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Morphometric and molecular studies on the populations of the damselflies *Chalcolestes viridis* and *C. parvidens* (Odonata, Lestidae)

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Morphometric and genetic differences were analysed for two closely related damselflies, *Chalcolestes viridis* and *C. parvidens*. A total of 305 male individuals were collected from six European countries (Austria, Croatia, Germany, Greece, Hungary and Portugal). Measurements from a total of 28 populations of *C. viridis* and *C. parvidens* and several intermediate forms were collected to determine if they can be definitely distinguished using simple morphometric characters. DNA sequences from two independent loci (nuclear ribosomal ITS region and mitochondrial cytochrome oxidase I gene) were analysed to test whether these taxa represent separate monophyletic groups as well as to compare the genetic distance with those found between well-accepted European *Lestes* species. Discriminant analysis revealed that *C. viridis* and *C. parvidens* are differentiated in morphometric space. Individuals with intermediate anal appendage traits overlapped with both *C. viridis* and *C. parvidens* which raised the possibility that they are merely subspecies of a single species. However, genetic analysis of both investigated DNA regions showed that the two *Chalcolestes* taxa did not share haplotypes, indicating their status as true species. Furthermore, they formed a monophyletic group separated from the investigated *Lestes* species, supporting the recognition of the genus *Chalcolestes*. The two *Chalcolestes* species are very closely related compared with European *Lestes* species, suggesting that their divergence occurred relatively recently.

Keywords: Zygoptera; damselfly; *Chalcolestes viridis*; *Chalcolestes parvidens*; morphometric differentiation; nuclear ribosomal ITS region; mitochondrial cytochrome oxidase I gene

Introduction

Traditionally, taxonomy is based on phenotypic analyses; although in many taxa this approach is impossible due to the lack of sufficient morphological characters (Chilton et al., 1995; Floyd et al., 2002; Wilkerson et al., 1993). For many aquatic insect orders such as Ephemeroptera

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(Alexander et al., 2009; Ball et al., 2005; Williams et al., 2006), Diptera (Pfenninger et al., 2007), Coleoptera (Balke et al., 2007; Dutton & Angus, 2007) and Trichoptera (Pauls et al., 2010) morphological characters alone do not allow reliable distinction. Hence molecular genetic techniques have become widespread in taxonomic studies. Although the number of studies combining DNA sequences and morphology are increasing, relatively few studies have focused on odonates (e.g. Pilgrim et al., 2002; Pilgrim & von Dohlen, 2007; Stoks et al., 2005). Not surprisingly, many debated taxonomic affiliations still remain in this order (Dijkstra, 2003; Dijkstra & Lewington, 2006; Schmidt, 2001).

One of these issues concerns *Chalcolestes viridis* (Vander Linden, 1825) and *C. parvidens* Artobolevskii, 1929 (Jödicke, 1997; Schmidt, 2001). Traditionally, both taxa were placed in the genus *Lestes* Leach, 1815, because adults are similar to other European *Lestes* species. However, given that their larvae differ by having a broad instead of a greatly narrowed (spoon-shaped) prementum, Kennedy elevated them to a separate genus *Chalcolestes* in 1920. Nevertheless this new genus name has not been generally accepted. Another debated issue is the rank of the taxon *parvidens* which was originally described by Artobolevskii in 1929 as a subspecies of *C. viridis* but later has been considered to be a valid species based on phenotypic traits and electrophoretic analyses (Cobolli et al., 1994). Both taxa live in similar habitats, including ponds, lakes, canals and slow-flowing rivers, with overhanging bushes required for oviposition (Askew, 2004). Where the two co-exist they segregate in their daily timing of the reproductive periods, indicating some level of reproductive isolation (Dell'Anna et al., 1996). Yet, the species status of *C. parvidens* has not been widely accepted (Jödicke, 1997; Sternberg & Buchwald, 1999; Wildermuth et al., 2005) although some authors have treated it as a good species (Askew, 2004; Dijkstra & Lewington, 2006; Olias et al., 2007).

The size and the general appearance of the two taxa are very similar. Size differences have been described but the literature is equivocal (e.g. Utzeri et al., 1995; Marinov, 2000). The most important discriminatory traits for males are found on the anal appendages: *C. parvidens* has a smaller tooth on the inner border of the cerci, which is placed slightly more dorsal than in *C. viridis*, and its paraprocts also have more slender and strongly up-curved tips (Askew, 2004; Dijkstra & Lewington, 2006). Furthermore, the anal appendages differ in colour pattern: the cerci of male *C. parvidens* are yellow with a sharply defined black tip, while those of *C. viridis* are more diffusely darkened at the apex and also at the base (Askew, 2004). These differences may, however, represent intraspecific variation instead of stable species-level differences (figure 54 in Jödicke, 1997) and may also be affected by factors such as the age of the specimen. Overlapping characters (Matushkina, 2006; Olias et al., 2007) and hybrids with intermediate male traits have been reported between these two taxa (Cobolli et al., 1994; Dell'Anna et al., 1996; Olias et al., 2007).

We compared morphometry of body, head, wings and cerci of males from several populations to determine if the two taxa can be unequivocally distinguished using simple measurements. We also included males with intermediate traits (based on appendage colour and shape) assumed to be hybrids (according to Olias et al., 2007) to test whether these parameters show intermediate morphometry. Additionally, we analysed DNA sequences from nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase I (COI) regions to test whether these taxa represent separate monophyletic groups. These DNA sequences were also used to compare the genetic distance between the two taxa with those found among well-accepted European *Lestes* species.

Material and methods

For morphometric comparison, 305 adult male damselflies were collected from Austria (AT), Croatia (HR), Germany (DE), Greece (GR), Hungary (HU) and Portugal (PT). A subset of 30

individuals was used for DNA analyses, including the following outgroup taxa: *Lestes barbarus* (Fabricius, 1798), *Lestes dryas* Kirby, 1890, *Lestes macrostigma* (Eversmann, 1836), *Lestes sponsa* (Hansemann, 1823), *Lestes virens vestalis* Rambur, 1842, *Lestes virens virens* (Charpentier, 1825) and *Sympecma fusca* (Vander Linden, 1820) (see Table 1 for details). At four of the 28 sampling localities both taxa were present. Here, we collected males with intermediate traits which may represent hybrids based on the colour and shape of the anal appendages (according to figure 1 in Olias et al., 2007). Specimens were collected and preserved in 70% ethanol for measurements and absolute ethanol for DNA isolation. Taxa were identified using Askew (2004) and Dijkstra and Lewington (2006).

Sixteen continuous characters were measured with digital caliper (DC) and a stereomicroscope using an ocular micrometer (SM) (Figure 1). These characters (Figure 1) were: the total length of the body (BL, from the frons to the tip of the cerci), the abdomen (AL), both pairs of fore and hind wings (RFw, RHw, LFw, LHw) and the width of the head (HW) and labrum (LW); the distance between the compound eyes (CED), the antenna scapes (ASD), and the nodus and the pterostigma on the right wings (RFwNP, RHwNP); and the characteristic dimensions of the cerci (C1–C4; Figure 1d). To allow accurate measurements, the head, wings and abdomen were separated for each individual with scissors after measurement of BL and AL, and body parts were placed at the same fixed position. To identify traits that separate the taxa and to evaluate the position of intermediate forms we performed a forward stepwise discriminant function analysis using Statistica version 9.1. (StatSoft, Tulsa, OK, USA). In each step of such an analysis all variables are evaluated to determine which contributes most to the discrimination of groups. All measurements were used as independent variables.

For genetic analysis muscular tissue was excised from the thoracic muscle and total DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was applied to amplify the nuclear ribosomal ITS region (containing the ITS1, 5.8S rRNA gene and ITS2) with the primers ETTS1 [5′-TGC TTA AGT TCA GCG GGT-3′] and ETTS2 [5′-TAA CAA GGT TTC CGT AGG TGA A-3′] (Kane & Rollinson, 1994). To amplify the mitochondrial COI gene we used the primers CW-3031 [5′-TTT GC(A/C) CT(A/T) ATC TGC C(A/C)T ATT-3′] (Heinze et al., 2005) and C1-J-2195 [5′-TTG AAT TTT TGG TCA TCC AGA AGT-3′] (Simon et al., 1994). Reactions were performed in a final volume of 50 µl using approximately 2 µl of purified genomic DNA, 0.2 mM of each deoxynucleotide, 2 mM $MgCl_2$, 1 U LC *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1 × PCR buffer (Fermentas), 0.325 µM of each primers. The thermal profile consisted of 5 min at 96°C for initial denaturation, followed by 35 cycles of 1 min at 48°C in case of COI PCR or at 54°C in case of the ITS PCR for annealing, 1 min at 72°C for extension and 1 min at 94°C for denaturation and with a final step of 10 min at 72°C for final extension. Amplified products were visualised on agarose gel stained with ethidium bromide. PCR products were purified using PCR-M™ Clean Up System (Viogene, Sijhih, Taiwan). DNA fragments were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The Chromas software v1.45 (Technelysium, Brisbane, QLD, Australia) was used for the manual correction of automatic base calling on chromatograms and for the removal of primer sequences. Sequence alignments were generated with ClustalW (Thompson et al., 1994). Alignments were corrected manually using the MEGA5 software (Tamura et al., 2011), and only unambiguously aligned positions were retained for subsequent phlyogenetic analyses. Maximum likelihood (ML) and maximum parsimony (MP) analyses were performed with the MEGA5 software. Nucleotide substitution models were selected based on the results calculated with Modeltest version 3.7 (Posada & Crandall, 1998). For COI sequences the Hasegawa-Kishino-Yano model using gamma distribution with invariable sites (HKY+I+G) and for ITS sequences the Kimura 2-parameter nucleotide substitution model using gamma distribution (K80+G) was applied. Bayesian analysis was performed with MrBayes version 3.1 (Huelsenbeck & Ronquist, 2001) applying the settings

Table 1. Collection information of the investigated specimens. Country codes: AT – Austria, DE – Germany, GR – Greece, HR – Croatia, HU – Hungary, PT – Portugal. Locality codes are given [in square brackets] for the specimens that were subjected to molecular biological analysis. Collector codes:[1] Attila Ferenc Kalmár,[2] György Dévai,[3] Hajnalka Anna Gyulavári,[4] Henrietta Beáta Nagy,[5] Margit Miskolczi,[6] Theodor Benken,[7] Tibor Jakab,[8] Valér Horvai,[9] Zoltán Varga. *n1*: number of individuals used for morphometric analysis, *n2*: number of individuals used for genetic analysis.

Specimens	Locality	Country	Coordinates	Date & collector	*n1*	*n2*
Chalcolestes parvidens	Apetloner Wäldchen	AT	N47°44′,E16°48′	16 September 2009[6]	1	0
	Kiesgrube, N Illmitz	AT	N47°46′,E16°48′	15 September 2009[6]	4	0
	Drava floodplain, N Botovo [B]	HR	N46°14′,E16°56′	28 October 2009[8]	2	1
	Stream, E Tavronitis, Crete [T]	GR	N35°31′,E23°49′	28 May 1994[2,5]	2	1
	Bédai-Holt-Duna, Erdőű (Kölked) [BK]	HU	N45°54′,E18°45′	30 August 2009[3,8]	0	1
	Diás-sziget, Kis-Balaton (Keszthely)	HU	N46°40′,E17°13′	12 August 2009[3]	10	0
	Fancsikai-mocsár (Debrecen)	HU	N47°30′,E21°44′	29 August 2008[2,5]	17	0
	Fenyves-tömpöly (Debrecen) [FeD]	HU	N47°30′,E21°45′	07 August 2009[1,3]	8	1
	Fertő (Fertőboz) [FF]	HU	N47°38′,E16°42′	02 September 2009[2,5]	6	1
	Halápi-tározó (Debrecen)	HU	N47°30′,E21°47′	25 September 2006[4,9]	33	0
	Halápi-tározó (Debrecen)	HU	N47°30′,E21°47′	29 August 2008[2,5]	63	0
	Kis-mező-szegi-Holt-Tisza (Kisar) [KK]	HU	N48°03′,E22°26′	16 August 2007[2,5]	18	1
	Marázs (Egyek)	HU	N47°40′,E20°51′	09 August 2007[7]	6	0
	Mentett-rét (Tiszaalpár)	HU	N46°49′,E19°59′	31 July 2008[2,5]	37	0
	Mentett-rét (Tiszaalpár)	HU	N46°49′,E19°59′	02 August 2008[2,5]	4	0
	Mentett-rét (Tiszaalpár) [MT]	HU	N46°49′,E19°59′	03 July 2009[2]	11	1
	Nagy-berek (Darány) [ND]	HU	N45°59′,E17°33′	27 August 2009[3,8]	11	1
	Suhonya (Pörböly) [SP]	HU	N46°11′,E18°50′	15 August 2009[3]	10	2
Chalcolestes viridis	Kiesgrube, N Illmitz	AT	N47°46′,E16°48′	15 September 2009[6]	1	0
	Kiesgrube, NE Wallern	AT	N47°44′,E16°57′	19 September 2009[6]	1	0
	Rosalia Kapelle	AT	N47°46′,E16°50′	15 September 2009[6]	1	0
	Schwarzseelacke	AT	N47°44′,E16°53′	19 September 2009[6]	1	0
	Backwater, NW Rheinbischofsheim [R]	DE	N48°39′,E07°54′	21 August 2009[3,6]	11	2
	Bacsó-nyak-alji-mocsár (Aggtelek) [BA]	HU	N48°28′,E20°29′	06 September 2009[2,5]	3	1
	Barbacsi-tó (Barbacs) [BB]	HU	N47°37′,E17°19′	02 September 2009[2,5]	0	1
	Kőzet-parki-tanösvény (Ipolytarnóc) [KI]	HU	N48°13′,E19°39′	19 August 2009[1]	1	1
	Vörös-tó (Aggtelek)	HU	N48°28′,E20°32′	06 September 2009[2,5]	6	1
	Rio Anqueira, Algoso	PT	N41°27′,W06°35′	08 July 2010[3]	7	0
	Rio Sabor, Gimonde	PT	N41°48′,W06°41′	07 July 2010[1]	1	0
	Rio Anqueira, Uva	PT	N41°29′,W06°31′	08 July 2010[3]	3	0
	Vila Chã de Braciosa	PT	N41°26′,W06°19′	11 July 2010[3]	1	0
Intermediate forms	Graben, W Pamhagen	AT	N47°42′,E16°52′	16 September 2009[6]	5	0
	Kiesgrube, N Illmitz [I]	AT	N47°46′,E16°48′	15 September 2009[6]	2	1
	Kiesgrube, NE Wallern	AT	N47°44′,E16°57′	19 September 2009[6]	2	0
	Fertő (Fertőboz) [FF]	HU	N47°38′,E16°42′	02 September 2009[2,5]	4	1
Lestes barbarus	Fancsikai-mocsár (Debrecen) [FD]	HU	N47°30′,E21°44′	13 July 2010[2]	0	2
Lestes dryas	Nagy-szik (Balmazújváros) [NB]	HU	N47°35′,E21°20′	12 July 2010[2]	0	2
Lestes macrostigma	Kelemen-szék (Fülöpszállás) [KF]	HU	N46°47′,E19°10′	22 July 2010[2,7]	0	2
Lestes sponsa	Halápi-tározó (Debrecen) [HD]	HU	N47°30′,E21°47′	14 July 2010[5]	0	2
Lestes virens vestalis	Nagy-szik (Balmazújváros) [NB]	HU	N47°36′,E21°22′	15 July 2010[2]	0	2
Lestes virens virens	Pond, SW Duas Igrejas [D]	PT	N41°27′,W06°22′	11 July 2010[3]	0	1
Sympecma fusca	Halápi-tározó (Debrecen) [HD]	HU	N47°30′,E21°47′	14 July 2010[2]	0	1

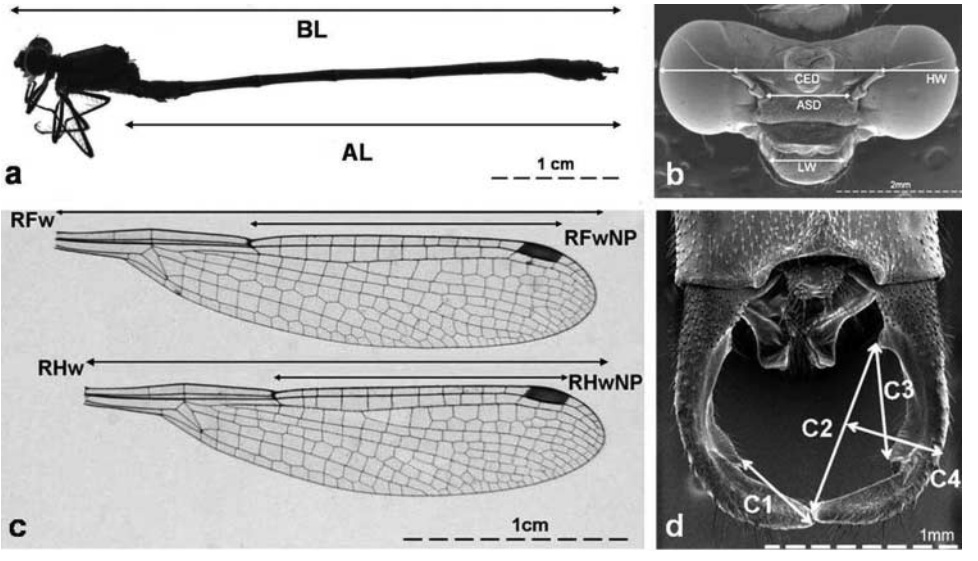

Figure 1. Characters used in the morphometric analysis of the two *Chalcolestes* taxa. (a) Total length of the body and the abdomen of a male *C. viridis*. (B) Measurements of the head of a male *C. viridis*. (C) Right wing measures of a male *C. parvidens*. (D) Anal appendages of a male *C. viridis* (dorsal view). See Material and methods for definition of the morphometric characters.

nst = 6 and rates = invgamma for 500,000 (ITS) and 1,000,000 generations (COI). Pairwise distances were calculated using the Kimura 2-parameter model in MEGA5. Sequences determined in this study were deposited in GenBank (accession numbers for ITS: HQ830270-HQ830298; for COI: HQ830299-HQ830321).

Results

Discriminant function analysis showed that traits contributing significantly to the overall discrimination were measurements of the cerci (C3, C2 and C1), sizes of the head (CED, LW and HW), the distances between the nodus and pterostigma on the forewing (RFwNP) and the total body length (BL). Overall, Wilks' lambda was low (0.339), which means that most of the total variability was attributable to differences among the means of the groups [$F(32, 550) = 12.312$, $p < 0.001$].

Different independent discriminant functions were computed in a canonical analysis to see how the variables discriminate between the different groups (Figure 2). The first discriminant function represented most (96.51%) of the discriminatory power and was weighted most heavily by some wing measurements (RFwNP and LFw), body length (BL) and one dimension of the cerci (C3). This function was marked by positive coefficients for the body length and one cerci size and negative coefficients for the wing measurements (Table 2). Thus, the longer the body and the cerci, and the shorter the wings, the more likely it is that the individual is *C. parvidens*. The second function accounted for a minor (3.49%) portion of the variance and seemed to be marked mostly also by wing sizes (RHw, RFw and LHw), the labrum width (LW) and the length of the abdomen (AL). The first discriminant function discriminated mostly between *C. viridis* and the other two groups. The second function was most useful to separate the intermediate forms; however, the magnitude of the discrimination was much smaller. Squared Mahalanobis distances (D^2) between *C. viridis* and *C. parvidens* were more than two times larger than distances between the intermediate forms and the other two taxa. The Mahalanobis distance

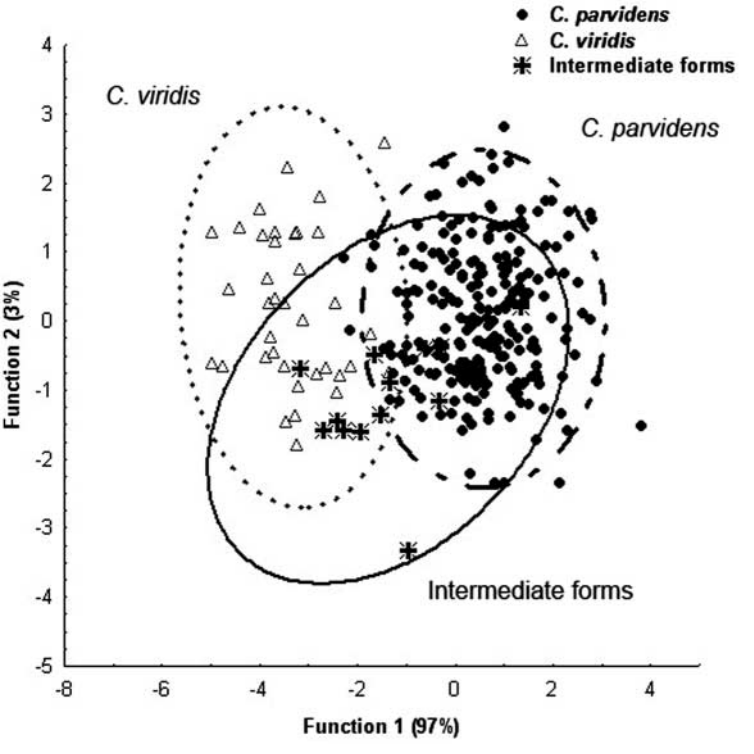

Figure 2. Plot of the discriminant analysis based on the morphometric measurements of *C. viridis*, *C. parvidens* and the intermediate forms.

is the distance of a case from the centroid in the multidimensional space, defined by the correlated independent variables. The distance matrix also showed that intermediate forms are almost as close to *C. viridis* as *C. parvidens* ($D^2_{C.\ viridis,\ C.\ parvidens} = 15.21$; $D^2_{C.\ viridis,\ intermediate\ forms} = 5.62$; $D^2_{C.\ parvidens,\ intermediate\ forms} = 5.13$). In general, males of *C. viridis* and *C. parvidens* are separated well from each other whilst intermediate forms were not differentiated well from *C. viridis* and *C. parvidens*.

In total, 13 cases were misclassified. The percentage of cases that were correctly classified for *C. parvidens* was 99.18% and for *C. viridis* was 91.89%. From the 13 intermediate forms six males were classified as *C. parvidens* and two males as *C. viridis* (only 38.46% of the total cases were correctly classified) which demonstrated that there were no obvious differences to separate these intermediate forms from the supposed parental taxa. Males with intermediate traits of the anal appendages had intermediate sizes (Table 3). The group of intermediate forms also showed higher standard errors for most measured characters and possessed remarkable morphometric variability compared with the two groups formed by the unequivocally identified *C. viridis* and *C. parvidens* specimens.

The phylogenetic relationships of the investigated damselflies were recovered using two different DNA regions: COI and ITS (Figure 3). All examined specimens of *C. parvidens* and of *C. viridis* formed separate monophyletic clades with high bootstrap support for both independent loci (>95 in the case of COI and >80 in the case of ITS based on three different analyses). Maximal pairwise sequence divergences for COI and ITS within taxa were 0.8% and zero among *C. parvidens* individuals, 0.6% and 0.2% among *C. viridis* individuals, respectively. In contrast, divergences between the two taxa for these DNA regions were approximately an order of magnitude higher: 12.4–13.8% and 1.0–1.2%. *Chalcolestes parvidens* and *C. viridis* together formed a monophyletic

Table 2. Results of the forward stepwise discriminant function analysis. Canonical vector coefficients for the two first axis (CV_1 and CV_2) and correlations between the values of the discriminant function and the values of variables (r_1 and r_2) are given.

Character	CV_1	CV_2	r_1	r_2
BL	0.592	0.559	0.108	0.076
AL	0.303	−0.802	0.124	0.000
RFw	0.188	−1.197	−0.490	−0.066
LFw	−0.727	0.533	−0.502	−0.026
RHw	−0.184	1.429	−0.458	−0.001
LHw	0.043	−0.689	−0.471	−0.024
RFwNP	−0.744	−0.138	−0.484	−0.029
RHwNP	0.548	0.155	−0.395	0.062
HW	0.249	−0.400	−0.254	−0.247
CED	−0.461	−0.245	−0.454	−0.080
ASD	0.307	−0.419	−0.117	−0.020
LW	−0.119	1.071	−0.237	0.484
C1	−0.227	−0.419	−0.391	−0.329
C2	−0.342	0.172	−0.119	−0.206
C3	0.615	−0.430	0.234	−0.247
C4	0.110	−0.084	0.066	−0.114

Table 3. Results of the measurements of the two *Chalcolestes* taxa and the intermediate forms. Mean and standard error (SE) are given. See Material and methods for definition of morphometric characters and measurement technique (Mt) abbreviations. Sizes are given in millimetres.

	C. parvidens		*C. viridis*		Intermediate forms		
Character	Mean	SE	Mean	SE	Mean	SE	Mt
BL	45.410	0.097	44.797	0.223	44.962	0.410	DC
AL	36.796	0.081	36.203	0.169	36.500	0.354	DC
RFw	24.156	0.049	25.649	0.147	24.965	0.253	DC
LFw	24.120	0.048	25.648	0.149	24.907	0.276	DC
RHw	23.336	0.046	24.677	0.142	24.005	0.244	DC
LHw	23.274	0.046	24.648	0.144	23.981	0.256	DC
RFwNP	14.126	0.030	15.053	0.091	14.606	0.183	SM
RHwNP	13.496	0.029	14.237	0.091	13.828	0.173	SM
HW	5.033	0.011	5.182	0.020	5.156	0.029	SM
CED	2.421	0.003	2.519	0.011	2.476	0.020	SM
ASD	1.356	0.005	1.391	0.006	1.375	0.009	SM
LW	1.293	0.002	1.332	0.008	1.288	0.008	SM
C1	0.414	0.002	0.455	0.004	0.446	0.005	SM
C2	0.987	0.002	1.003	0.007	1.005	0.013	SM
C3	0.644	0.003	0.605	0.005	0.637	0.008	SM
C4	0.470	0.001	0.464	0.004	0.470	0.005	SM
HW/BL	0.111	0.000	0.116	0.000	0.115	0.001	SM/DC

group with high bootstrap support (≥99) clearly separated from the investigated *Lestes* species. In the case of the *Lestes* species, within-species dissimilarity values ranged between zero (*L. barbarus*) and 1.3% (*L. sponsa*) for COI sequences and between zero (*L. dryas* and *L. macrostigma*) and 0.3% (*L. sponsa* and *L. virens*) for ITS sequences. These divergence values were similar to those observed in the *Chalcolestes* species. Between *Lestes* species nucleotide sequence divergence ranged from 7.3% (*L. dryas* and *L. sponsa*) to 16.9% (*L. virens virens* and *L. barbarus*) based on COI and from 1.8% (*L. dryas* and *L. sponsa*) to 12.9% (*L. sponsa* and *L. virens*) based on ITS data. These dissimilarity values are also very similar to those in *Chalcolestes* (∼13% and ∼1.1%, respectively). Divergence in the ITS region between the two subspecies *L. v. virens* and *L. v. vestalis* only ranged from 0.2% to 0.3%; similar to the variation within the *Chalcolestes* taxa.

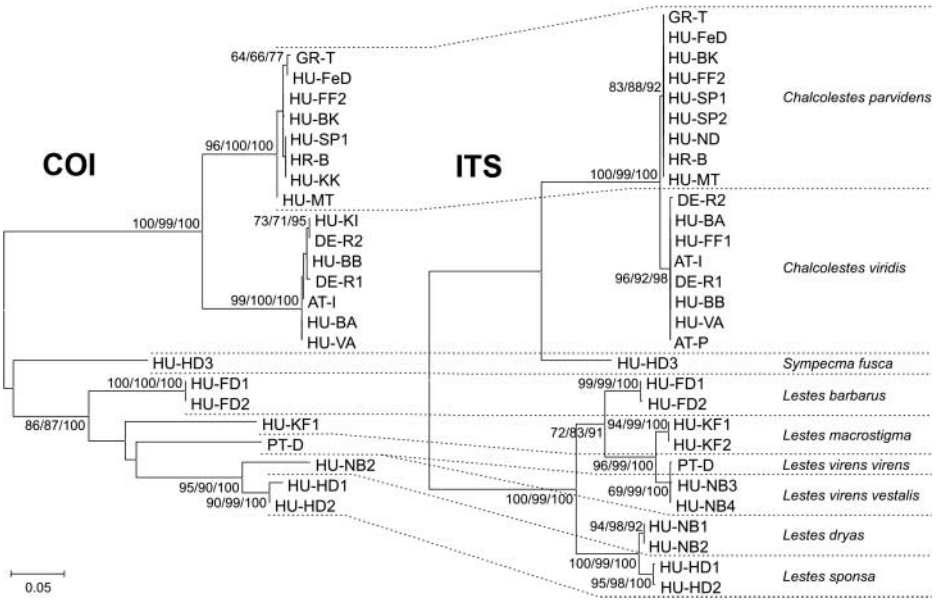

Figure 3. Phylogenetic relationship of *Chalcolestes* and *Lestes* taxa investigated in this study using mitochondrial cytochrome oxidase subunit I (COI) gene and genomic ribosomal ITS sequences. Sequence analyses were performed based on 534 and 643 unambiguously aligned nucleotide positions, respectively. Phylogenetic trees were constructed according to the maximum likelihood (ML) method. Bayesian (B) posterior probabilities and bootstrap values greater than 50 [based on 500 and 1000 replicates for ML and maximum parsimony (MP), respectively] are shown (order: ML/MP/B). Specimen codes were generated from the abbreviations of sampling sites: the first two letters are the country code and the additional letters indicate sampling locality, and numbers are serial numbers (if at least two specimens were sequenced from the same locality). See country and locality codes in Table 1.

On the whole, sequence dissimilarity between the two *Chalcolestes* species was similar to those of the investigated *Lestes* species based on COI while in the ITS region interspecies sequence dissimilarity was slightly lower than among *Lestes* species, although considerably higher than among the studied *Lestes* subspecies. Furthermore, all species and genus level categories investigated in this study were supported with high bootstrap values (usually >95). Finally, *Sympecma*, rather than *Lestes*, was recovered as sister taxon to *Chalcolestes* in the ITS tree and as sister to *Lestes* in the COI tree, i.e. in neither analysis were *Chalcolestes* and *Lestes* sister taxa to one another.

Discussion

Our genetic data support the generic status of *Chalcolestes*. The two *Chalcolestes* taxa formed a monophyletic group separated from other investigated *Lestes* species based on both the mitochondrial COI gene and the nuclear ribosomal ITS region. Dumont et al. (2010) analysed the 5.8S and 18S rRNA genes augmented with ITS1 and ITS2 sequences and concluded that *Chalcolestes* formed a monophyletic group with *Sympecma* and *Indolestes* distantly from the true *Lestes* species. In the present study, similar grouping patterns were observable in the phylogenetic trees; however, present sampling is far from exhaustive. Because *Lestes* is a large, cosmopolitan and heterogeneous genus with 84 species (Schorr et al., 2011), further study is needed from the family *Lestidae* to establish the exact position of *Chalcolestes*.

Even though we were unable to find morphometric characters that would always conclusively distinguish *C. viridis* from *C. parvidens* they were differentiated in morphometric space. Our

quantitative analysis of specimens from widely different regions showed that *C. parvidens* males usually had a longer body and smaller wings than *C. viridis* males. Contrary to this, Utzeri et al. (1995) reported not only smaller wings but also a shorter body in two Italian populations of *C. parvidens*, while Marinov (2000) reported equal body lengths but smaller wings. Because we did not include Italian and Bulgarian specimens in our study it remains to be tested whether animals from these regions differ morphometrically.

Female damselflies have mechanoreceptors on the mesostigmal plate that are stimulated by male cerci during tandem linkage (Robertson & Paterson, 1982). Smaller males may have difficulties grasping the thorax of a larger female with the cerci, while males that are too large may not be able to position their cerci correctly on the thoracic plates of the females. Loibl (1958) provided evidence that females of *Lestes* recognize conspecific males due to the tactile stimulation of the pronotum by the male appendages which are distinctively shaped. Females refused to copulate with conspecific males having experimentally altered inferior appendages, indicating mechanical or tactile isolation. Therefore further studies are recommended that focus not only on the cerci but also on the paraprocts.

McPeek et al. (2011) tested species identity traits in mate choice among six species of *Enallagma* damselflies (Coenagrionidae). They concluded that cerci sizes overlapped among species. In contrast, cerci shapes were non-overlapping among species, and five of six *Enallagma* species showed no population variation across their entire range. This suggests that cerci shape is the primary feature to discriminate conspecifics from heterospecifics during mating. This reflects the view that anal appendages are determinative for species recognition, hence reproductive isolation, in some odonates (Loibl, 1958; McPeek et al., 2008; Paulson, 1974).

Therefore, the differences in size and shape may reduce the probability of heterospecific copulation, but this mechanism apparently does not completely separate the two *Chalcolestes* taxa. This was supported with another study, in which we examined 20 pairs in copula in NW Hungary (where both species live syntopically), and found two pairs of male *C. viridis* with female *C. parvidens* and one reverse combination and some individuals with intermediate traits (Gyulavári et al., personal observation).

Probably after the Würm and earlier glaciations, the two *Chalcolestes* taxa re-advanced into Europe from different refugia in the western (*C. viridis*) and eastern Mediterranean (*C. parvidens*) (Dévai, 1976; Samraoui et al., 2003). In the Carpathian basin and in the Mediterranean region, the western and eastern invaders presumably mixed (Stewart & Lister, 2001), which resulted in a contact zone with intermediate forms. The intermediate forms suggest that the two *Chalcolestes* taxa are capable of interbreeding, but further studies are needed to determine the fertility of such offspring.

Genetic analysis showed that the two *Chalcolestes* taxa are closely related compared to other European *Lestes* species, but also that they form two monophyletic groups without shared haplotypes, suggesting that lineage sorting was complete and supporting the species status of *C. parvidens*. However, interspecies sequence dissimilarity values suggest a close relationship and a relatively recent divergence of the two taxa. This may also explain the occurrence of individuals with intermediate morphological traits presumed to be hybrids. Although we find the data supportive of recognizing *C. parvidens* as a distinct species, detailed morphometric (including shape analysis of the cerci and paraprocts) and genetic analysis of individuals of possibly hybridizing populations in the contact zone, and fertility studies on potential hybrids are recommended.

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