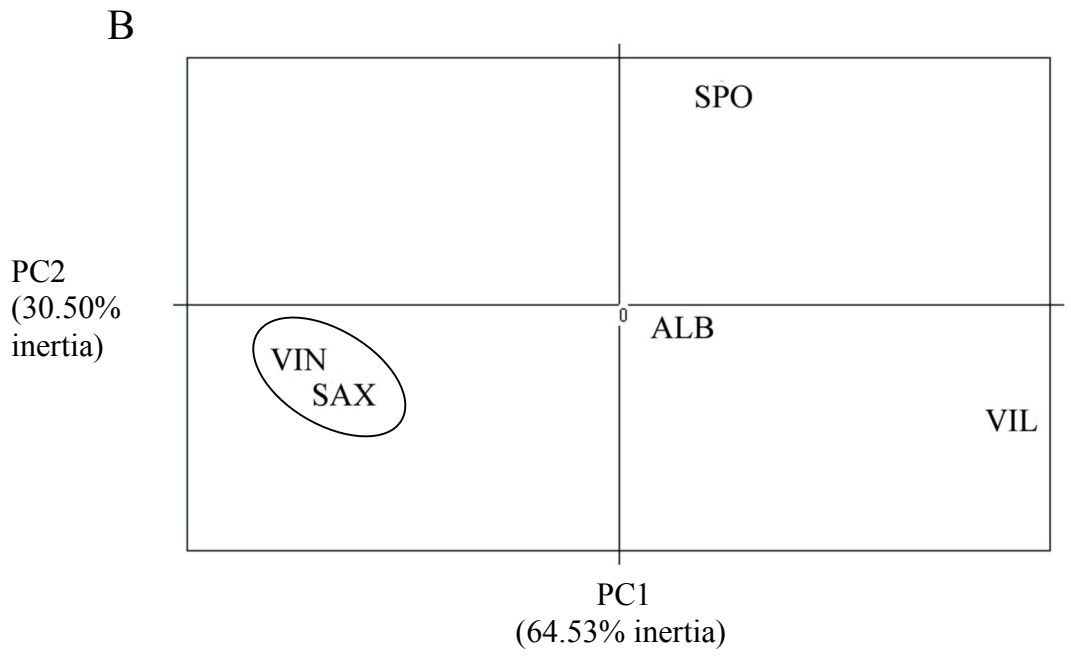
Fig. SIII - ΔK values (Evanno et al., 2005) calculated for K numbers of clusters using the STRUCTURE programme (Pritchard et al., 2000). A) *A. fasciatus* populations. B) *A. fasciatus* and *A. iberus* populations. ΔK is calculated as the mean of the absolute values of the second derivative of L(*K*), (L" (*K*)) average over five runs divided by the standard deviation of L(*K*) (Evanno et al. 2005).

Fig. SIV - Graph showing mtDNA control region haplotypic (h) and nucleotidic (π) diversity values for *A. fasciatus* (grey) and *A. iberus* (black).

LE
Adriatic
Sea
SR
Iberia
Peninsula
TRA
VE
TR
ALB
VIL
SAX
VIN
SPO
Mediterranean Sea
40 km
N

Figure


A
LE
SR
PC2
(35.95%
inertia)
0
VE
TRA
TR
PC1
(44.76% inertia)


B
SPO
PC2
(30.50%
inertia)
0
ALB
VIN
SAX
VIL
PC1
(64.53% inertia)

Figure
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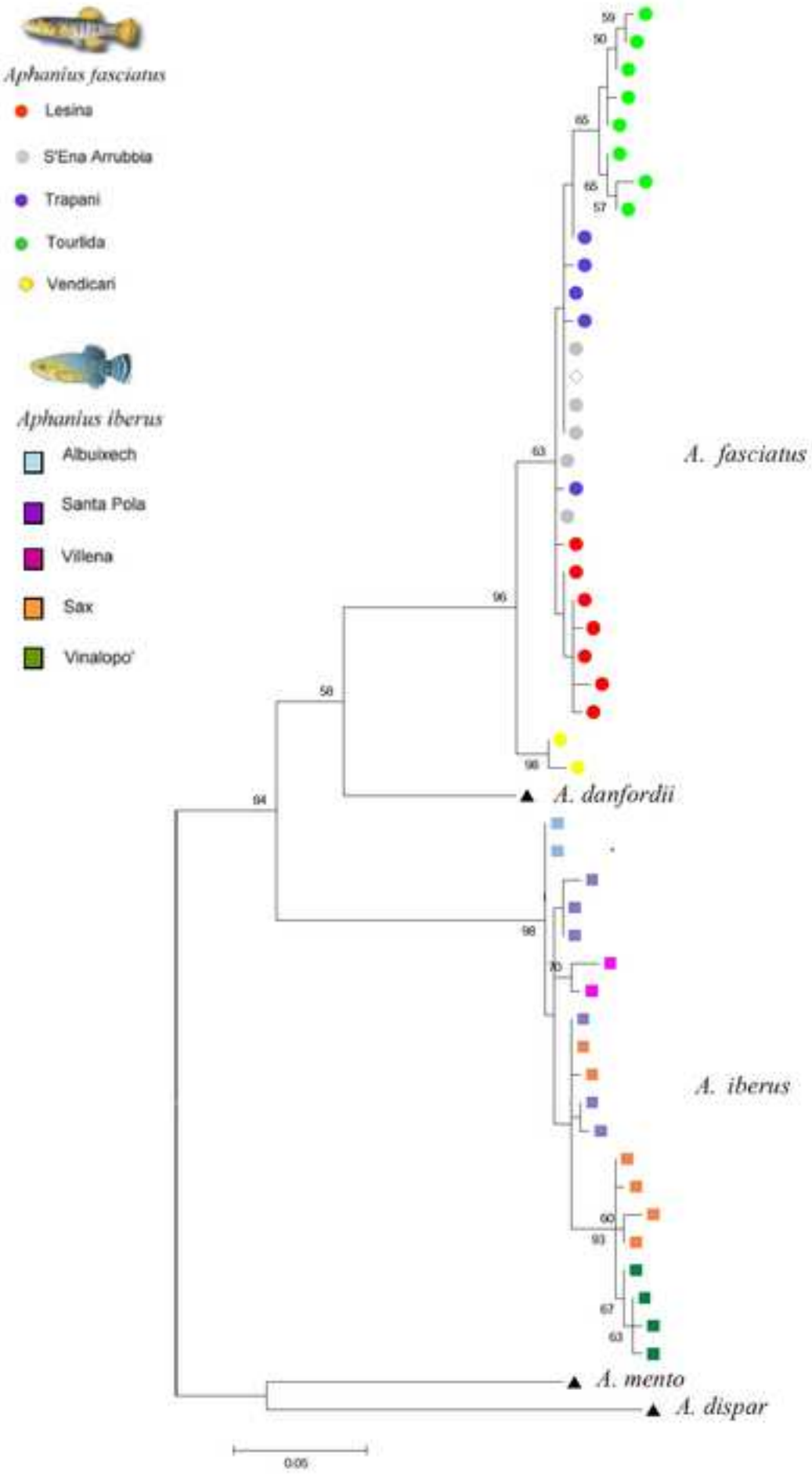

Figure
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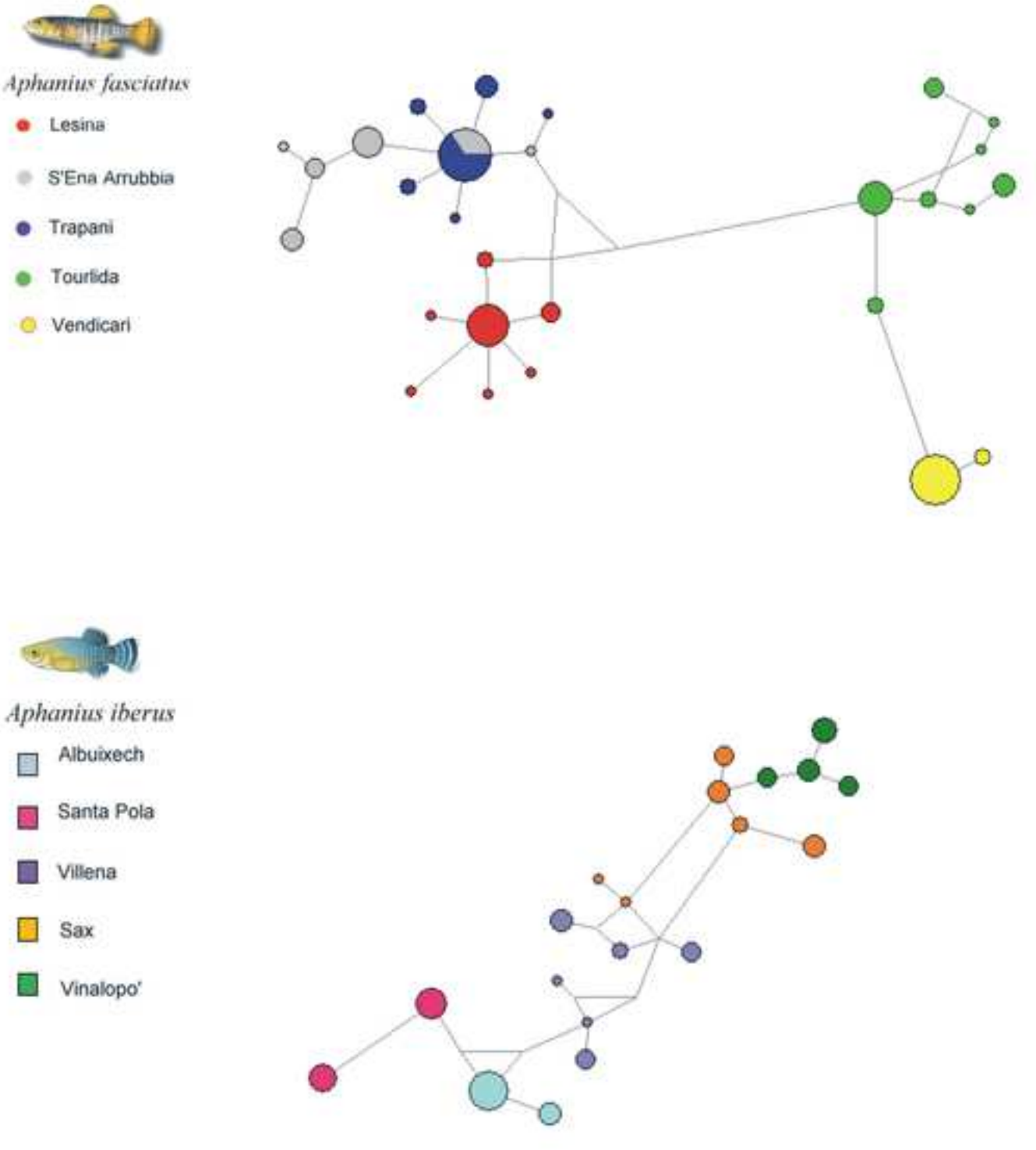


Table SI - Bottleneck analysis. Heterozygote excess (significant *P* values in bold), deviated allele frequency classes

	Heterozygote excess and deviated allele frequency classes			
Sampling site	IAM*	TPM*	SMM*	L-shape
SR	**0.031**	**0.031**	0.062	deviation
LE	0.062	0.437	0.906	no deviation
VE	0.062	0.437	0.437	no deviation
TRA	**0.031**	0.062	0.937	no deviation
TR[§]	0.125	0.250	0.875	deviation
VIL	0.062	0.125	0.125	deviation
ALB[§]	0.062	0.062	0.062	deviation
VIN[§]	0.125	0.125	0.125	deviation
SAX[§]	0.125	0.125	0.125	deviation
SPO[§]	0.062	0.062	0.062	deviation

*Significance of heterozygote excess according to the Wilcoxon test under the IAM, TPM and SMM for each population, and deviation of allele frequency classes from a normal L-shaped
[§]note that the tests for these populations are only based on three polymorphic loci (Fig. 2).

Table SII – Analysis of Molecular Variance (AMOVA) calculated for the populations examined in this work with Arlequin ver. 3.0.

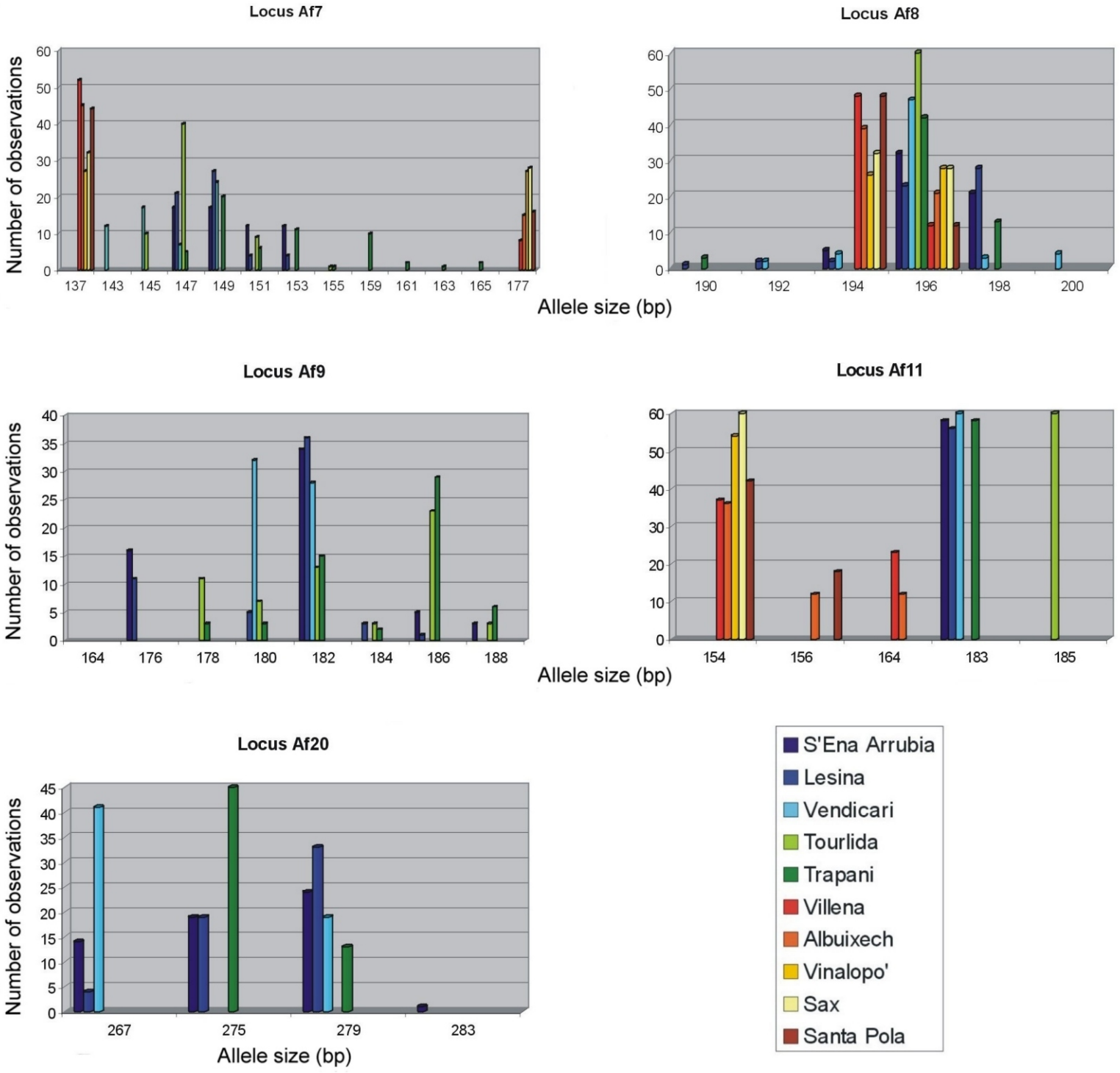
		nuclear DNA				mtDNA			
Structured tested	Source of variation	F-statistics	Sum of squares	Variance	*P*	Φ-statistics	Sum of squares	Variance	*P*
(S'Ena Arrubia, Lesina, Vendicari, Tourlida, Trapani Villena, Albuixech, Sax, Vinalopò, Santa Pola)		F_{ST} = 0.35			**0.000**				
A. fasciatus vs. *A. iberus*	Among groups	F_{CT} = 0.37	100,260	37.37	0.008		2305.714	84.50	**0.000**
	Within groups	F_{SC} = 0.14	37,329	8.20	**0.000**		550.774	12:09	**0.000**
	Within populations	F_{ST} = 0.46	273,691	54.43	**0.000**	Φ_{ST} = 0.97	181.643	3:41	**0.000**
A. fasciatus	Among populations			18:19				83.13	
	Within populations	F_{ST} = 0.18		81.81		Φ_{ST} = 0.83		16.87	
A. iberus	Among populations			7:21				72.60	
	Within populations	F_{ST} = 0.07		92.80		Φ_{ST} = 0.72		27.40	

* Bold *P* numbers are significant values (P <0.005)

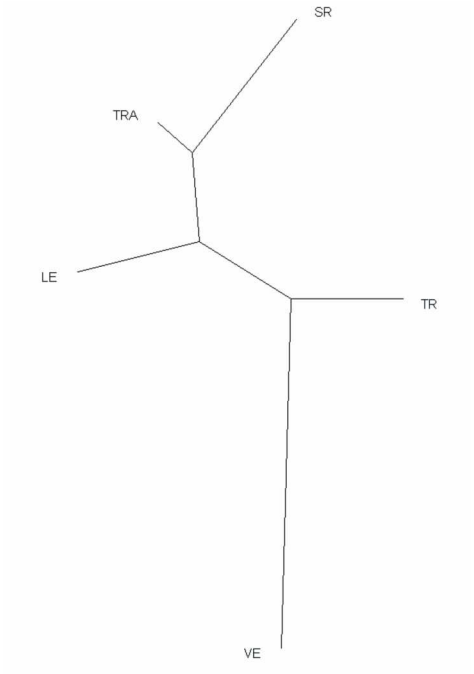
Table SIII - Per cent sequence divergence (Kimura 2 parameter distances) based on the mtDNA CR.

	SR	LE	VE	TRA	TR	VIL	ALB	VIN	SAX	SPO
SR										
LE	1.5									
VE	3.8	3.1								
TRA	0.5	1.3	3.4							
TR	2.2	1.9	3.7	1.9						
VIL	17.1	17.1	17.6	16.7	17.9					
ALB	16.6	16.5	17	16.1	17	1.3				
VIN	18.5	18.4	17.6	18	18.9	2.7	3			
SAX	17.8	17.7	17.1	17.4	18.4	2	2.5	1.3		
SPO	18.2	18.1	18.5	17.8	18.6	2.2	1.5	3.7	3.3	

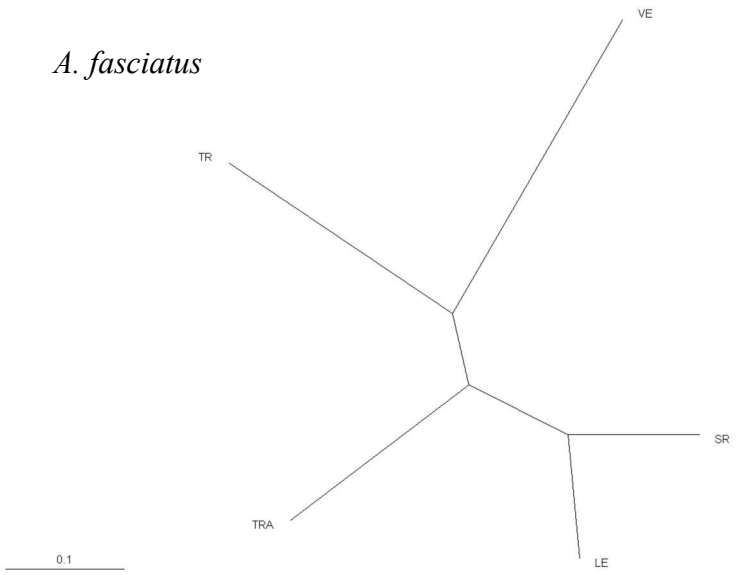
Supporting information e.g. additional data



Supporting information e.g. additional data



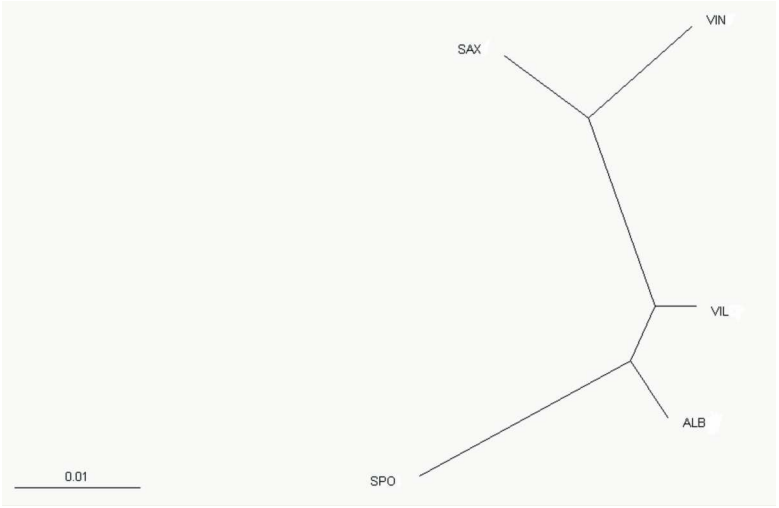
A) *A. fasciatus*

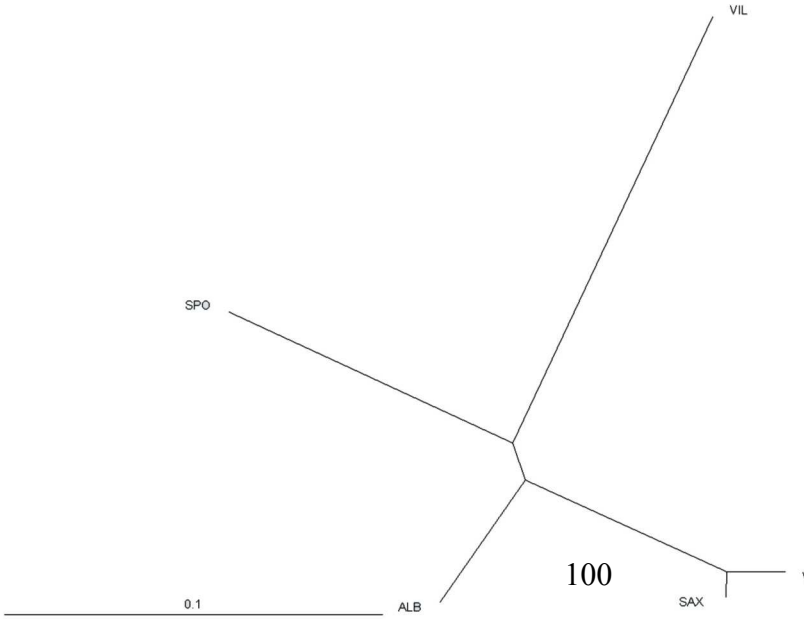


D-loop

Microsatellite

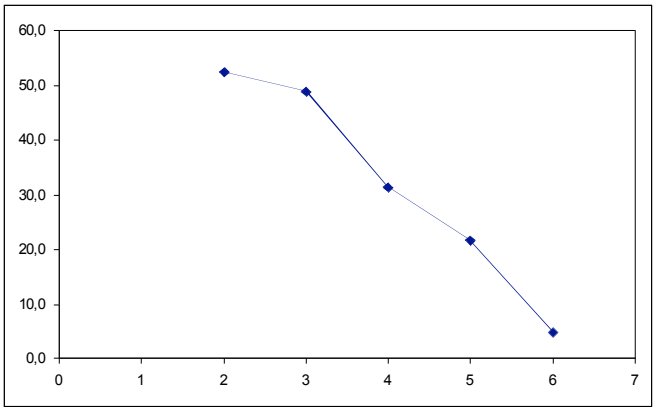
B) *A. iberus*





Supporting information e.g. additional data

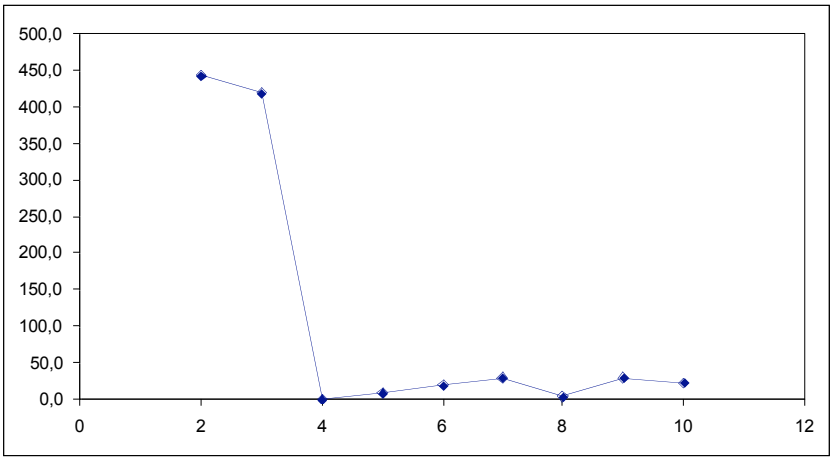
A)



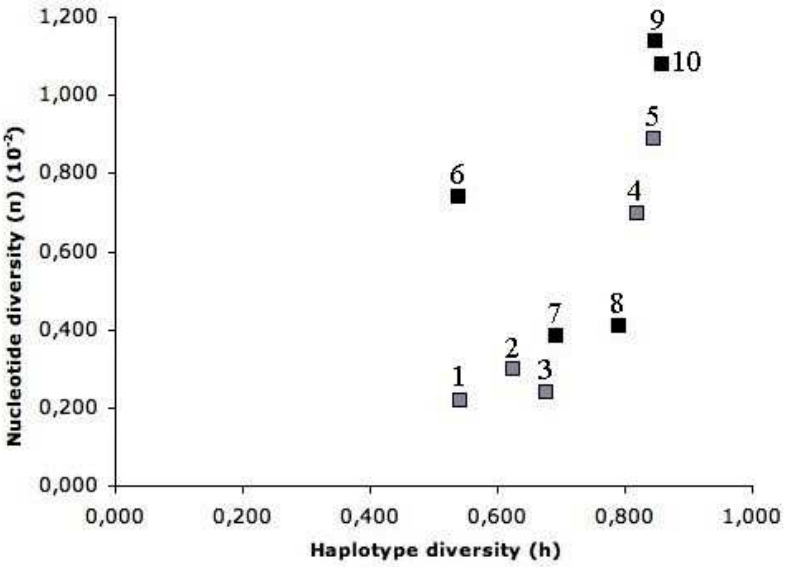
ΔK

K

B)



ΔK

K

Supporting information e.g. additional data