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The limits of cryptic diversity in groundwater: phylogeography of the cave shrimp *Troglocaris anophthalmus* (Crustacea: Decapoda: Atyidae)

VALERIJA ZAKŠEK,* BORIS SKET,* SANJA GOTTSTEIN,† DAMJAN FRANJEVIĆ† and PETER TRONTELJ*

**Department of Biology, Biotechnical Faculty, University of Ljubljana, PO Box 2995, SI-1001 Ljubljana, Slovenia, †Department of Zoology, Faculty of Science, University of Zagreb, Roosveltov trg 6, 10000 Zagreb, Croatia*

Abstract

Recent studies have revealed high local diversity and endemism in groundwaters, and showed that species with large ranges are extremely rare. One of such species is the cave shrimp *Troglocaris anophthalmus* from the Dinaric Karst on the western Balkan Peninsula, apparently uniform across a range of more than 500 kilometres. As such it contradicts the paradigm that subterranean organisms form localized, long-term stable populations that cannot disperse over long distances. We tested it for possible cryptic diversity and/or unexpected evolutionary processes, analysing mitochondrial (COI, 16S rRNA) and nuclear (ITS2) genes of 232 specimens from the entire range. The results of an array of phylogeographical procedures congruently suggested that the picture of a widespread, continuously distributed and homogenous *T. anophthalmus* was wrong. The taxon is composed of four or possibly five monophyletic, geographically defined phylogroups that meet several species delimitation criteria, two of them showing evidence of biological reproductive isolation in sympatry. COI genetic distances between phylogroups turned out to be a poor predictor, as they were much lower than the sometimes suggested crustacean threshold value of 0.16 substitutions per site. Most results confirmed the nondispersal hypothesis of subterranean fauna, but the southern Adriatic phylogroup displayed a paradoxical pattern of recent dispersal across 300 kilometres of hydrographically fragmented karst terrain. We suggest a model of migration under extreme water-level conditions, when flooded poljes could act as stepping-stones. In the north of the range (Slovenia), the results confirmed the existence of a zone of unique biogeographical conflict, where surface fauna is concordant with the current watershed, and subterranean fauna is not.

Keywords: cryptic species diversity, ITS, phylogeography, subterranean, *Troglocaris anophthalmus*

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Introduction

The extent of discovered cryptic species (morphologically unrecognized species) is increasing, even in well-known taxa, with wider use of molecular techniques and increasing number of phylogeographical studies. Those studies have revealed cryptic diversity in most groups of organisms and most of the habitats examined (e.g. Bickford *et al*. 2007). Subterranean fauna is no exception. Moreover, nominal groundwater species tend to harbour numerous cryptic species and highly subdivided populations leading to high endemism in different regions around the world (e.g. Leys *et al*. 2003; Lefébure *et al*. 2006a, 2007; Witt *et al*. 2006; Finston *et al*. 2007; Page *et al*. 2008; Trontelj *et al*. 2008). The area hosting the richest subterranean fauna in the world is the Dinaric Karst of the Balkan Peninsula (Fig. 1; Sket *et al*. 2004; Culver *et al*. 2006) with numerous cryptic species revealed by molecular analyses (e.g. Verovnik *et al*. 2004; Gorički & Trontelj 2006; Zakšek *et al*. 2007; Trontelj *et al*. 2008).

Under the prevailing model, high local diversity of groundwater organisms is explained by their tendency to form stable populations that cannot disperse over long distances, and are thus prone to genetic isolation and

Correspondence: Valerija Zakšek, Fax: +386 1 257 33 90; E-mail: valerija.zaksek@bf.uni-lj.si

ultimately speciation (Barr & Holsinger 1985; Sbordoni *et al*. 2000; Lefébure *et al*. 2006a; Culver *et al*. 2008). Endemism is boosted by strong fragmentation of aquatic subterranean habitats in karstic areas (Marmonier *et al*. 1993; Verovnik *et al*. 2004) where drainages and thus potential range limits rarely exceed 200 kilometres (Trontelj *et al*. 2008). Groundwater species with larger ranges challenge this model. One of those species is the cave shrimp *Troglocaris anophthalmus* (Kollar, 1848) distributed over almost the entire Dinaric Karst. In the past two decades, increasing cryptic diversity within the genus *Troglocaris* has been found, followed by diminution of species ranges (Cobolli Sbordoni *et al*. 1990; Zakšek *et al*. 2007; Sket & Zakšek 2009; Trontelj *et al*. 2008). However, none of these studies refuted the 500-kilometre range of the type species *T. anophthalmus*, which continues to present a challenge to the current paradigm.

Here, we examine the diversity and distribution of *T. anophthalmus* haplotypes in order to address two hypotheses resulting directly from the current paradigm of ecology and evolution in subterranean biota: (i) *T. anophthalmus* is constituted of cryptic taxa, or phylogroups, within its large range, and (ii) the current distribution of phylogroups is allopatric and geographically defined. In support of these tests, we studied the demographic events, and searched for possible gene flow barriers and/or pathways that might have shaped the current distribution of phylogroups in the Dinaric Karst. We used DNA sequence data from two independent molecules: mitochondrial cytochrome oxidase I (COI) and 16S rDNA, and the second internal transcribed spacer (ITS2) from the nuclear ribosomal gene region.

Materials and methods

Samples

Specimens were collected from populations across the entire range (Fig. 1). From one and up to 17 specimens per population were analysed, adding up to a total of 232 specimens from 52 localities including 35 specimens from Zakšek *et al*. (2007). Localities of all samples and the resulting DNA haplotypes (per locality) are listed in Table 1. The Kras/Carso plateau in Slovenia and Italy was

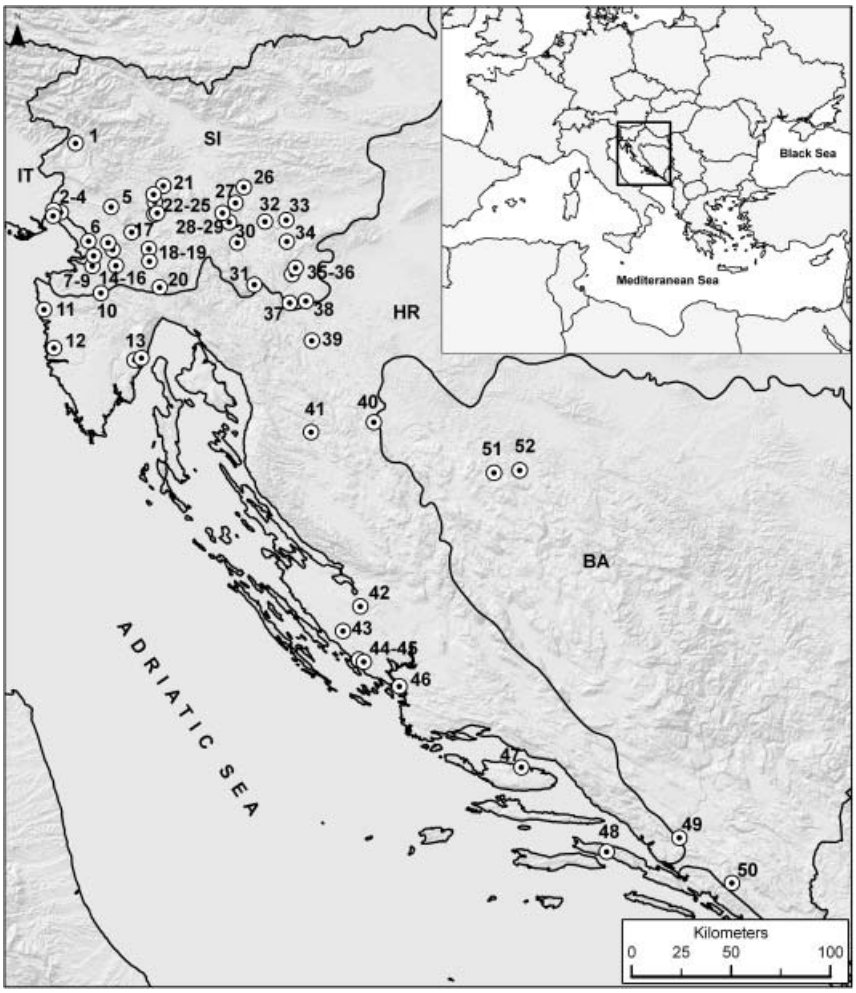

Fig. 1 Sampling sites for *Troglocaris anophthalmus* included in this study. Numbers correspond to locality numbers listed in Table 1. Country abbreviation codes: SI (Slovenia), IT (Italy), HR (Croatia), BA (Bosnia and Herzegovina).

Table 1 List of sampling localities with corresponding population identifiers (ID, used in Fig. 1), sample size and GenBank Accession numbers for the sequences obtained for the study

No.	Clade	Locality*	Haplotypes COI (no. of specimens)	COI	ITS	16S
1	Soča	Vogršček (= Babja jama), cave, Most na Soči, SI	VO(3)	FJ426035	FJ426047	FJ426045
2	W-Slo	Grotta presso Comarie/Brezno pri Komarjih, cave, Jamiano/Jamlje, IT	ID(5)	FJ425924	FJ426082	DQ641573
			IN(2)	FJ425929		
			CP(1)	FJ425925		
			IT2(1)	FJ425931		
3	W-Slo	Pozzo presso S. Giovanni di Duino/Brezno pri Štivanu, cave, Monfalcone, IT	IT1(3)	FJ425936	FJ426070	DQ641573
			ID(2)	FJ425935		
			IN(6)	FJ425934		
			IT2(1)	FJ425937		
			DU1(1)	FJ425945		
			DU2(1)	FJ425943		
4	W-Slo	Dolenca jama, cave, Komen, SI	IT1(2)	FJ425964	—	DQ641573
			ID(5)	FJ425962		
			IN(7)	FJ425930		
			IT2(2)	FJ425931		
5	Soča	Vipavska jama, cave, Vipava, SI	VI(3)	FJ426032	FJ426049	—
6	W-Slo	Labodnica/Grotta di Trebiciano, Trebiciano/Trebče, cave, IT	IT1(5)	DQ641530	FJ426069	DQ641575
			ID(3)	FJ425924		DQ641573
			IN(1)	DQ641534		
			IT2(1)	FJ425937		
7	W-Slo	Fonte Oppia, springs, Val Rosandra/Dolina Glinščice, IT	OSO(3)	FJ425950	FJ426091	—
			GL(1)	FJ425954		
			OSS(1)	FJ425952		
8	W-Slo	Antro delle Ninfe, cave, Val Rosandra/Dolina Glinščice, IT	OSO(2)	FJ425951	FJ426079	—
9	W-Slo	Osapska jama (= Grad), cave, Osp, SI	OSO(2)	DQ641532	FJ426072	DQ641573
			OS(1)	DQ641533		
			OSS(2)	DQ641535		
10	W-Slo	Jama pod Krogom, cave, Sočerga, SI	OSS(5)	FJ426020	—	DQ641573
			SO(1)	DQ641536		
11	W-Slo	Jama kod Komune, cave, Novigrad, Istra, HR	NO(2)	FJ426001	FJ426085	—
12	Istra	Klarića jama, cave, Vrsar, HR	VR(1)	FJ426038	FJ426101	—
13		spring in the Tunnel čepić, spring, Kršan, HR	KR1(2)	FJ425975	FJ426081	DQ641576
			KR2(2)	FJ425973	FJ426103	DQ641587
			KR3(1)	DQ641538		
			KR4(4)	FJ425971		
			KR5(1)	DQ641552		
14	W-Slo	Kačna jama, cave, Divača, SI	DI1(1)	DQ641531	FJ426080	DQ641571
			ITDI(5)	FJ425958		DQ641574
			DI2(1)	FJ425957		
15	W-Slo	Mejame, cave, Kačiče, Divača, SI	ID(5)	FJ425990	FJ426074	DQ641573
			ME(1)	FJ425991		
16	W-Slo	Ponikve v Odolini, cave, Materija, SI	IN(1)	FJ425924	FJ426084	—
			OSO(1)	FJ425950		
			OD(2)	FJ426003		
17	W-Slo	Markov spodmol, cave, Sajevče, Postojna, SI	ID(5)	FJ425986	FJ426066	—
18	W-Slo	Kozja luknja, cave, Podtabor, Šembije, SI	KL(1)	FJ425970	FJ426067	—
			PINO(1)	FJ425969		
19	W-Slo	Jama v Mlaki, cave, Parje, Pivka, SI	PINO(3)	FJ425996	FJ426075	—
			ML(1)	FJ425995		
20	W-Slo	Novokrajska jama, cave, Novokračine, Ilirska Bistrica, SI	PINO(6)	FJ426002	FJ426090	DQ641576
21	W-Slo	Malo okence, cave-resurgence, Retovje, Vrhnika, SI	MO(1)	FJ426037	FJ426078	DQ641573
22	W-Slo	Gašpinova jama, cave, Logatec, SI	PINO(1)	FJ425949	FJ426088	DQ641576
23	W-Slo	Najdena jama, cave, Laze, Planina, SI	IN(4)	FJ425998	FJ426068	—
24	W-Slo	Škratovka, cave, Planina, SI	PINO(3)	FJ425969	FJ426077	—

Table 1 *Continued*

No.	Clade	Locality*	Haplotypes COI (no. of specimens)	COI	ITS	16S
25	W-Slo	Planinska jama, cave, Pivka (W branch), Planina, SI	CP(2)	DQ641537	FJ426087	DQ641576
			PINO(5)	FJ426009		
	W-Slo	Planinska jama, cave, Rak (E branch), Planina, SI	CP(1)	DQ641537	FJ426086	—
			PR1(1)	FJ426013		
			PR2(1)	FJ426015		
26	E-Slo	Šimenkovo brezno, cave, Stična, Grosuplje, SI	ST(3)	FJ426022	FJ426062	—
27	E-Slo	Poltarica, cave, Krka, SI	POP(2)	FJ426018	FJ426061	DQ641580
			PO(1)	FJ426017		
28	E-Slo	Podpeška jama, cave, Videm-Dobrepolje, Grosuplje, SI	POP(5)	FJ426016	FJ426059	DQ641580
29	E-Slo	Kompoljska jama, cave, Kompolje, Grosuplje, SI	KOMB(1)	FJ425967	FJ426058	DQ641583
			KOM(3)	FJ425968		
30	E-Slo	Mobi brezno v Vrtačah, cave, Ribnica, SI	KOMB(1)	FJ425997	FJ426060	DQ641580
			MBCO(4)	FJ425922		DQ641582
			KOM(2)	FJ425968		
31	E-Slo	Jelovička jama, cave, Colnarji, SI	MBCO(5)	FJ42522	FJ426064	DQ641581
			CO(1)	FJ425923		
32	E-Slo	Črničkova jama, cave, Dvor, SI	DV1(1)	FJ425946	FJ426063	DQ641580
			DV2(1)	FJ425948		
			DV3(3)	FJ425947		
33	E-Slo	Jama pod gradom Luknja, cave, Novo mesto, SI	LU1(3)	FJ425979	FJ426065	—
			LU2(1)	FJ425980		
34	E-Slo	Sušica, spring, Dolenjske Toplice, SI	DT(1)	FJ425933	FJ426055	FJ426046
35	E-Slo	Stobe, cave, črnomelj, SI	SB(2)	FJ426025	FJ426056	FJ426046
36	E-Slo	Jelševnik, spring, črnomelj, SI	JE1(2)	FJ425955	FJ426051	DQ641585
			JE2(2)	DQ641550		DQ641580
			JE3(1)	FJ425956		
37	E-Slo	Kobiljača, cave, Špeharji, SI	KB(2)	DQ641548	FJ426054	DQ641584
38	E-Slo	Jama v kamnolomu, cave, Vinica, SI	VI1(3)	FJ426029	FJ426053	—
			VI2(3)	FJ426031		
39	E-Slo	Mikašinovića pećina (= Zala), cave, Ogulin, HR	MI1(3)	FJ426039	FJ4266050	DQ641586
			MI2(1)	FJ426042		
			MI3(1)	DQ641551		
40	E-Slo	Kuruzovića pećina (= Kukuruzovićeva), cave, Rakovica, HR	KU1(1)	FJ425977	FJ426057	FJ426043
			KU2(1)	FJ425978		
41	Adriatic	Markovac, spring, Podum, Otočac, HR	OT(1)	FJ426004	FJ426104	—
42	Adriatic	Karišnica, cave, Donji Karin, HR	KA(1)	FJ425961	FJ426100	—
			KAME(1)	FJ425960		
43	Adriatic	Pećina kod Vrane, cave, Vrana, Pakoštane, HR	SI(1)	DQ641543	—	DQ641577
44	Adriatic	Bikovica, cave, Pirovac, HR	PI1(1)	FJ425918	FJ426096	DQ641577
			PI2(2)	FJ425919		
			PI3(2)	FJ425920		
			PI4(1)	FJ426007		
45	Adriatic	Izvor kod Pirovca, spring, Pirovac, HR	PI3(1)	FJ426006	—	—
			PI4(2)	FJ426007		
46	Adriatic	Mandalina špilja, cave, Šibenik, HR	SI(5)	FJ425982	FJ426097	—
47	Adriatic	Jama na Dučacu, cave, Postira, Brač, HR	BR(1)	FJ425921	FJ426098	—
48	Adriatic	Špilja kod Jurjevića, cave Pelješac, HR	PE	FJ426005	FJ429099	—
49	Adriatic	Spring near Metković, HR	MEVJ(1)	FJ425993	FJ426095	FJ426044
			KAME(1)	FJ425994		
50	Adriatic	Vjetrenica, cave, Zavala, Popovo polje, BA	VJ(2)	FJ426034	FJ426093	DQ641579
			MEVJ(1)	FJ426993		
51	*T. bosnica*	Suvaja pećina, cave, Lušci Palanka, BA	SP(1)	FJ426027	—	DQ641588
			DPSP(2)	DQ641554		
52	*T. bosnica*	Dabarska pećina, cave, Sanski most, BA	DP(1)	DQ641555	FJ426105	DQ641589
			DPSP(1)	DQ641554		

*country abbreviation codes: SI (Slovenia), IT (Italy), HR (Croatia), BA (Bosnia and Herzegovina).

sampled especially thoroughly in order to check for the co-occurrence of sympatric sibling species in that area, as proposed by an earlier allozyme study (Cobolli Sbordoni *et al*. 1990).

DNA extraction, amplification and sequencing

Pieces of muscles were cut of the dorsal part of the shrimp pleon and the rest of the animal was stored as a voucher for morphometrical analysis. Genomic DNA was isolated using the GenElute Mammalian Genomic DNA from Sigma-Aldrich. The nearly complete cytochrome oxidase I (COI) was amplified for 173 specimens using primers LCO 1490 (Folmer *et al*. 1994) and the newly designed COIdes2 5′-TTTCANGNWGTGTANGCGTCTGG-3′. From the specimens already included in the previous study (Zakšek *et al*. 2007), a shorter but overlapping COI fragment was used. On the basis of divergent COI haplotypes, we selected specimens for further 16S and ITS2 amplification and sequencing. Primers and polymerase chain reaction (PCR) procedure for 16S are described in Zakšek *et al*. (2007). The ribosomal internal transcribed spacer ITS2 was amplified for 59 specimens using a combination of the newly designed primer 5′-TTGATCATCGACACTTCGAACGCAC-3′ and ITS4 described by White *et al*. (1990).

PCR was performed using the following cycling settings: 45 s at 94 °C, 60 s at 48 °C and 120 s at 72 °C, for 34 cycles followed by 72 °C for 3 min for COI (LCO and COIdes2 primers) and 45 s at 94 °C, 45 s at 50 °C and 90 s at 72 °C, for 35 cycles followed by 72 °C for 3 min for ITS2. PCR products were purified using the Multiscreen PCR (Millipore) according to the manufacturer's instructions. Each fragment was sequenced in both directions using PCR amplification primers by Macrogen. Additional sequencing primers were used for COI to reach complete sequence overlap: HCO (Folmer *et al*. 1994) and the newly designed primer COIlev3 5′-TTTGGTCACCCAGAAGTGTAT-3′. Contigs were assembled and edited using ChromasPro 1.32 (Technelysium Pty). Sequences were aligned using Clustal_X (Thompson *et al*. 1997) for COI and Muscle (Edgar 2004) for 16S and ITS2. The correctness of the COI alignment was verified at the amino acid level. The sequences were tested for saturation using Xia's test implemented in DAMBE version 4.2.13 (Xia & Xie 2001).

Phylogenetic analyses

Phylogenetic analyses were carried out on each data set (COI, 16S, ITS2 and total) using maximum-likelihood (ML), maximum parsimony (MP) and Bayesian methods. In order to reduce computing time, only unique haplotypes were used for phylogenetic analyses. MP analyses were conducted with heuristic searches using random sequence addition with 100 replicates and tree-bisection–reconnection (TBR) branch swapping in PAUP* version 4.0b10 (Swofford 2002). MP bootstrap analyses were carried out using 500 bootstrap pseudoreplicates. A general time-reversible model with a proportion of invariant sites and a gamma distribution of rate heterogeneity (GTR + I + Γ) assuming six discrete gamma categories was selected for the combined data set to be the most appropriate using the program ModelTest 3.7 (Posada & Crandall 1998). Bayesian analyses were performed using the program MrBayes 3.1 (Ronquist & Huelsenbeck 2003). For each dataset, Markov chain Monte Carlo (MCMC) searches were run with four chains for 2×10^6 generations, sampled every 100 generations. The burn-in was graphically determined from the MS Excel plot of the likelihood values of the trees. The trees visited by the chains before the likelihood values reached a plateau were discarded as burn-in. The remaining trees were used to calculate posterior probabilities. The ML phylogeny was obtained using PHYML (Guindon & Gascuel 2003). All parameters of the nucleotide substitution model and the gamma shape parameter were simultaneously estimated during the ML search. The robustness of the topology was tested with 1000 bootstrap replicates. Monophyly of the major cave shrimp phylogroups was tested on the total data set (COI, 16S, and ITS2 combined) by comparing the most likely tree against the best tree with the group of interest constrained to nonmonophyly using the approximately unbiased (AU) test proposed by Shimodaira (2002) implemented in the CONSEL program (Shimodaira & Hasegawa 2001).

A likelihood ratio test for *Troglocaris anophthalmus* did not reject the clock-like behaviour of the COI sequences, permitting the use of a global-clock approach to provisionally date splitting events. As available rates of divergence for quite closely related shrimp taxa differ substantially, e.g. 1.4% (divergence rate per million years) for *Alpheus* (Knowlton & Weigt 1998), 1.3–5.2% for the atyid *Stygiocaris* (Page *et al*. 2008), and up to ~20% for the atyid *Halocaridina* (Craft *et al*. 2008), we only attempted to estimate the order of magnitude of divergence time. We tentatively took the Knowlton & Weight (1998) rate, which is the most commonly used rate for decapods.

Haplotype networks and nested clade analysis

All analyses reported in this section were performed on the COI data set (610 bp) that was available for all sampled individuals. ML, MP and BI all reconstruct the evolutionary history of sequences under assumption that a tree best represents their relationships. However, intraspecific data usually consist of many very similar sequences, some of which may be ancestral, and whose phylogenetic relationships are therefore often more clearly and accurately represented by networks (Posada & Crandall 2001). We used Network version 4.5.0 to create a median-joining network

(MJN), available at www.fluxus-engineering.com. The method is particularly suitable for sequence data sets with incomplete geographical coverage and larger genetic distances (Bandelt *et al*. 1999). Equal weights were assigned to all positions and the homoplasy level parameter (ε) was set to zero. Furthermore, to provide insight into historical processes of major phylogroups, we employed a nested clade analysis (NCA; Templeton *et al*. 1995; Templeton 1998) of the two major and thoroughly sampled clades designated W-Slo and E-Slo. The relationships among populations were visualized, and networks constructed using the program TCS 1.21 (Clement *et al*. 2000), which utilizes the cladogram estimation algorithm of Templeton *et al*. (1992). The analysis was conducted using default settings, which provide a 95% parsimoniously plausible branch connection between haplotypes. In resolving ambiguous connections, we followed the criteria described by Crandall & Templeton (1993) and Templeton & Sing (1993). Clades were nested using standard nesting rules (Templeton *et al*. 1987; Crandall 1996), and a nested clade analysis was computed using the program GeoDis version 2.5 (Posada *et al*. 2000) to test for significant associations between geographical locations and genetic distances over 5000 random permutations. For the geographical analysis, latitude and longitude coordinates at the cave entrance were used. Localities were mapped with the help of the program package ArcGIS version 9.1 (ESRI). Inferences were based on statistically significant distances within nesting clades and geographical distribution of clades using Templeton's key available at http://darwin.uvigo.es/download/geodisKey_11November 2005.pdf. There is ongoing debate in some recent works and comments that have cautioned against the uncritical use of the NCA with a single gene tree, and which discuss about the usefulness of NCA (Knowles 2004; Panchal & Beaumont 2007; Garrick *et al*. 2008; Petit 2008; Templeton 2008). However, NCA still offers a powerful tool to explore patterns related to population history and complex historical scenarios. We tried to minimize the danger of mis- or over-interpreting our results by using several other analytical approaches besides NCA, as well as independent molecular loci.

Historical demography

Historical demography was inferred taking three different approaches. Demographic analyses were carried out separately for each of the major phylogroups recognized by phylogenetic analyses described above. Nucleotide (π) and haplotype (h) diversity estimates for the cave shrimp phylogroups were calculated according to the method of Nei (1987) using the program DnaSP version 4.10 (Rozas *et al*. 2003). We also investigated historical patterns of population structure using the mismatch distribution of pairwise nucleotide differences between haplotypes in three main genetically and geographically distinct groups (W-Slo, E-Slo and Adriatic) using the same program. If the population has undergone rapid expansion, a unimodal mismatch distribution approximating Poisson curve is expected (Rogers & Harpending 1992), whereas populations approaching mutation–drift equilibrium are expected to produce a multimodal mismatch distribution. For each phylogroup, theoretical distribution under the assumption of constant population size and the sudden expansion model were compared to the observed data. Additionally, the possible occurrence of past demographic expansions was investigated by neutrality statistics Tajima's D (Tajima 1989), Fu's F_S (Fu 1997) and R_2 (Ramos-Onsins & Rozas 2002). The latter has been shown as the most powerful test statistics for detecting population growth (see Ramos-Onsins & Rozas 2002 for review). These statistics were evaluated using DnaSP by running 10 000 coalescent simulations.

A Mantel test was performed using Alleles in Space (AIS; Miller 2005) to test for isolation by distance (IBD; Wright 1943) by calculating the correlation coefficient between two pairwise matrices of genetic (calculated as uncorrected (p) distances between sequences and geographical distances within and between phylogroups). Geographical distances between sampling localities were determined from latitudinal and longitudinal coordinates at the cave entrance.

Results

Phylogroups

A total of 610 base pairs of the COI fragment were obtained from each of the 232 *Troglocaris anophthalmus* and *Troglocaris bosnica* specimens included in the study. Out of these, 173 specimens were sequenced for additional 625 bases of COI, altogether 1235 bases. The lengths of the other two alignments used for further analyses were: 836 bases of ITS2 rDNA and 472 bases of 16S rDNA. From the shorter part of COI, 72 unique haplotypes were identified. Thirty-seven COI haplotypes were sampled more than once while the other 35 occurred as singletons (Table 1). Approximately one half of the sampled haplotypes were site specific, and the other half were shared between at least two populations. Among individuals with the most divergent COI haplotypes, we selected and sequenced 59 specimens (47 localities) for ITS2 and 58 specimens (31 localities) for 16S rDNA. Twenty-six different ITS2 sequences and 32 16S haplotypes were found.

The major tree topologies with four big phylogroups: *W-Slo*, *E-Slo*, *Adriatic* and *Soča* (Fig. 2) were identical with all data sets (COI, 16S, ITS2 and total). To avoid ambiguity, the names of the phylogroups already tentatively identified by Zakšek *et al*. (2007) were kept (W-Slo, E-Slo and Adriatic). The W-Slo phylogroup, which is the most basal one, includes populations distributed in the Italian Carso,

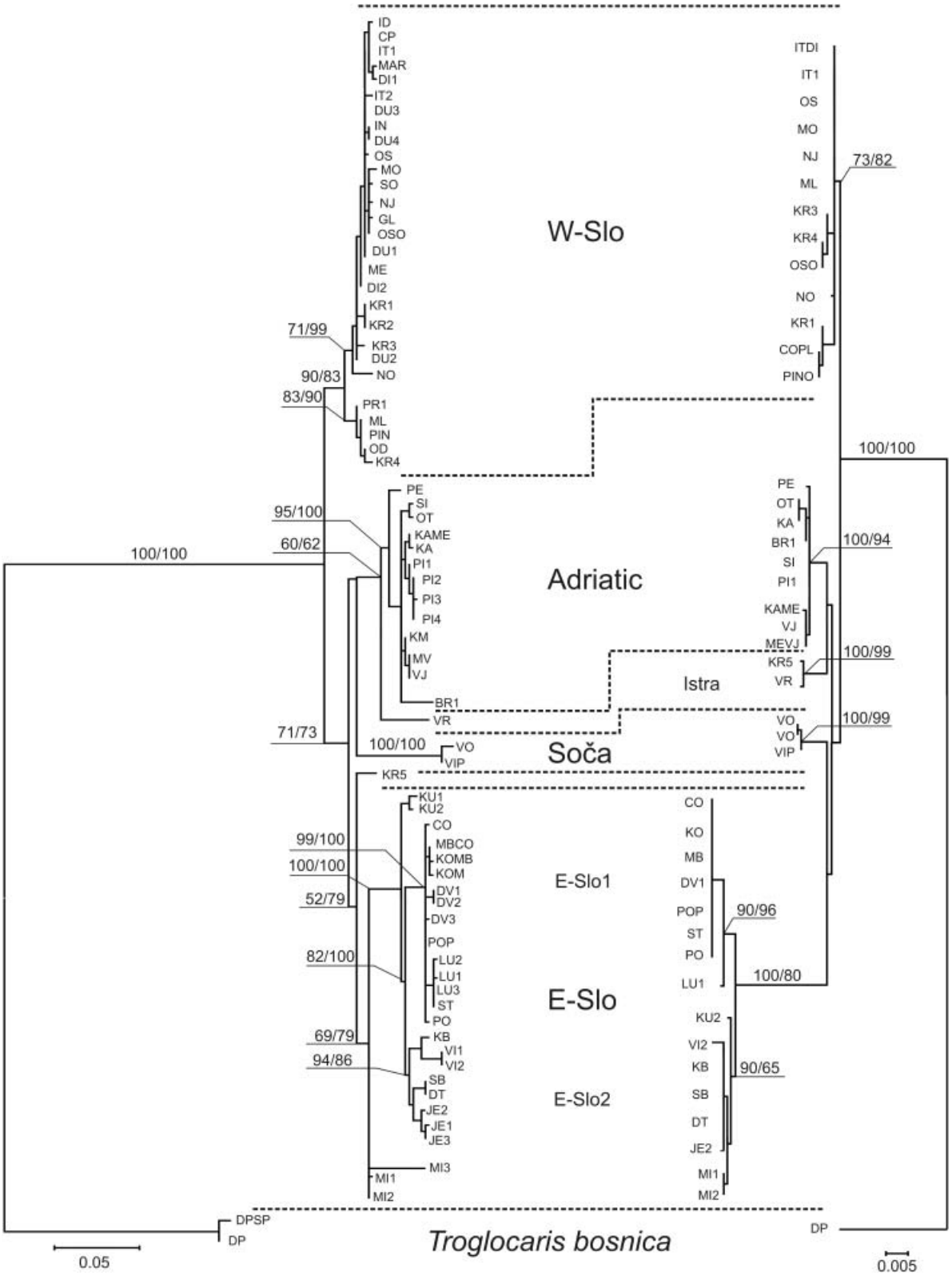

Fig. 2 Phylogenetic relationships within *Troglocaris anophthalmus* derived by maximum likelihood based on COI (left tree) and ITS2 (right tree) sequences under the GTR + I + γ model of evolution. The topology of the 16S rRNA tree is concordant with the COI topology, but less resolved and therefore not shown. Numbers above main branches indicate nonparametric bootstrap values of main splits based on 1000 pseudoreplicates for (1) likelihood and (2) posterior Bayesian probabilities in per cent.

southwestern and central Slovenia, and the northern Istra Peninsula; the E-Slo phylogroup contains populations from southeastern Slovenia to Kordun in Croatia; the Adriatic phylogroup extends from Lika along the Adriatic Sea coast to southern Herzegovina; the Soča phylogroup is restricted to the Soča and Vipava river basins in western Slovenia (north of the W-Slo). Although the four phylogroups were consistently recovered with all three gene fragments and different tree-building methods (Fig. 2), the relationships between them were weakly supported. The monophyly tested by the AU test (Shimodaira 2002) proved significant ($P < 0.05$) for three phylogroups: W-Slo, E-Slo and Adriatic (Table 3). For the Soča phylogroup, the result was significant according to the approximation of posterior probabilities by the BIC and close to significance by the *P* value of the AU test. Moreover, detailed sampling recovered a further split of the E-Slo phylogroup into two subgroups: E-Slo1 and E-Slo2. All phylogroups and subgroups are geographically associated (Fig. 3). Another newly discovered, putative phylogroup (*Istra*) was supported by ITS2 sequences (Fig. 2), and is geographically limited to the Istra Peninsula. Conversely, it could not be recovered by the COI phylogenetic tree (Fig. 2; Table 3). The small sample size of only two specimens renders conclusions about a possible fifth phylogroup unreliable.

In the network analysis, the most parsimonious ($\varepsilon = 0$) median-joining network contained 31 median vectors (Fig. 3). The four phylogroups (see above) were separated from one another by more than 13 mutational steps. The W-Slo phylogroup — basal in the phylogenetic tree — was closest to the outgroup. The locality Kršan in Istra was the only one where we found the co-occurrence of representatives of two phylogroups: W-Slo and Istra. It should be noted that branch lengths of the phylogenetic tree and the network are not directly comparable because the latter is based on positions with binary states only. Many haplotypes occupied intermediate positions, suggesting the persistence of ancestral or close-to-ancestral haplotypes. The median-joining network for the W-Slo phylogroup (Fig. 3) showed star-like relationships which are suggestive of past demographic expansion (Slatkin & Hudson 1991).

Corrected divergence of the COI (short part) sequences between cave shrimp phylogroups ranged from 4.2 to 5.8%. A generalization of shrimp divergence rates (see methods) to our cave shrimp phylogroups suggested that they separated later than all other Dinaric cave shrimp taxa (Zakšek *et al*. 2007), probably during Pleistocene.

Genetic diversity and divergence

Genetic variation within the four major phylogroups is summarized in Table 2. The nucleotide diversity (π) among all sequences was relatively low. There were only slight differences in the level of genetic variation between phylogroups and no particular geographical trend was observed in the sequence variation among these samples, except for the high nucleotide diversity within the Istra phylogroup and the population from Mikašinovića pećina (Ogulin, Croatia).

Isolation by distance tested for the total data set and for the large phylogroups (W-Slo, E-Slo and Adriatic) separately, showed a significant positive correlation of genetic and geographical distances only for the total data set (Mantel test, $r = 0.37$, $P = 0.001$, 10 000 randomizations). Genetic and geographical distances were not (W-Slo, Adriatic) or were only slightly (E-Slo) correlated within each phylogroup. It was only in the E-Slo phylogroup that the test of IBD was close to significant ($r = 0.24$; $P = 0.083$). These results indicate that there is no significant IBD within phylogroups, and hence, no stable genetic substructure with limited gene flow. Conversely, significant IBD within the entire *T. anophthalmus* range can be attributed to the fact that mutually completely isolated phylogroups occupy geographically remote areas, i.e. complete cessation rather than restriction of gene flow.

Table 2 Mitochondrial COI genetic diversity in geographical areas of the cave shrimp *Troglocaris anophthalmus* along the Dinaric Karst

Geographical area	No. of specimens	No. of haplotypes	*h*	π	π/d
W-Slo	131	21	0.878	0.004	0.005
E-Slo	55	21	0.890	0.013	0.010
Adriatic	25	11	0.910	0.009	0.003
Istra	2	2	1.000	0.026	0.066
Soča	6	2	0.600	0.007	0.021
Dinaric Karst (total)	232	60	0.955	0.033	0.007

π/d, nucleotide diversity per 100 km (d, maximum diameter of geographical area).

Table 3 Results of the approximately unbiased test supporting the monophyly of *Troglocaris anophthalmus* phylogroups

Phylogroup	AU	BIC
W-Slo	0.026	< 0.001
E-Slo	0.030	< 0.001
Adriatic	0.043	< 0.001
Soča	0.156	0.001
Istra	0.512	0.025

AU, *P*-value of the Approximately Unbiased test; BIC, approximation of the posterior probability of the same test.

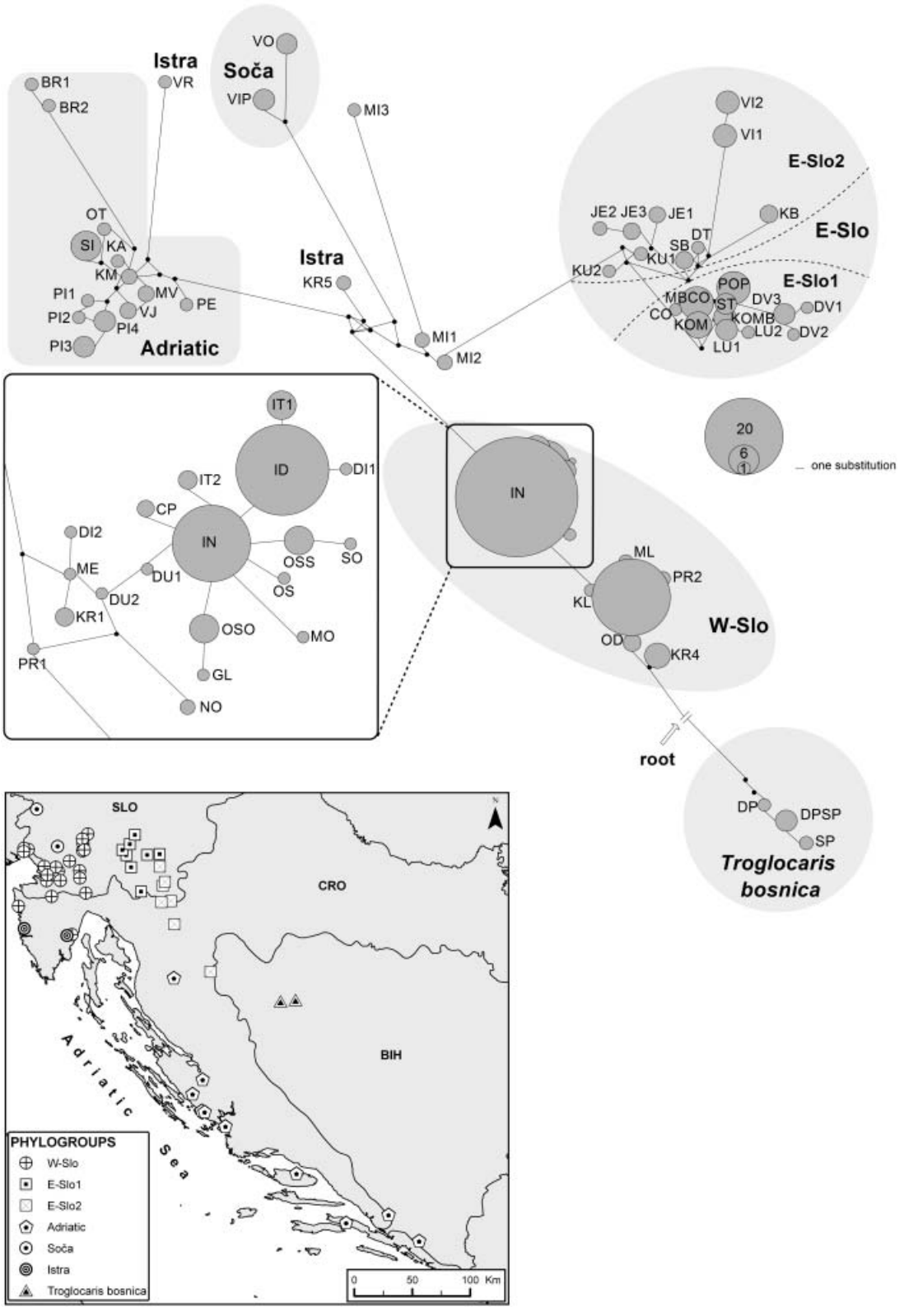

Fig. 3 Most parsimonious median-joining network (ε = 0) for *Troglocaris anophthalmus* COI haplotypes. The size of the circles is proportional to the frequencies of the respective haplotype. Black dots symbolize median vectors that represent hypothetical missing or unsampled haplotypes. The arrow indicates the position of the root as identified by the phylogenetic analysis. Between *Troglocaris bosnica* and the rest, 46 haplotypes are missing. The distribution map of phylogroups is included.

Table 4 Inferred evolutionary processes from NCA (nested contingency results from GeoDis). Only clades with significant values are represented

	Chi-squared	Probability	Inference chain	Inferred pattern
W-Slo haplotype network				
1-1	22.075	0.010*	No significant values	
1-5	58.703	0.002*	No significant values	
2-6	249.410	0.000*	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but with some long-distance dispersal
3-1	130.502	0.000*	1-2-11-12-No	Contiguous range expansion
3-3	28.000	0.000*	1-2-11-12-No	Contiguous range expansion
Total	142.959	0.015*	1-2-3-4-No	Restricted gene flow with IBD
E-Slo haplotype network				
2-2	11.000	0.008*	1-19-No	Allopatric fragmentation
3-1	16.000	0.000*	1-19-No	Allopatric fragmentation
3-6	16.000	0.000*	1-19-No	Allopatric fragmentation
4-1	32.000	0.000*	1-19-No	Allopatric fragmentation
4-2	10.000	0.021*	1-19-No	Allopatric fragmentation
4-3	13.000	0.001*	1-19-No	Allopatric fragmentation
Total	94.273	0.000*	1-2-3-4-9-10-Yes	Allopatric fragmentation

*indicates significance at the $P < 0.05$ level.

Nested clade phylogeographical analysis

To uncover the major historical processes and patterns, nested clade analysis (NCA) was used. NCA was conducted only on the northern part of the range (W-Slo and E-Slo) where the sampling was dense enough. Due to the large genetic divergence between haplotypes in the network (see Fig. 3) and the fact that no ancestral haplotypes were found between the well-sampled W-Slo and E-Slo phylogroups, subsequent NCAs were conducted for both regions separately. The NCA of the W-Slo *T. anophthalmus* populations, including 27 haplotypes from 23 localities, revealed 14 1-step clades, six 2-step clades and three 3-step clades (Fig. 4). The enigmatic KR5 haplotype was excluded from the network by the 95% parsimony plausibility criterion. The most frequent ancestral haplotype is haplotype IN connected by several independent mutational steps to more localized ones. Therefore, this part of the W-Slo network is showing a star-like pattern, which is characteristic of recent range expansions from a small number of founders (Avise 2000). The E-Slo population network, connecting 20 haplotypes from 14 localities, revealed 14 1-step clades, nine 2-step clades, six 3-step clades and three 4-step clades (Fig. 4). Within the E-Slo network, two divergent groups of haplotypes separated from one another by 11 steps were recognized. According to the 95% parsimony plausibility criterion, haplotypes from Mikašinovića pećina were not connected to other haplotypes in the E-Slo network. The two sets of haplotypes (E-Slo1 and E-Slo2) are found in geographically separated areas (Fig. 3) and were also supported as two separate groups by the independent nuclear ITS2 marker. Haplotypes DV1-KOM were found exclusively in the northwestern part of the range, while other haplotypes from this area are found exclusively in the southern part of the range. The E-Slo network is characterized by long branches between haplotypes, and haplotypes from this area are much more localized and narrowly distributed as compared to haplotypes in the W-Slo network. The fact that no haplotypes were shared between the W-Slo and E-Slo network suggests that the populations of the cave shrimps from both phylogroups had independent evolutionary histories for a relatively long time. The evolutionary processes inferred from the nested contingency test revealed significant correlation with geography (Table 4). Within the W-Slo haplotype network two of the nested clades showed contiguous range expansion while the total W-Slo network was best explained by restricted gene flow with isolation by distance. Historical routes in the W-Slo network using the 3-step clades indicated contiguous range expansion with some long distance dispersal and restricted gene flow followed by secondary contact between Pivka and Reka drainages (Fig. 6). The haplotype PR1 sampled in the Postojna-Planina Cave System (PPJS) is connecting both 3-step clades. Within the E-Slo network, allopatric fragmentation is the only historical process inferred from the NCA (at all nesting levels) (Table 4). Strong clustering of haplotypes per geographical region (Fig. 6), particularly when the haplotype clusters are separated by long branches with missing intermediates, can be considered as evidence of past fragmentation (Templeton 1998).

The haplotype network for the Adriatic phylogroup was not further investigated using NCA due to the small

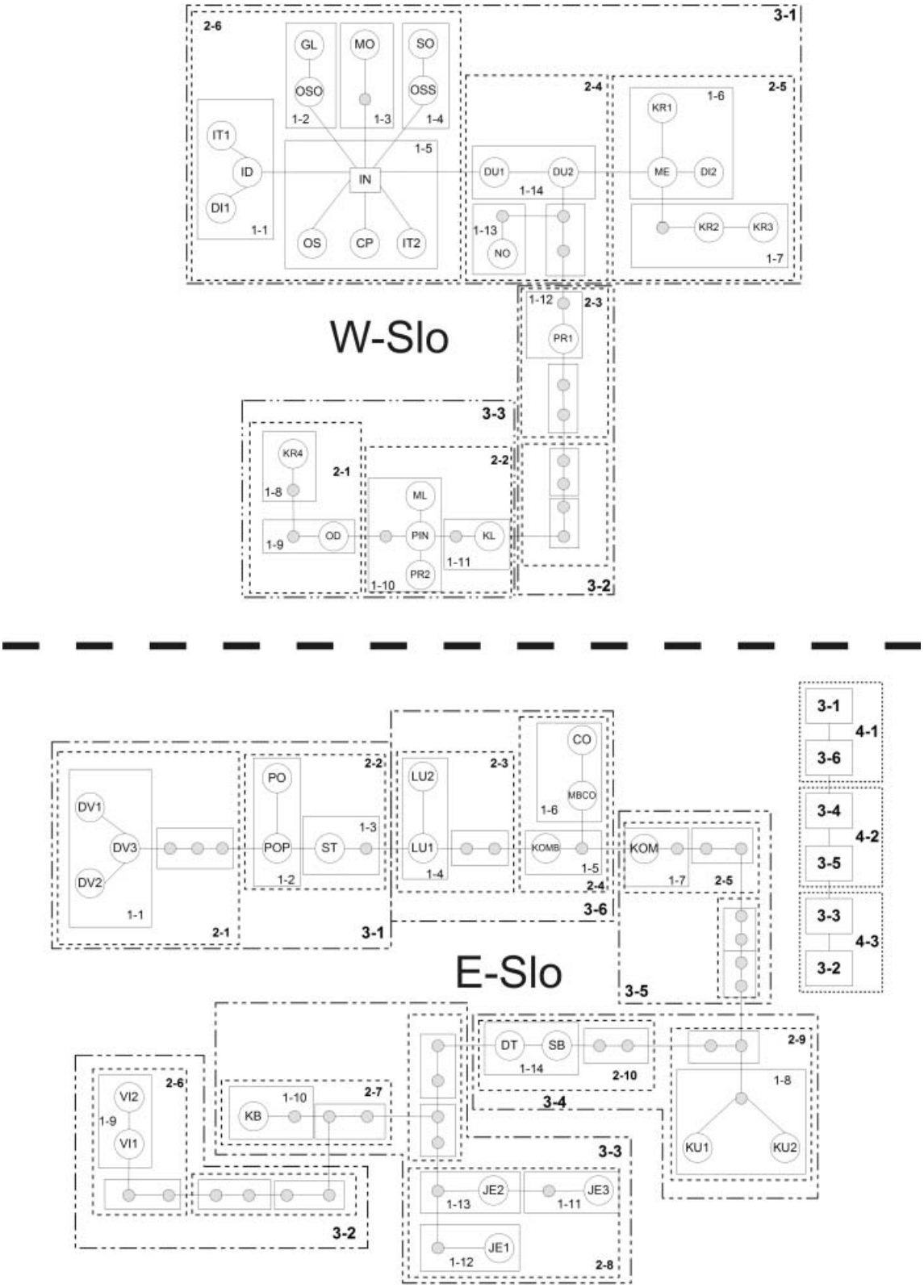

Fig. 4 Haplotype networks depicting the nesting levels used to infer historical processes in the cave shrimp populations from W-Slo and E-Slo phylogroups. Haplotype names and numbers are as per Table 1. Haplotype with the highest outgroup probability according to the TCS analysis (Clement *et al*. 2000) are depicted by rectangles. Grey circles represent missing haplotypes. Note that in spite of variable branch lengths, each branch represents a single mutational step.

number of samples and ambiguous connections. The Adriatic phylogroup has the greatest distribution area within *T. anophthalmus* with the shortest genetic distance between haplotypes from localities extending 310 kilometres (from Otočac to Popovo polje). In general, genetic distances between sampled haplotypes within the Adriatic clade are shorter, and geographical distances between sampled localities are much longer than in any other phylogroup. For example, the haplotypes from caves Karišnica and Vjetrenica (235 kilometres apart) are separated by only one mutational step. For comparison, in the W-Slo phylogroup, also showing some widespread haplotypes, the longest distance between localities with the same COI haplotype is approximately 50 kilometres.

Demographic history

Considerable genetic differentiation between three major phylogroups led us to evaluate historical demography for each group separately. None of the mismatch distributions (Fig. 5) followed a Poisson distribution exactly, with no clear signs of recent demographic expansions. A rough fit was observed for the Adriatic phylogroup pointing to a possible recent demographic expansion. The bimodal mismatch profile for the E-Slo phylogroup is probably a consequence of restricted gene flow between E-Slo1 and E-Slo2, already indicated by all preceding analyses (Figs 2 and 3).

Tajima's test only showed a significant deviation from neutrality in the Soča phylogroup ($D = 2.153$, $P < 0.05$), providing evidence of either selection acting on the sequences analysed, rate heterogeneity, population growth or bottleneck effect. The F_S and R_2 statistics provided no evidence of possible population expansion for any of the main phylogroups.

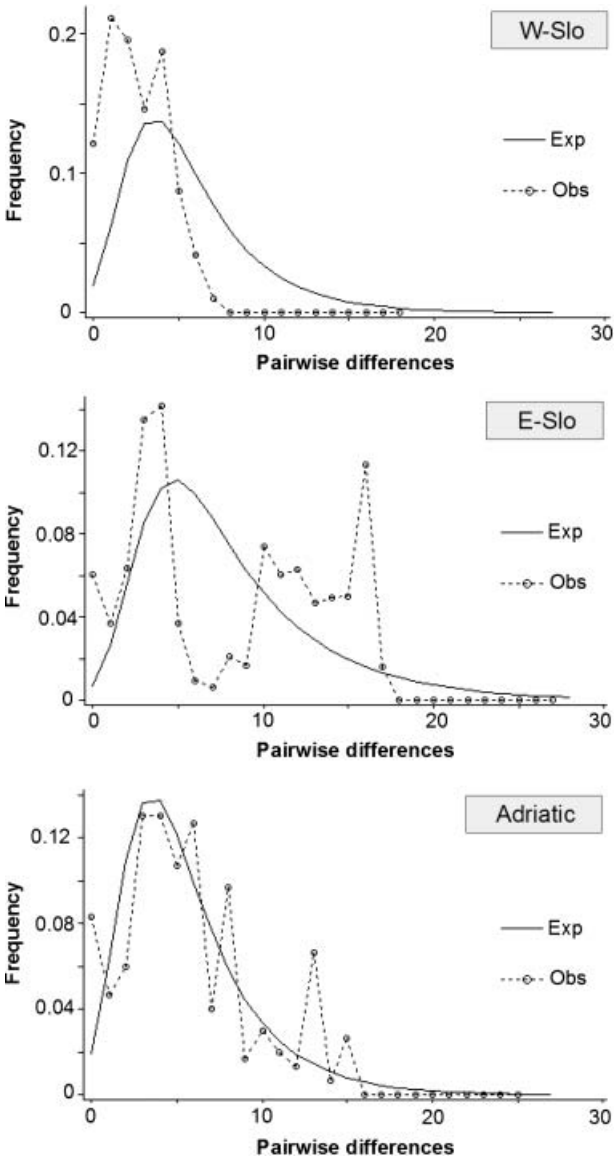

Fig. 5 Mismatch distribution histograms for the 'short' COI haplotypes from the three main phylogenetically and geographically defined *Troglocaris anophthalmus* phylogroups. Dotted lines represent the actual distribution of pairwise differences, and solid lines the frequency distribution expected under the hypothesis of population expansion.

Discussion

Phylogeographical patterns and demographic events

With this study, it became evident that the picture of a widespread, continuously distributed, homogenous and uniform *Troglocaris anophthalmus* was wrong. The geo-hydrographical factors causing extreme fragmentation in all other obligate subterranean species scrutinized so far in the Dinaric Karst and other karst areas worldwide (e.g. Verovnik *et al*. 2004; Lefébure *et al*. 2006a; Finston *et al*. 2007), are apparently universal enough to have acted also upon *T. anophthalmus*. In the following, we consider some possible scenarios of ecological and evolutionary events that might have lead to the observed complex patterns.

Remarkably, contrasting phylogeographical patterns and evolutionary processes were identified within a small geographical area in the northern Dinaric Karst occupied by the W-Slo and E-Slo phylogroups. While contiguous range expansion (CRE) followed by periods of isolation by distance (IBD) appear to be the main mechanism within the W-Slo phylogroup, allopatric fragmentation has shaped the genetic structure of E-Slo (Table 4). Two inferred centres from which CRE might have started are the large underground rivers Reka/Timavo (clade 3-1) and Pivka (clade 3-3). The haplotype PR1 connecting both 3-step clades is pointing to an area of secondary contact in the world-famous Postojna-Planina Cave System, where haplotypes of all 3-step clades can be found (Fig. 6, Table 4). The

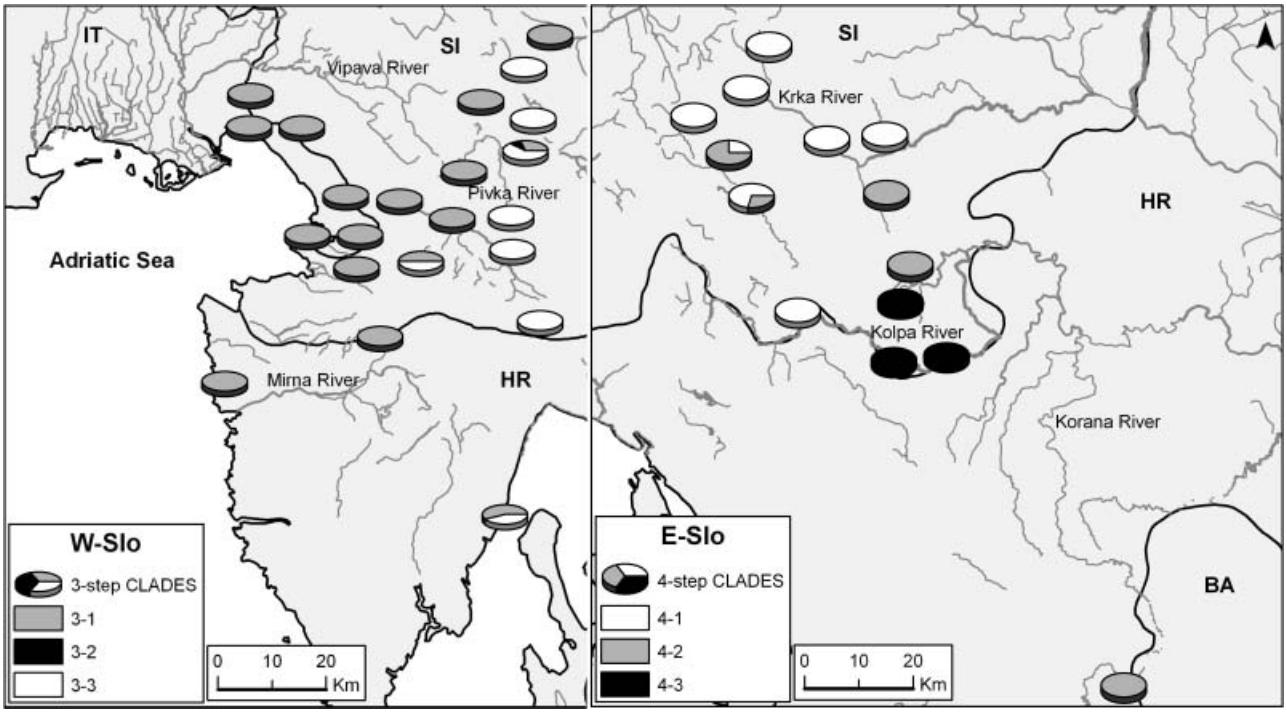

Fig. 6 Geographical distribution of higher-level clades for the W-Slo and E-Slo phylogroups inferred from nested clade analysis. Mixed pie charts symbolize sites of secondary contact with sympatrically occurring higher level clades.

Reka/Timavo River is draining to the Adriatic Sea, whereas the Pivka, including the Postojna-Planina Cave System, belongs to the Black-Sea drainage. The two drainages have distinct surface fish and invertebrate faunas, whereas the distribution of subterranean aquatic fauna seems to be in conflict with the obvious ecological boundary. This unique biogeographical disagreement between epigean and subterranean freshwater faunas was first suggested by the phylogeography of subterranean isopods (Verovnik *et al*. 2004). The most unexpected finding, however, was that of a distinct phylogroup (Soča) in the northern part of the Adriatic Sea drainage, despite its hydrological interconnection with the Pivka (Black Sea) drainage. The split between Soča and W-Slo phylogroups is phylogenetically and chronologically much older than any of the events within W-Slo. Put together, these three observations — (i) hydrological connection, (ii) gene flow in younger phylogroup, (iii) unique genetic setup of Soča phylogroup — represent circumstantial evidence of a biological reproductive barrier between both phylogroups.

Haplotypes of the E-Slo phylogroup are much more localized as compared to W-Slo, although their ranges are similar in size. The inferred patterns of allopatric fragmentations are partly in agreement with the extant hydrology of the two Dinaric basins of rivers Krka and Kolpa. This subdivision corresponds to the two subphylogroups E-Slo1 and E-Slo2, respectively. Altogether, the phylogeographical pattern of the E-Slo phylogroup matches our current view of how subterranean biodiversity is shaped. Vicariant fragmentation of habitat, associated with reduced or absent dispersal is the biogeographical interpretation most often invoked during the past decades of intensive research on subterranean fauna (review in Porter 2007).

A contrasting pattern has been found for the Adriatic phylogroup. Here, the same COI haplotype has been discovered in populations 200 kilometres apart. There can be hardly another explanation to this than dispersal. Yet, the two populations belong to the drainages of two Adriatic rivers that are at present completely separated. In addition, the mismatch distribution indicated the possibility of recent demographic expansion for the whole Adriatic phylogroup. Dispersal between Otočac (Croatia) and Trebinje (Bosnia Herzegovina) needs to be explained, but no continuity of karstic groundwater or superficial freshwater exists. An explanation can be sought in the combination of the specific geomorphology of the Dinaric Mountains in combination with karst hydrology. The limestone ridges in which the caves with *T. anophthalmus* habitat were formed are narrow (down to a few kilometres) and long (up to about 100 kilometres), stretching in a southeast–northwest direction. Between the ridges, there are flat depressions (poljes) on insoluble ground, often traversed by sinking rivers, and periodically flooded. Their hydrology is highly dynamic, changing within historical time, and extreme floods can

Table 5 Species delimitation criteria (according to Wiens & Penkrot 2002; Sites & Marshall 2004) met by different *Troglocaris anophthalmus* phylogroups

Criterion	Phylogroup				
	W-Slo	E-Slo	Adriatic	Soča	Istra
Haplotype exclusivity	Yes	Yes	Yes	Yes	Yes
Mutual monophyly (AU)	Yes	Yes	Yes	No	No
Concordance (mitochondrial vs. nuclear DNA)	Yes	Yes	Yes	Yes	No

lead to local water level upsurge of several tens of metres (Nikolić 1972), temporarily levelling out the water table between two adjacent poljes and the caves in the ridges between. During periodical floods, aquatic cave animals are frequently washed out of their subterranean habitat and can survive for several days. On such occasions, they could reach caves at other parts of a polje and eventually reach a subterranean connection to an adjacent polje. The flooded poljes and interconnecting subterranean passages could act as occasional stepping stones where no permanent hydrological connection exists. The range of the Adriatic phylogroup is long and narrow, tightly associated with a series of large poljes and combination of geomorphology and hydrology like this cannot be found in the ranges of other phylogroups. It might be the unique explanation for a rare case of a groundwater animal with a range of several hundred kilometres.

Cryptic diversity and implications for taxonomy

An accurate delimitation of species is essential as species are basic units for distributional and habitat studies in biodiversity assessment, and provide the framework for conservation strategies. The four major phylogroups of the cave shrimp *T. anophthalmus* (W-Slo, E-Slo, Adriatic and Soča) inferred from both mitochondrial and nuclear DNA (Fig. 2) should be delimited as separate species according to different species delimitation criteria (e.g. Wiens & Penkrot 2002; Sites & Marshall 2004) as summarized in Table 5. Moreover, these phylogroups could be treated as species as they are in agreement with all four aspects of genealogical concordance species concept (see Avise 2000). Sympatry of two phylogroups in Istra indicates that we also could consider them as biological species. Nevertheless, our COI patristic distances between phylogroups are much lower (0.05–0.08) than the patristic COI distance of 0.16 substitutions per nucleotide position found to optimally separate intra-from interspecies divergence in many other crustaceans by Lefébure *et al*. (2006b). In any instance, we are dealing with distinct 'evolutionary significant units' (ESUs) for conservation (Moritz 1994).

At present, it is still unclear whether an accurate and consistent definition based on morphological characters can be found for the four phylogroups. Failing to do so would mean that we have a true case of cryptic species diversity, and not just 'overlooked diversity' as happens often to be the case after molecular hints aid the search for diagnostic morphologies, for example in some other cave shrimp species (Sket & Zakšek 2009). Morphometric studies report on high variability within *T. anophthalmus* populations and phylogroups (e.g. Fabjan 2001; Jure Jugovic, personal communication). The lack of morphological characters in subterranean cryptic species is often explained by convergence in the extreme subterranean environment over long periods (Caccone & Sbordoni 2001; Lefébure *et al*. 2006b). Since the main splits found in this study are relatively young, morphological stasis following a certain degree of morphological adaptation in the ancestor might be an alternative explanation.

Although four well-supported phylogroups can be recognized within *T. anophthalmus*, multiple groups were not found in the Kras/Carso, as might be expected by the results of an earlier allozyme study that indicated the sympatric occurrence of at least two cryptic species in this area (Cobolli Sbordoni *et al*. 1990). Northeastern Italy was extensively sampled, and specimens from three of the populations analysed also by Cobolli Sbordoni *et al*. (1990) were included. All of them belong to the W-Slo phylogroup, which despite extensive sampling shows rather low haplotype diversity (Table 2). This part of our results does not support the notion of several sympatric shrimp species in the Kras/Carso area. However, close to the range of W-Slo, the new Soča phylogroup was discovered (Fig. 3). According to an entirely hypothetical scenario, at much higher groundwater levels, individuals from both phylogroups might become syntopic. Perhaps one of the species found by Cobolli Sbordoni *et al*. (1990) is identical with the Soča phylogroup.

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The authors are interested in biogeographic, evolutionary, taxonomic and conservation aspects of subterranean fauna and karst territories. Their metodological approach is multidisciplinary, combining morphological, distributional and various kinds of molecular data.
