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MORPHOMETRIC CHARACTERISTICS AND COI HAPLOTYPE DIVERSITY OF *ARCTODIAPTOMUS SPINOSUS* (COPEDODA) POPULATIONS IN SODA PANS IN HUNGARY

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Arctodiaptomus spinosus (Daday, 1891) is a characteristic species of the soda pan zooplankton in the Great Hungarian Plain. The biogeographical distribution of the species is interesting, since its range expands from the Pannonian Biogeographic region to the other side of the Carpathians, occurring in saline lakes in Eastern Anatolia, Armenia, Iran and in temporary waters in Ukraine. Our investigations focused on the morphometric characteristics and the COI haplotype diversity of four Hungarian populations in the Kiskunság area. We detected substantial morphological differences between the Böddi-szék population and the rest of the sampling sites, however considerable differences were not observable in the COI haplotypes in the populations. The 20 animals investigated for COI haplotypes belonged to the same haplotype network. Tajima's D indicated departures from the neutral Wright – Fisher population model and suggested population expansion. The genetic composition of *Arctodiaptomus spinosus* populations in the Kiskunság area is rather uniform.

Keywords: Arctodiaptomus spinosus – morphology – COI haplotypes – sodic waters

INTRODUCTION

Arctodiaptomus spinosus is a characteristic zooplankton species of alkaline waters, its westernmost distribution area reaches the Neusiedler See, Seewinkel and surrounding soda pans in Austria. It is widespread and abundant in the soda pans of the Pannonian Plain (including the Great Hungarian Plain and the Little Hungarian Plain) and it can be found on the other side of the Carpathian mountain range in Ukraine. From the Balkan Peninsula no records are available but the species is known from Eastern Anatolia, Armenia and Iran [13, 18, 30]. The species has originally been described

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based on specimens collected in Lake Fertő (Neusiedler See) and in soda pans near Bugac [9].

Soda pans are typical habitats in the Pannonian Plain and they are present in high number in the Kiskunság area, in the middle of Hungary. Soda pans are characterised by low to medium salinity with marked seasonal changes throughout the year, excess of Na^+ and HCO_3^- ions and alkaline pH (7.5–10.5). The geology and biology including composition, abundance and seasonal changes of zooplankton of the soda pans has been intensively studied previously [4, 12, 22, 27, 34]. Characteristic crustacean species in the soda pans are fairy shrimps like *Branchinecta orientalis* Sars, 1901, *Branchinecta ferox* (Milne–Edwards, 1840), *Chirocephalus carnuntanus* (Brauer, 1877), copepods like *Arctodiaptomus spinosus* and *Arctodiaptomus bacillifer* (Koelbel, 1885) and cladocerans like *Daphnia magna* Straus, 1820, *Moina brachiata* (Jurine, 1820) and *Daphnia atkinsoni* Baird, 1859. Cryptic speciation has been shown in the latter two species recently [24, 25].

Arctodiaptomus spinosus represents an important food source for aquatic bird species in the Kiskunság soda pans [2, 3] and this abundant species has a prominent role in the food web, providing a bottom-up effect and structuring aquatic bird assemblages in spring [17]. The colour of *Arctodiaptomus spinosus* is bright red due to carotenoids (G.-Tóth L. unpublished data) in the studied soda pans. These pigments serve either as UV-photoprotection in the habitats without fish predators [15], while other factors like wind-induced turbulence and crowding may play role in their accumulation [32].

The number of the detected cryptic species increased to a great extent in the recent decades [26], and cryptic species discoveries among copepod crustaceans that were previously believed to be cosmopolitan are frequent, too. Support for the presence of cryptic taxa comes from molecular analyses [8, 14, 19, 21] and crossbreeding studies [23].

With this study we aimed to investigate morphological and genetic diversity within and between *Arctodiaptomus spinosus* populations from a geographically small region (Kiskunság) in the Great Hungarian Plain. We hypothesised that this area is a diversity hotspot in the Pannonian Plain since the suitable alkaline habitats for *Arctodiaptomus spinosus* are present in high number with diverse physicochemical characteristics in the Kiskunság area within the Carpathian basin. We also aimed to reveal the possible presence of cryptic taxa in *Arctodiaptomus spinosus*, or the opposite, to prove that genetically uniform populations are present in this region.

MATERIAL AND METHODS

Our study focused on the investigation of *Arctodiaptomus spinosus* populations from four soda pans in Central Hungary in the Kiskunság area: Kelemen-szék (120 ha, N46°49’ E19°11’), Zab-szék (100 ha, N46°50’ E19°10’), Büdös-szék (30 ha, N46°52’ E19°10’) at Szabadszállás and Böddi-szék (60 ha, N46°46’ E19°08’), size data taken from Tajima [35]. Zooplankton samples were collected with random netting by a

85 µm mesh sized net from the pelagial zone of Zab-szék, Kelemen-szék, Büdös-szék and Böddi-szék (labelled wilh Z, K, BU and BO in the text) on the 4th August 2011. Samples were conserved in 95% ethanol on site and stored at 5 °C until further analysis.

Arctodiaptomus spinosus females were randomly selected from the total zooplankton samples for morphometric analysis. We measured 40 individuals from the Zab-szék, 30 individuals from the Kelemen-szék, 30 individuals form the Büdös-szék and 30 individuals from the Böddi-szék under a NIKON YS2 light microscope. The measured variables were total body length (not including the setae at the end of the furca), body width (measured at the widest part of the first segment), length of the abdomen (measured from the genital segment to the end of the furca), body length (subtraction of the abdomen length from the total body length), length of the genital segment (measured alongside the longitudinal axis of the body), width of the genital segment (measured under the lateral spines), furca length, furca width, length of the inner seta on the furca, length of the outer seta on the furca, furthermore the number of carried eggs and the number of spermatophores attached to the females.

We performed Shapiro–Wilk normality tests on the data to check for parametric assumptions. Normally distributed data were further analysed by ANOVA and Tukey's test to discriminate between groups. Data that did not meet the assumption of normality were analysed by the Kruskal–Wallis test. All morphometric statistical analyses were performed in R [29].

For molecular analysis, adult females without eggs were randomly picked, but if the female carried eggs these were removed by needles from the individuals under a stereo microscope before proceeding to the DNA isolation. Total genomic DNA from single individuals was isolated using the H3 method [33]. Prior to DNA isolation, animals were soaked in TE buffer overnight. We used 50 µl H_3 buffer in a grinded glass microtube to squash the animals gently with a grinded glass pestle. After this, the homogenate was transferred into a 2-ml microcentrifuge tube and the grinded glass tube and pestle were flushed into the same microcentrifuge tube with an additional 50 µl H_3 buffer. We added 20 µl proteinase-K (Fermentas, 18.5 mg/ml) to each homogenate and incubated the mixture at 60 °C with mild shaking for approximately 24 hours. Proteinase-K was denaturated by placing the tubes into a 95 °C waterbath for 10 minutes. PCR reactions to amplify the mitochondrial cytochrome oxidase I (COI) region were performed in 2 × 25 µl volumes with 10 µl of the DNA template, 5.775 µl sterile milliQ water, 2.5 µl 10× buffer with 20 mM $MgCl_2$, 3.125 µl 2 mM dNTP mix, 1.75 µl 5 µM primer each and 0.1 µl 5 U/µl DreamTaq polymerase (Fermentas). The universal primers HCO1490 and LCO2198 [10] were used in the reactions. Settings for the amplification were: 94 °C – 1 min, 40 cycles (94 °C – 1 min, 42.9 °C – 1 min 30 sec, 72 °C – 1 min 30 sec) and 72 °C – 6 min.

Following the PCR reactions, products were cleaned (Roche Diagnostics, High Pure PCR Product Purification Kit) and sequencing was carried out from both directions according to the manufacturer's instructions by using BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 Genetic Analyser with the same primer pair that was used in the PCRs.

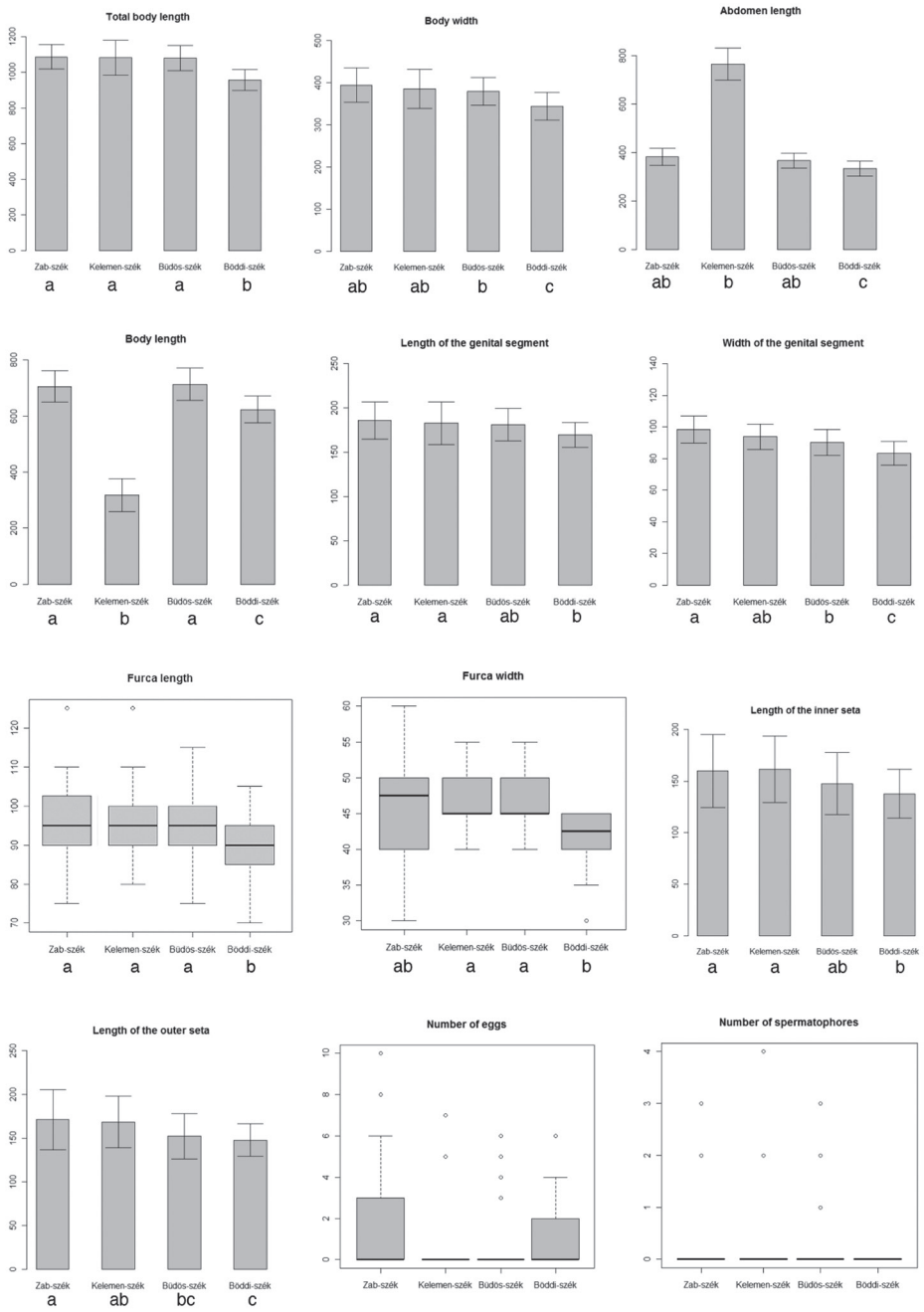

Fig. 1. The measured morphological variables with standard deviations of *Arctodiaptomus spinosus* individuals collected from soda pans in 2011. Left side scalebar indicates length in μm except for the number of eggs and spermatophores. Different letters indicate significant differences according to Tukey's test ($p < 0.05$)

Manual check of automatic base calling and alignment of the sequences were performed in BioEdit 7.0.5.3 [15]. A minimum spanning network at 99% connection limit was generated in TCS 1.21 [5] based on the statistical parsimony cladogram estimation method [35]. Descriptive statistics and Tajima's D [33] to test for the neutrality of the mutations were calculated in DnaSP v5 [20]. Significance level of the latter statistics was calculated based on coalescent simulations without recombination with 5000 replicates.

RESULTS

Morphological analyses

The ANOVA for the total body length (Fig. 1) showed a significant difference ($F_{3,126} = 21.97$, $p<0.001$) and Tukey's test pointed out that the Böddi-szék population was different from all the other sites ($p<0.001$). ANOVA for the body width was significant ($F_{3,126} = 10.40$, $p<0.001$, Fig. 1) and Tukey's test differentiated between the Büdös-szék and the Böddi-szék ($p<0.01$), Kelemen-szék and Böddi-szék ($p<0.001$), furthermore the Zab-szék and the Böddi-szék ($p<0.001$) populations. The length of the abdomen was significantly different ($F_{3,126} = 702.8$, $p<0.001$) between sites and Tukey's test indicated the separation of the Böddi-szék from the other sites ($p<0.05$) while the Kelemen-szék was also different from the Büdös-szék and Zab-szék ($p<0.001$) population. In the case of the body length (Fig. 1), the ANOVA was also siginificant ($F_{3,126} = 250.90$, $p<0.001$) and Tukey's test differentiated among populations of Böddi-szék, Kelemen-szék and all the other sites ($p<0.001$). The length of the genital segment (Fig. 1) was significantly different ($F_{3,126} = 4.04$, $p<0.01$) and Tukey's test indicated the differentiation of the Zab-szék and Böddi- szék populations. The ANOVA for the width of the genital segment was significant ($F_{3,126} = 20.42$, $p<0.001$) while Tukey's test pointed out the Böddi-szék as different from all the other sites ($p<0.01$) and indicated differentiation between the Zab-szék and the Büdös-szék ($p<0.001$) populations. For the furca length the Kruskal–Wallis test showed significant differences between the populations ($X^2 = 12.08$, $df = 3$, $p<0.01$) and also the furca width proved to be significantly different among sampling sites ($X^2 = 23.52$, $df = 3$, $p<0.001$) (Fig. 1). The length of the inner seta on the furca differed significantly (ANOVA: $F_{3,126} = 4.10$, $p<0.01$) and Tukey's test differentiated between the Böddi-szék and Kelemen-szék ($p<0.05$) and the Böddi-szék and Zab-szék ($p<0.05$) populations. The length of the outer seta on the furca was also different between sites ($F_{3,126} = 5.58$, $p<0.01$) and Tukey's test indicated differentiation between the Böddi-szék and Zab-szék ($p<0.01$), Böddi-szék and Kelemen-szék ($p<0.05$) furthermore between the Büdös-szék and Zab-szék ($p<0.05$) populations. The Kruskal–Wallis test did not indicate a difference between populations collected from different sites in the number of eggs carried ($X^2 = 5.74$, $df = 3$, $p = 0.12$) and in the number of the spermatophores attached to the females ($X^2 = 5.36$, $df = 3$, $p = 0.15$) (Fig. 1).

DNA analysis

After the alignment of the forward and reverse sequences we obtained 656 bp fragments of the mitochondrial COI gene with 9 singleton sites (unique occurrence of different character state of the nucleotides), 2 parsimony informative site (each distinct character state occurring at least with a frequency of two) and 10 different haplotypes among the 20 investigated individuals. GenBank Accession numbers can be found in Table 1. Descriptive statistics, diversity indices and Tajima's D for the populations are reported in Table 2.

All the 10 detected haplotypes contributed to the same haplotype network (Fig. 2) The A1 haplotype was detected in nine animals, it had the highest number of connections and this haplotype was present in each population. Haplotype A2 was detected in the Böddi-szék and Zab-szék populations while haplotype A3 was present in the

Table 1
The studied haplotypes of *Arctodiaptomus spinosus* from the Kiskunság area

Ind	HID	Site	GenBank AN
1	A1a	BU	KC660055
2	A1b	BU	KC660058
3	A1c	BU	KC660059
4	A1d	K	KC660060
5	A1e	K	KC660061
6	A1f	BO	KC660065
7	A1g	BO	KC660066
8	A1h	BO	KC660067
9	A1i	Z	KC660071
10	A2a	BO	KC660069
11	A2b	Z	KC660074
12	A3a	BU	KC660057
13	A3b	Z	KC660070
14	A10	K	KC660063
15	A8	K	KC660062
16	A9	K	KC660064
17	A6	Z	KC660072
18	A5	BO	KC660068
19	A4	BU	KC660056
20	A7	Z	KC660073

Ind = individual, HID = haplotype identification, the numbers correspond to haplotypes in Figure 2. Site = site codes: BO – Böddi-szék, K – Kelemen-szék, Z – Zab-szék, BU – Büdös-szék. GenBank Accession Numbers are given in the last column.

Table 2
Descriptive statistics, diversity indices and Tajima's D for the populations

Pop	*n*	*h*	*S*	Hd ±1S.D.	π ±1S.D.	D_T	pD_T
All	20	10	11	0.8 ± 0.089	0.00217 ± 0.00048	–1.915	0.0146
BO	5	3	2	0.7 ± 0.218	0.00122 ± 0.00046	–0.973	0.4494
K	5	4	6	0.9 ± 0.161	0.00396 ± 0.00112	–0.668	0.4190
BU	5	3	2	0.7 ± 0.218	0.00122 ± 0.00046	–0.973	0.4618
Z	5	5	4	1 ± 0.126	0.00244 ± 0.00047	–1.094	0.3052

Pop = population identifier, *n* = number of individuals sequenced in a population, *h* = number of haplotypes, *S* = number of segregating sites, Hd ± 1 S.D. = haplotype diversity plus or minus one standard deviation, π ± 1 S.D. = average pairwise distance, plus or minus one standard deviation, D_T = Tajima's D, pD_T = probability of $D_T \neq 0$ as determined by coalescent simulation.

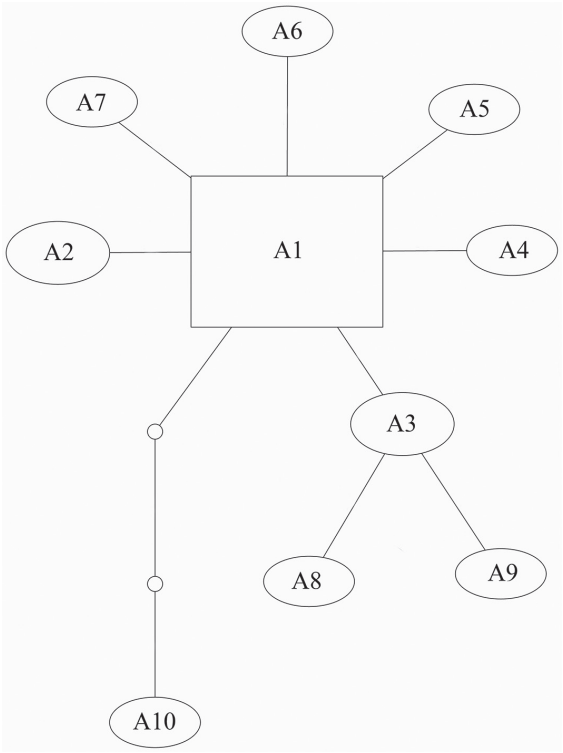

Fig. 2. COI haplotype network of twenty *A. spinosus* specimens at 99% connection limit. A1–A10: ten different *A. spinosus* haplotypes. The haplotypes with the highest mutational step numbers (A8, A9 and A10) originated from the Kelemen-szék population. The size of the ellipsoids and the rectangle is proportional to the number of occurrences of the given haplotype

Büdös-szék and Zab-szék. The other haplotypes (A4–A10) were revealed only form one individual/site each. Haplotypes A8, A9 and A10 that were in the farthest distance from the A1 haplotype regarding the number of mutational steps were found in the Kelemen-szék population.

DISCUSSION

The Böddi-szék population differed strikingly from the other populations in morphometric characteristics. Females in the Böddi-szék were shorter and narrower than in the other sites, their abdomen, body length and width of the genital segment was shorter as well. After all the Böddi-szék individuals were smaller than individuals from the other sampling sites. It can happen that the size of the animals increases throughout the breeding season but in our case the samples originated from the same sampling dates, so this explanation for the difference between the Böddi-szék and other populations can be excluded. Animals in the Kelemen-szék had exceptionally long abdomens and short body length. This phenomenon is rather interesting and requires further investigation.

The discrepancy among the Böddi-szék, Kelemen-szék and the rest of the populations can be either genetically encoded or simply the result of phenotypic plasticity explained by between-site differences of environmental factors that were not investigated in the present study.

Despite the morphological differences, we did not detect substantial DNA divergence within the species, therefore we can conclude that *Arctodiaptomus spinosus* populations within the Kiskunság area are probably not affected by cryptic speciation. Regarding haplotype occurrences, the population is genetically diverse in the Kiskunság. Haplotype diversity in the total population was higher than and comparable to Ponto-Caspian cladoceran and amphipod populations, respectively [7]. Presumably with increased sample size we would detect even more haplotypes.

At the same time, the mean number of differences between pairwise haplotype comparisons (nucleotide diversity) was low, therefore the population can be considered as rather uniform. Nucleotide diversity was lower in the *Arctodiaptomus spinosus* than in cladoceran and amphipod populations from the Ponto-Caspian region [7].

Dormancy in copepods is a widespread phenomenon [10]. They produce subitaneous eggs in stable environments, while diapausing eggs with a different chorion structure [31] are produced to survive desiccation, freezing and other harsh conditions. Direct evidence for dormant stages of *Arctodiaptomus spinosus* was not yet reported in the literature but since it occupies habitats that occasionally dry out, presumably dormant propagules are produced by the species frequently and in high amounts. Copepod subitaneous eggs are able to maintain viability after passing through larval and adult fish guts [1, 6] and viable copepod eggs were recovered from the faeces of killdeer (*Charadrius vociferus* Linnaeus, 1758) [28]. Dormant propagules of *Arctodiaptomus spinosus* are presumably capable of passive dispersal via

avian vectors and this might have been the way of colonisation of the Pannonian Plain or the other habitats in Ukraine and Turkey. It is unclear from where the species originates but the significant Tajima's D for the total Kiskunság population indicates that we can reject the neutral Wright–Fisher model and the negative value of it suggests population expansion and/or purifying selection. Supposedly Kiskunság populations of *Arctodiaptomus spinosus* still increases genetic diversity after the arrival of the founding population.

Herewith we clarified the COI haplotype characteristics of some *Arctodiaptomus spinosus* populations in the Kiskunság and provided data for comparison with future studies that should focus on a phylogenetic and population genetic comparison between the Pannonian populations and the populations from Turkey and from the Eastern side of the Carpathian mountain range.

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