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ORIGINAL PAPER

Conservation genetics in hypersaline inland waters: mitochondrial diversity and phylogeography of an endangered Iberian beetle (Coleoptera: Hydraenidae)

P. Abellán · J. Gómez-Zurita · A. Millán · D. Sánchez-Fernández · J. Velasco · J. Galián · I. Ribera

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Abstract Saline inland waters are globally threatened habitats harbouring many specialised endemic species, which often have restricted geographic ranges, and occur as highly isolated populations. We studied the genetic variation and phylogeography of *Ochthebius glaber* Montes and Soler, a rare and endangered water beetle endemic to hypersaline streams in the South and Southeast of the Iberian Peninsula. We used a 633 bp fragment of cytochrome oxidase subunit 1 gene to determine the genetic diversity and phylogeographic structure within this species, and interpret this in the light of the species' conservation requirements. Thirteen populations were sampled across the species' geographic range, and genetic diversity found to be very high, with 37 haplotypes across the 71 specimens examined (*p*-distance 0.2–7.3%, average 3.1±0.4). Phylogeographic analyses revealed a surprisingly high degree of geographical structure, detectable among populations separated by relatively short geographical distances, with three main groups of haplotypes which have apparently been isolated for significant periods of time. Past fragmentation and contiguous range expansion events were inferred as the main causes of the detected geographical associations of haplotypes. The establishment of independent evolutionary lineages as conservation units is particularly important for species inhabiting saline habitats such as *O. glaber*, which is endangered by habitat loss across most of its distribution. However, given the natural instability of hypersaline environments, the conservation of a network of populations and potential habitats would be necessary to enable the preservation of the process generating and maintaining the diversity of the species.

Keywords Iberian Peninsula · Hypersaline streams · Coleoptera · Habitat fragmentation · ESU

P. Abellán (✉) · A. Millán · D. Sánchez-Fernández · J. Velasco
Departamento de Ecología e Hidrología, Universidad de Murcia, Campus de Espinardo, 30100, Murcia, Spain
e-mail: pabellan@um.es

J. Gómez-Zurita · J. Galián
Departamento de Biología Animal, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain

I. Ribera
Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal 2, 28006 Madrid, Spain

Introduction

Saline inland waters are uncommon and, in many cases, threatened environments, which contain communities particularly rich in rare or endemic species (Williams 1999; Gómez et al. 2005). Considering the dramatic loss of saline habitats as a consequence of the rapid changes in land uses taking place in some Mediterranean areas, moving from extensive to intensive agriculture (Martínez-Fernández et al. 2000; Stoate et al. 2001), the evaluation of genetic variation and phylogeographical patterns in inland saline water taxa is of immediate importance if appropriate conservation measures are to be undertaken.

Although our knowledge of the genetic diversity and phylogeography of freshwater invertebrates has increased significantly in the last decade (e.g., Hughes et al. 1996; King et al. 1999; Baker et al. 2003; Hughes et al. 2003; Kauwe et al. 2004), information concerning invertebrate populations in saline environments is scarce and limited to a few species (Diogo et al. 1999; Gómez et al. 2000;

2002), despite their widely acknowledged conservation value (Bamber et al. 1992; Foster 2000; Gómez et al. 2005). Among the saline aquatic habitats, hypersaline running waters are particularly interesting due to their rarity and the high number of endemic species they sustain, especially species of aquatic Coleoptera (Moreno et al. 1997; Millán et al. 2002; Sánchez-Fernández et al. 2003). In the Mediterranean region, the hydraenid genus *Ochthebius* includes many species living exclusively in saline or hypersaline waters with very restricted ranges (Hansen 1999), over 20 of them in the Iberian Peninsula alone (Ribera et al. 1998; Ribera 2000).

This study focuses on the genetic structure of *Ochthebius glaber* Montes and Soler, a small (<3 mm) water beetle endemic to the south of the Iberian Peninsula and one of the most characteristic species of the macroinvertebrate community in hypersaline streams (Abellán et al. 2005a). Although it may be locally abundant, the species is limited to only a few saline streams or ''ramblas'', grouped mainly in three geographical areas, the basins of the rivers Guadalquivir, Segura and Júcar (in the last with a single known population).

The species shows high habitat specificity, occurring only in hypersaline running waters (generally associated with marl soils) with salinity ranging from 40 to over 300 g l^{-1} (Montes and Soler 1988; Abellán et al. 2005a). Many of these hypersaline environments are highly threatened (Sánchez-Fernández et al. 2003; Gómez et al. 2005), and some populations of *O. glaber* are known to have disappeared in recent decades (Abellán et al. 2005a). The main threats to this species, as with many other saline species, are related to intensive agriculture practices, such as over-exploitation of aquifers, non-point source pollution and reductions in salinity due to the input of freshwater used for irrigation (Abellán et al. 2005a). Despite the lack of formal recognition of its endangered status (no Iberian aquatic Coleoptera are as yet covered by any national or international protection legislation, Ribera 2000), *O. glaber* is a vulnerable species because of its restricted and fragmented distribution, high habitat specificity and continued habitat loss. It has been proposed for inclusion in the IUCN red list (Abellán et al. 2005b) and is included in the Spanish red list (Verdú and Galante 2005).

We used partial sequences of the mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene of *O. glaber* to determine the genetic diversity and phylogeographic structure of populations throughout its range. Data on the genetic diversity of these populations and their degree of geographical subdivision may help decision making in conservation management and the design of recovery strategies (Avise 2000; Crandall et al. 2000) for this and other water insects adapted to saline environments.

Materials and methods

Sampling and DNA sequencing

Specimens were collected from 13 localities through the known range of *O. glaber* (south–southeast of the Iberian Peninsula) during 2003 and 2004 (Fig. 1, Table 1). With the exception of some possible additional populations in Jaén (Guadalquivir basin), these are all the known populations of the species as determined by a recent survey in suitable habitats in southern Spain (Abellán et al. 2005a). Each sampling site (i.e. individual stream) constitutes an isolated habitat patch for *O. glaber*, and we therefore consider each sample as a separate population.

Outgroups for the phylogenetic analyses included *O. notabilis* Rosenhauer, a closely related species according to morphology (Jäch 1992), *O. montesi* Ferro and *O. quadrifossulatus* Wollaston, plus two other more distantly related species (*Catops fuliginosus* Erichson, family Leiodidae, and *Aleochara bilineata* Gyllenhal, family Staphylinidae), both in the same superfamily as Hydraenidae (Lawrence and Newton 1995). The sequence of *A. bilineata* was obtained from Maus et al. (2001) (accession number AJ293086).

Samples were stored in absolute ethanol and DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Mannheim). The mtDNA fragment used in this study was a partial sequence of approximately 800 bp of the protein coding *cox1* gene that was PCR-amplified using the primers C1-J-2183 and L2-N-3014 (Simon et al. 1994). Sequencing was done using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City) following manufacturer's instructions, and sequenced

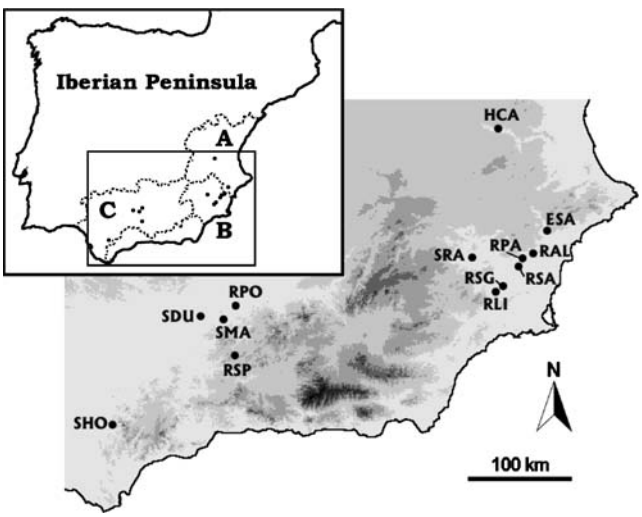

Fig. 1 Sampling sites of *Ochthebius glaber* in the Iberian Peninsula. The grey shades represent 500 m isoclines. See Table 1 for the codes of the localities. The three river basins (Júcar, A; Segura, B; Guadalquivir, C) are indicated in the inset map

Table 1 Locality data, number of individuals (N), number of haplotypes (h), nucleotide diversity (π) and haplotype frequencies (number of individuals with the same haplotype in parenthesis) of the studied populations of *Ochthebius glaber* and other *Ochthebius* outgroups. The main land-use classes in the surrounding area to sampling localities are also indicated

Code	Locality[a]	N	h	π	Haplotypes	Land-uses[b]
O. glaber						
HCA	Rambla Hoces del Cabriel (V)	6	4	0.002	Ju1(1), Ju2(3), Ju3(1), Ju4(1)	A
ESA	Estrecho de la Salineta (A)	6	3	0.001	Se5(4), Se6(1), Se7(1)	A, C
RAL	Rambla de Albatera (A)	6	2	0.002	Se8(4), Se9(2)	A
RPA	Rambla de la Parra (MU)	5	3	0.003	Se8(3), Se10(1), Se11(1)	C
RSA	Rambla Salada Fortuna (MU)	7	2	0.001	Se8(5), Se12(2)	C
SRA	Salinas de la Ramona (MU)	5	4	0.003	Se8(2), Se13(1), Se14(1), Se15(1)	A
RSG	Rambla de Sangonera (MU)	7	2	0.000	Se16(1), Se17(6)	C
RLI	Rambla de Librilla (MU)	6	3	0.005	Se8(2), Se17(3), Se18(1)	A
RPO	Rambla en Porcuna (J)	5	5	0.013	Gu19(1), Gu20(1), Gu21(1), Gu22(1), Gu23(1)	B
SMA	Salinas de la Maturra (CO)	5	5	0.011	Gu21(1), Gu22(1), Gu24(1), Gu25(1), Gu26(1)	B
SDU	Salinas de Duernas (CO)	4	4	0.012	Gu21(1), Gu27(1), Gu28(1), Gu29(1)	B
RSP	Río Salado de Priego (CO)	4	4	0.011	Gu30(1), Gu31(1), Gu32(1), Gu33(1)	B
SHO	Salinas de Hortales (CA)	5	4	0.009	Gu34(1), Gu35(2), Gu36(1), Gu37(1)	B
O. notabilis						
SHO	Salinas de Hortales (CA)	1	1	–	n1(1)	B
SDU	Salinas de Duernas (CO)	1	1	–	n2(1)	B
CVE	Ayo. salino Casa de Ves (AB)	6	2	0.002	n3(5), n4(1)	A
SAN	Salinas de Añana (AV)	4	3	0.002	n5(2), n6(1), n7(1)	A
O. montesi						
RAG	Rambla de Algüeda (A)	6	4	0.003	m1(2), m2(2), m3(1), m4(1)	B
O. quadrifossulatus						
	Oued Kenndek, Morocco	4	4	0.005	q1(1), q2(1), q3(1), q4(1)	–

[a]Spanish province (in brackets): A Alicante; AB Albacete; AV Álava; CA Cádiz; CO Córdoba; J Jaén; MU Murcia; V Valencia

[b]Land-uses in the surrounding area: A Forestry; B Extensive agriculture and ganaderie; C Intensive agriculture

products were electrophoresed on ABI 310 and 3700 automated sequencers (Applied Biosystems, Foster City).

Newly obtained sequences for this study were submitted to EMBL gene database (accession numbers AJ890027-AJ890079).

Genetic variability and population structure

MtDNA sequences were edited and aligned by eye using the program BioEdit 7.0 (Hall 1999). Nei's (1987) nucleotide diversity (π) was calculated for different groups of haplotypes using DnaSP 3.53 (Rozas et al. 1999) and the measures of genetic divergence within and between groups of haplotypes were obtained with Mega 2.2 (Kumar et al. 1994). Mitochondrial genetic differentiation between populations was assessed by calculating pairwise F_{ST} values and testing their significance by 10,000 permutations using Arlequin 2.0 (Schneider et al. 2000). The association between population pairwise geographical and genetic distances (F_{ST} values) was assessed by the nonparametric Mantel test implemented in Arlequin 2.0 using 1,000 permutations. Tajima's D statistic (Tajima 1989), which can identify the effects of demographic changes under the assumption of neutrality, was calculated to test for evidence of population past expansions or contractions. The statistical significance of the observed D-value was obtained by generating samples under the null hypothesis of selective neutrality and population at equilibrium, using a coalescent simulation algorithm in DnaSP 3.53 adapted from Hudson (1990). In this case, the simulations were run using the same number of segregating sites observed in the sample, and a sample size identical to that of each tested scenario.

To identify genetically distinct geographical groupings of populations we used a test implemented in SAMOVA 1.0 (Dupanloup et al. 2002). The method is based on a simulated annealing procedure that aims to maximise the proportion of total genetic variance due to differences between groups of populations (F_{CT}). Unlike classical tests of population genetic structure (e.g., AMOVA), this method does not require a priori definition of populations, but instead searches for emergent group structures based only on the genetic data. We defined the number of populations (K) and ran 100 simulated annealing processes for each possible K, ranging from $K = 2$ through $K = 12$, recording the progressive split of the populations according to their genetic variance.

Phylogenetic analyses

Phylogenetic trees were constructed using Bayesian analyses with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). We used ModelTest 3.06 (Posada and Crandall 1998) to choose

the best fit model of molecular evolution for our data set. We ran four chains of 2,000,000 generations each and sampled the chain every 100 generations. After confirming that the estimated likelihood of the trees reached a plateau, we discarded the first 1,000 trees as the burn-in phase and used the remaining trees to estimate Bayesian posterior probabilities for each of the parameters of the model and the tree (including branch lengths). For comparison with the Bayesian-based results, we also conducted parsimony (as implemented in PAUP 4.0b, Swofford 2002) and maximum likelihood analyses (ML) (with Phyml 2.4, Guindon and Gascuel 2003), the latter using the same evolutionary model as in the Bayesian analyses. We used bootstrap values and Bayesian posterior probabilities to assess node support. Bootstrap values were calculated in PAUP and Phyml using 1,000 and 100 bootstrap pseudoreplicates, respectively.

Nested clade analysis

We used nested clade analysis (NCA) on the mtDNA sequence data to disentangle the influence of historical events and ongoing population processes on the genetic structure of the populations of *O. glaber* (Templeton et al. 1995; Templeton 1998). NCA tests the null hypothesis of no association between sample locality and haplotype variation and provides an interpretation of statistically significant patterns (Templeton et al. 1995). Homoplasy free (with a 95% confidence interval) haplotype networks for NCA based on the most parsimonious connections of haplotypes were obtained using TCS 1.18 (Clement et al. 2000), which incorporates the cladogram estimation algorithm described by Templeton et al. (1992). Independent networks were linked *a posteriori* considering only the most parsimonious connections between any two haplotypes by relaxing the probability of linking haplotypes in the samples (i.e., increasing the number of possible mutational steps between haplotypes) in TCS 1.18. The data were then converted into a hierarchical nested series of grouped haplotypes (henceforth called ''clades'') following the rules proposed by Templeton et al. (1995). These clades were analysed for significant associations with geography using Geodis 2.0 (Posada et al. 2000). Demographic events were inferred according to the most recent inference key provided on the Geodis web page (14 Jul 2004; http://www.zoology.byu.edu/crandall_lab/geodis.htm), modified from Templeton (1998).

Results

MtDNA diversity and population structure

We obtained *cox1* sequences from 71 specimens of *O. glaber*, harbouring 37 distinct haplotypes (Table 1). The sequences (after removing incomplete ends) had 633 bp, including 62 polymorphic sites, 57 (92%) at third, 4 (6%) at first, and only one (2%) at second codon positions. The only amino acid changes were a Valine to Isoleucine in three of the four Júcar haplotypes, and a Thyrosine to Isoleucine in all the haplotypes from the Júcar and Guadalquivir basins. Sequence divergence ranged from 0.2 to 7.3% (average $3.1 \pm 0.4\%$) with an overall nucleotide diversity $\pi = 0.026$. Most haplotypes (33 out of the 37) were restricted to single localities, and only four (Se8, Se17, Gu21 and Gu22) were observed in two or more localities (Table 1). There were no shared haplotypes among the three main geographical areas considered in the study (Guadalquivir, Segura and Júcar basins).

Nucleotide diversity within *O. glaber* was an order of magnitude higher than its likely sister species, *O. notabilis* (0.026 and 0.004, respectively), despite the larger area occupied by the sampled populations of the latter (from the south and north of the Iberian Peninsula). On the contrary, nucleotide diversities within populations of *O. glaber* were very similar to those obtained for the other three *Ochthebius* species, with the only exception of the populations in the Guadalquivir basin, which were more diverse (Table 1). The number of haplotypes within the Guadalquivir basin samples (19) was higher than that corresponding to the Segura basin (14), despite the higher number of sampled individuals for the latter (Table 1). Similarly, the nucleotide diversity within the Guadalquivir populations was approximately an order of magnitude higher than that of the other populations, generally with as many haplotypes as individuals (Table 1).

Although population genetic approaches to evaluate population structure might not be appropriate for data showing high diversity and few individuals sampled per population, in the broader context of this study they may help us to understand our results. Thus, the comparison of the population pairwise F_{ST} was also indicative of high levels of differentiation. Most populations were significantly differentiated from all others except those within the same geographical area (Table 2). The highest values of differentiation were found between the population in the Júcar basin and the others, although all values between the main geographical areas were high (Table 2). F_{ST} values between populations within the same geographical areas were in general much lower, with a few exceptions: ESA and RSG within the Segura basin, and SHO within the Guadalquivir basin (Table 2). The level of pairwise population differentiation as measured by F_{ST} values was significantly correlated with geographical distance (Mantel test, $r = 0.63$; $P = 0.001$).

The values obtained with Tajima's *D*-test were negative for the species overall (−0.008), and for the sequences from the Guadalquivir (−0.653) and the Segura basins (−1.142).

Table 2 Population pair-wise F_{ST} values (in bold, values with a $P < 0.01$ as measured with 10,000 permutations)

Code	ESA	RAL	RPA	RSA	SRA	RSG	RLI	RPO	SMA	SDU	RSP	SHO
HCA	**0.97**	**0.97**	**0.96**	**0.98**	**0.96**	**0.98**	**0.95**	**0.87**	**0.88**	**0.89**	**0.89**	**0.91**
ESA		**0.71**	**0.65**	**0.79**	**0.64**	**0.93**	**0.62**	**0.79**	**0.82**	**0.83**	**0.85**	**0.86**
RAL			−0.11	0.20	0.15	**0.87**	0.33	**0.80**	**0.81**	**0.83**	**0.85**	**0.86**
RPA				0.08	0.01	**0.81**	0.18	**0.77**	**0.79**	**0.80**	**0.82**	**0.84**
RSA					**0.16**	**0.91**	**0.37**	**0.82**	**0.84**	**0.85**	**0.87**	**0.88**
SRA						**0.81**	0.23	**0.76**	**0.77**	**0.78**	**0.81**	**0.83**
RSG							0.37	**0.84**	**0.85**	**0.87**	**0.89**	**0.91**
RLI								**0.76**	**0.77**	**0.77**	**0.81**	**0.83**
RPO									−0.08	−0.15	0.02	**0.31**
SMA										−0.06	0.11	**0.44**
SDU											0.14	**0.38**
RSP												**0.41**

However, none of the *D*-values showed statistically significant differences from the neutral coalescent model of Hudson (1990).

The SAMOVA algorithm (Dupanloup et al. 2002) did not allow us to unambiguously identify the number *K* of groups of populations displaying the highest differentiation among groups (Table 3), as F_{CT} values increased progressively with *K*, while those of F_{SC} decreased. This was not unexpected given the relationship between both parameters (Dupanloup et al. 2002). F_{CT} was clearly improved between $K = 2$ and 3, while all values for $K \geq 3$ were very similar, and all statistically significant, ranking from 0.81 ($K = 3$) to 0.85 ($K = 12$), possibly meaning that the information brought by the third group is more important than the information brought by the fourth and the following. The composition of groups for $K = 3$ corresponded to the geographical arrangement of localities according to river basin (Fig. 1). Progressively increasing *K* had the effect of adding new groups containing a single population, diluting the group structure.

Phylogenetic relationships

The optimal nucleotide substitution model for the *cox1* matrix including the outgroups, as selected by Modeltest, was the general time-reversible model (GTR, Rodríguez et al. 1990), with a proportion of invariable sites and heterogeneous rates following a gamma distribution (GTR + I + G). The result of the Bayesian analysis was very similar to those using other phylogenetic methods (Fig. 2), with *O. glaber* sister to *O. notabilis*, and high support for the monophyly of the haplotypes found in the Segura and Júcar basins, respectively (Fig. 2). However, the basal nodes defining the relationships among haplotypes of the three main geographical areas had very low support, defining what is in fact a basal polytomy including several lineages within the Guadalquivir basin plus the populations from the Segura basin and the single population of the Júcar basin (Fig. 2). The use of the ingroup only (with an estimated HKY85 + I + G model of evolution, Hasegawa et al. 1985) did not improve the resolution of the basal nodes within *O. glaber*. Within the Segura basin, three haplotypes of the same locality were sister to the rest (Se5, Se6 and Se7, Table 1), although with moderate support (Fig. 2). Some other clades within the Segura and Guadalquivir basins were also strongly supported (Fig. 2), defining a clear geographic structure (see below).

Nested clade analysis

The statistical parsimony networks (Templeton et al. 1992) connected *O. glaber cox1* haplotypes separated by ten or

Table 3 Results of SAMOVA tests for increasing K (number of groups) values. With stars (*), values with $P < 0.05$ based on 100 simulations. See Table 1 for the locality codes

K	F_{SC}	F_{ST}	F_{CT}	Group composition
2	0.76*	0.92*	0.68	(HCA) (ESA, RAL, RPA, RSA, SRA, RSG, RLI, RPO, SMA, SDU, RSP, SHO)
3	0.39*	0.88*	0.81*	(HCA) (ESA, RAL, RPA, RSA, SRA, RSG, RLI) (RPO, SMA, SDU, RSP, SHO)
4	0.33*	0.88*	0.82*	(HCA) (ESA, RAL, RPA, RSA, SRA, RSG, RLI) (RPO, SMA, SDU, RSP) (SHO)
5	0.22*	0.86*	0.82*	(HCA) (ESA, RAL, RPA, RSA, SRA, RLI) (RSG) (RPO, SMA, SDU, RSP) (SHO)
6	0.13*	0.85*	0.83*	(HCA) (ESA) (RAL, RPA, RSA, SRA, RLI) (RSG) (RPO, SMA, SDU, RSP) (SHO)
7	0.06*	0.85*	0.84*	(HCA) (ESA) (RAL, RPA, RSA, SRA, RLI) (RSG) (RPO, SMA, SDU) (RSP) (SHO)
8	0.04*	0.85*	0.84*	(HCA) (ESA) (RAL, RPA, RSA, SRA, RLI) (RSG) (RPO, SMA) (SDU) (RSP) (SHO)
9	−0.00*	0.83*	0.83*	(HCA) (ESA) (RAL, RPA, RSA) (SRA) (RSG) (RLI) (RPO, SMA, SDU) (RSP) (SHO)
10	−0.06*	0.83*	0.84*	(HCA) (ESA) (RAL, RPA, RSA) (SRA) (RSG) (RLI) (RPO, SDU) (SMA) (RSP) (SHO)
11	−0.10*	0.83*	0.84*	(HCA) (ESA) (RAL, RPA, RSA) (SRA) (RSG) (RLI) (RPO) (SMA) (SDU) (RSP) (SHO)
12	−0.16	0.82*	0.85*	(HCA) (ESA) (RAL, RPA) (RSA) (SRA) (RSG) (RLI) (RPO) (SMA) (SDU) (RSP) (SHO)

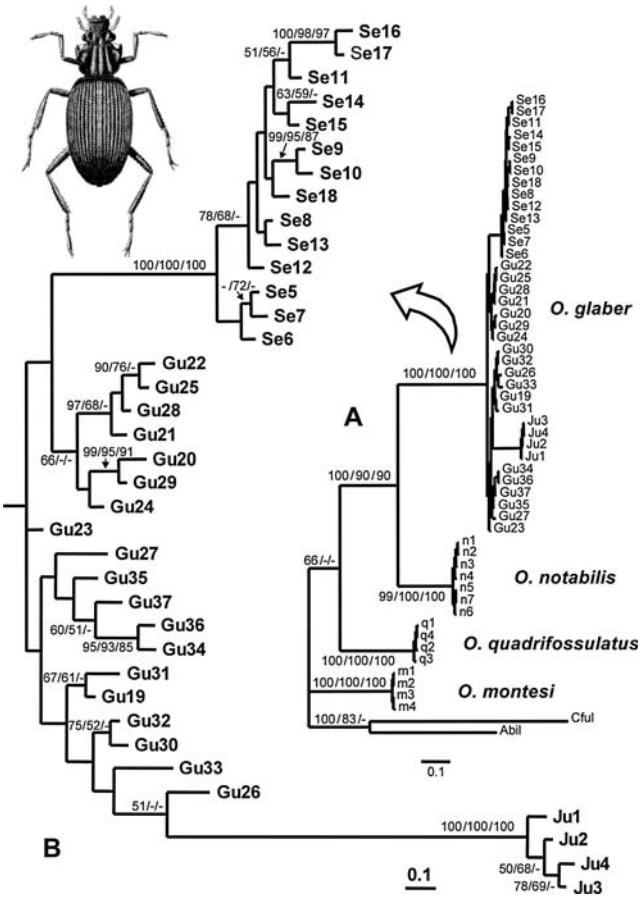

Fig. 2 Phylogenetic reconstruction of the cox1 haplotypes of *Ochthebius glaber*. (**A**) Majority rule consensus tree of the Bayesian analysis including outgroups; (**B**) analysis including only *O. glaber* haplotypes. Numbers above branches are (respectively) Bayesian posterior probabilities (×100)/ML bootstrap values/parsimony bootstrap values. For Bayesian and ML only support values greater than 50% are shown, for parsimony only values above 70% (under these criteria all supported nodes are congruent). Haplotypes are labelled as in Table 1, with the addition of Cful and Abil (*Catops fuliginosus* and *Aleochara bilineata*, respectively)

less non-homoplastic nucleotide substitutions with a 95% confidence probability. This threshold separated three independent networks, formed by the main three geographical areas: the basins of the Segura, Guadalquivir and Júcar rivers (Fig. 3A), in agreement with the phylogenetic results (see above). The Segura basin network had a typical star-shape, with the most abundant and widespread haplotype occupying a central position, while that of the Guadalquivir basin had a more diffuse structure, with very few haplotypes found in more than one population (Fig. 3A).

In an attempt to establish the best hypothesis for the geographical origin of the Segura and Júcar populations, a connection between the three haplotype networks was enforced by relaxing the 95% confidence threshold. The connection between Segura and Guadalquivir basin subnetworks required 14 mutational steps, four-steps beyond the confidence threshold for including homoplasious mutations (Templeton et al. 1995), while the connection between the Júcar and Guadalquivir basins required 31 steps. The study of historical/demographic inferences using NCA requires that the network is rooted (Posada et al. 2000), but in the absence of a robust hypothesis for the relationships among the three groups of haplotypes (see above), we rooted the *O. glaber* genealogy in the long branch connecting the haplotypes of the Júcar and Guadalquivir basins.

The connection between the Segura and Guadalquivir basins networks had a homoplastic loop, with two alternative equally parsimonious connections of the haplotype Se6 (Fig. 3). We considered the two alternative connections in performing the nested clade analysis, although this ambiguity did only affect the nesting design of the networks at the three-step level, resulting in two contrasting possibilities (Fig. 3). In one of them (Solution 1), clade 2–12 (involving the locality ESA) connected to clade 2–3 (including localities RPO and SHO), while in the other (Solution 2) clade 2–12 connected to clade 2–9 (SMA). Table 4 show the inferences of the nested clade analysis for the clades with significant geographical associations for the two alternative nesting solutions. In both cases, the main causes of the geographical associations, according to NCA, were restricted gene flow with isolation by distance, allopatric fragmentation and contiguous range expansion.

Discussion

Genetic diversity and population history of *Ochthebius glaber*

The most unexpected result of this study was the discovery of considerable genetic diversity of mitochondrial haplotypes within and between populations of *O. glaber*, especially within those from the Guadalquivir basin. These intraspecific genetic divergences are higher than those found among other morphologically well characterised Iberian endemic species of water beetles (Ribera and Vogler 2004), whose geographic ranges are much wider than that of *O. glaber*. The genetic variation of *O. glaber* has also revealed a well defined geographical population structure, in contrast to our expectations based on the taxonomy and biology of the species. Little is known regarding the dispersal capability of *O. glaber*, but it is able to fly and its small size could facilitate passive dispersal by the wind. Other species within the genus *Ochthebius* in similar habitats show massive swarming flights under certain environmental conditions (unpublished observations), although this has never been observed with *O. glaber*.

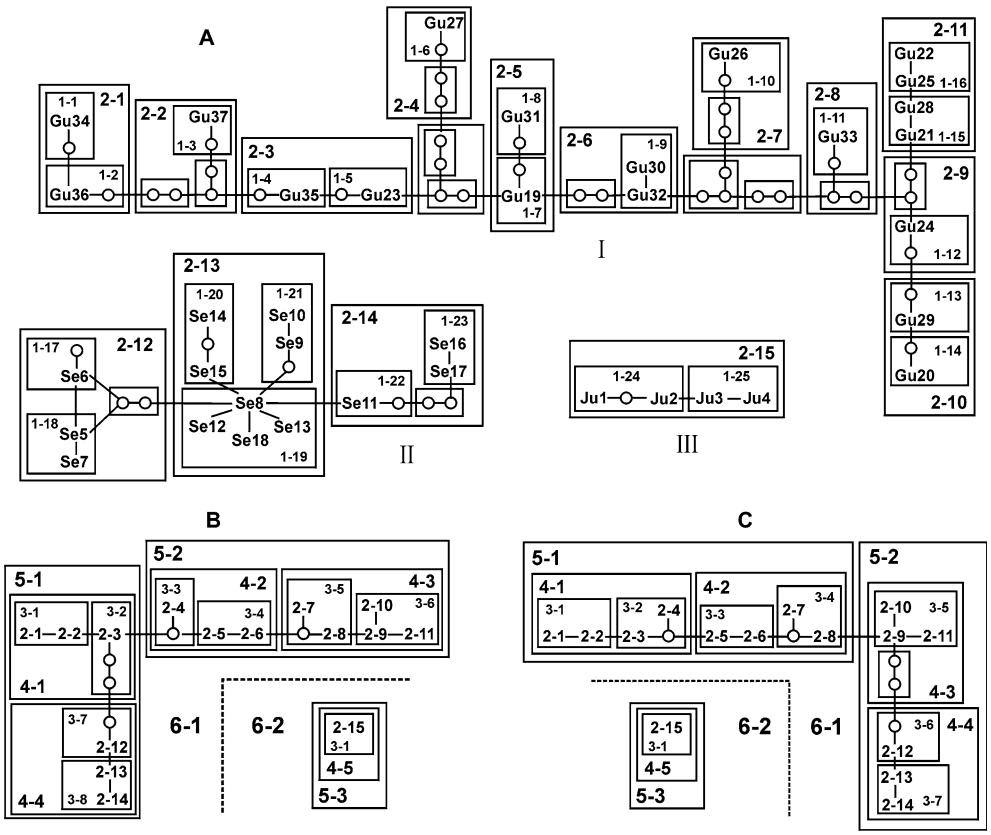

Fig. 3 Statistical parsimony networks for the *cox1* haplotypes of *Ochthebius glaber*. Haplotype codes refer to Table 1. Solid lines connect haplotypes with a single mutation, boxes indicate nested clades of increasing steps; missing intermediates are indicated by an open circle. (**a**) The three independent networks obtained with a 95% probability of being linked without homoplasy (connections ≤10 mutational steps). For clarity, three- to six-step clades are represented in separate networks (**b** and **c**). Networks I, II and III grouped the haplotypes from the Guadalquivir, Segura and Júcar basins, respectively. Fourteen mutational steps connect I and II, and 31 steps connect I and III. (**b**) and (**c**), alternative designs connecting networks I and II (Solutions 1 and 2, respectively; see Results). In **b** clade 2–12 connect to clade 2–3 and in **c** clade 2–12 connect to 2–9

Table 4 Inferences of the nested clade analysis for the clades with significant geographical associations for the two alternative nesting solutions (see main text and Fig. 3)

Solution 1			Solution 2			Key chain and inferred process
Clade	χ^2	*P*	Clade	χ^2	*P*	
3–8	25.161	0.000	3–7	25.161	0.000	1-2-3-4-NO Restricted gene flow
4–4	42.000	0.000	4–4	42.000	0.000	1-19-NO Allopatric fragmentation
5–1	47.000	0.000	5–2	52.000	0.000	1-19-NO Allopatric fragmentation
5–2	6.163	n.s.	5–1	10.988	0.009	1-2-11-12-NO Contiguous range expansion
6–1	60.858	0.000	6–1	47.813	0.000	1-2-11-12-NO Contiguous range expansion
Total cladogram	70.000	0.000	Total cladogram	70.000	0.000	1-19-NO Allopatric fragmentation

Our results clearly demonstrate the existence of three main geographical groups of mitochondrial haplotypes but with unresolved phylogenetic relationships, which have remained genetically isolated from each other for significant periods of time. The higher genetic diversity observed within the Guadalquivir populations, which in the phylogenetic trees appear as basal and paraphyletic with respect to the Segura and Júcar populations, suggests that *O. glaber* could have originated in this Tertiary basin, from where the other areas would have been colonised. Supporting this view is the star-like signature of the haplotype tree in the Segura basin, typical of rapid population expansions from a reduced number of founders (Slatkin and Hudson 1991; von Haeseler et al. 1996; Forster et al. 2001), with a high negative *D*-value (Aris-Brosou and Excoffier 1996; Rogers et al. 1996).

The earliest phylogeographic event inferred for *O. glaber* was a past fragmentation separating the population in the Júcar basin from the rest. Prominent genetic gaps define allopatric lineages, and these probably originated from long-term extrinsic barriers to gene exchange (Avise 2000), concordant with the high genetic distinctiveness shown by the Júcar population. However, it should be noted that the distance between the Júcar population and the nearest population of *O. glaber* in the Segura basin is only 110 km (Fig. 1) and, although there are no suitable habitats for the species in between, there are also no prominent orographic barriers.

The history of the remaining populations in the Guadalquivir and Segura basins, as reconstructed by the NCA, seems to be one of range expansion and subsequent fragmentation originating the isolation of Segura haplotypes in SE Spain. Within this last group, a new allopatric fragmentation event and restricted gene flow seem to have played a main role in the geographical associations of haplotypes.

A potential explanation for the considerable mtDNA divergence and genealogical structuring found in populations separated by relatively small geographical distances is the scattered, discontinuous occurrence of suitable habitats. In the light of the results presented here it seems that the patchy distribution of hypersaline streams (Abellán et al. 2005a) may be sufficient to restrict gene flow even among close populations within the same geographical area, this perhaps being reinforced in some cases by a rugged topography. An additional factor to be considered is the natural instability of hypersaline environments, resulting in likely frequent extinction/recolonisation events and large changes in effective population size due to the explosive population dynamics of *O. glaber*.

Implications for conservation

The recognition of conservation units below the species level is a crucial task if we are to avoid the loss of genetic diversity in species inhabiting threatened environments such as saline inland waters. One widely used framework to distinguish units for conservation purposes has been that of evolutionary significant units (ESU), originally proposed by Ryder (1986) but further developed by different authors (e.g., Waples 1991; Avise 1994; Moritz 1994b; Vogler and DeSalle 1994, see Fraser and Bernatchez 2001 for a review). Our results show the existence of three discrete genetic units within *O. glaber* at a relatively small geographical scale, which could be considered as ESUs.

We are aware of the potential effects of evolutionary stochasticity and of sampling limitations in defining ESUs based exclusively on mtDNA, which is also maternally inherited and therefore does not reflect male-mediated gene flow (Moritz 1994a; Avise 1995). Furthermore, some authors (e.g., Bowen 1999; Crandall et al. 2000) have recently called for incorporation of ecological and/or genetic data of adaptive significance into the formulation of ESUs. To date, there is no evidence that the populations of *O. glaber* exhibit any adaptive divergence (morphological or ecological), and collection of corroborating ecological, behavioural or life history data is beyond the original aims of this study. Nevertheless, the sort of deep genetic structuring found in *O. glaber* may have promoted as yet undetected local adaptation which may be important in maintaining the adaptive potential of the species. At the very least, historical isolation represents an accumulation of novel mutations between populations even though these may not be readily distinguishable by obvious external ''adaptive'' differences (Fraser and Bernatchez 2001).

Fraser and Bernatchez (2001) integrate the ESU in a more general and flexible concept, the Adaptive Evolutionary Conservation (AEC). In this theoretical framework, an ESU is a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level of the species, and the authors suggest to use the best available biological information in exercising ESU definitions on a case-by-case basis. In general, any criteria providing evidence of lineage sorting through highly reduced gene flow are potentially useful for conservation initiatives under the ESU definition of AEC, provided it is rigorously applied. The mtDNA data presented here suggest significant geographical structuring of genetic variation across the range of *O. glaber*, and the phylogenetic and phylogeographic analyses point to long-term historical isolation among the three geographical areas where this species occurs, corresponding to the main river basins. Because of the existence of large areas of habitat discontinuity between these three regions, their genetic singularity and the inferred history of the group, it seems unlikely that genetic exchange occurs. Thus, our study supports the recognition of these three lineages as different evolutionary significant units under the adaptive evolutionary conservation concept.

We believe that a definition of ESUs within *O. glaber* corresponding to the main river basins is also in good correspondence with their different conservation status (see Green 2005). Anthropogenic habitat transformations are not equally dramatic in the three areas where the populations of the species are found. Although inland saline habitats are in general socially under-valuated environments, land-use changes are particularly intense in the south-east of Spain, specially in the Segura basin (see Table 1), where the amount of land being irrigated has increased rapidly and saline streams are disappearing due to the irrigation of surrounding agricultural land (Martínez-Fernández et al. 2000; Sánchez-Fernández et al. 2004). In

conservation terms, these Segura populations are most threatened, and therefore require more urgent attention than those within other less transformed areas such as the Guadalquivir and Júcar basins.

Given the natural instability of hypersaline environments, it seems likely that efforts to preserve individual populations that could be considered to be representative of the different ESUs are futile, as this will not warrant the preservation of the process generating and maintaining their diversity. It could be argued that the conservation of a whole network of populations and potential habitats is necessary for preserving the processes underlying the generation of the observed pattern (see e.g., Ennos et al. 2005), and the important factor would be to preserve the ability to generate and maintain high levels of haplotype diversity within and between populations, whatever the originating mechanisms.

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