A new marker for molecular phylogenetic studies in Odonata including parts of conservative histone H3 and H4 genes and spacer between them

A.V. Mglinets[1], VS Bulgakova[1] and OE Kosterin[1, 2]

[1]Institute of Cytology and Genetics of the Siberian Branch of Russian Academy of Sciences, Acad. Lavrentyev ave. 10, Novosibirsk, 630090. Russia

[2]Corresponding author; E-mail: kosterin@bionet.nsc.ru; telephone: +79139220865; fax: +73833631267

Abstract. A new molecular marker, the histone H3-H4 region, containing partial coding sequences of the genes of histones H3 and H4 and the non-coding spacer between them, is proposed. This marker is potentially useful for molecular phylogenetic studies at generic, species, and even intra-species level in insects. The highly conservative histone coding sequences ensure universality of primers and the ease of primary alignment, while the highly variable non-coding spacer provides enough variation for analyses at short evolutionary distances. In insects, the histone genes reside in the histone repeat which is tandemly repeated in dozens to hundred copies forming the so-called histone cluster. However, the order and orientation of the histone genes in the histone repeat is variable among orders, which exerts some limitation for the new marker use. The marker efficiency is hereby shown for Odonata (dragonflies and damselflies), where it well resolved the families, genera and species involved and provided an insight into the relationship of *Sympetrum croceolum* (Selys, 1883) and *S. uniforme* (Selys, 1883).

Keywords: new molecular marker, molecular phylogeny, histone repeat, histone H3, histone H4, intergenic spacer, Odonata, dragonflies, damselflies, *Sympetrum*

Introduction

Analysis of DNA variation is a powerful tool in reconstructing phylogenetic history of living creatures, hence the use of molecular methods provided a profound progress in phylogenetic analysis, with applications in taxonomy, paleobiology, paleogeography and evolutionary theory. Particular sequences used for this purpose, traditionally called 'phylogenetic markers', differ in their rate of fixation of mutations thus permitting phylogenetic resolution at different time scales, with resolution of most recent divergences being possible only with most variable markers (for those applied to Odonata see CHENG et al. (2018)). Studying divergence of particular alleles within a species rather than divergence of orthologs between species and considering phylogenetic history of a species rather than higher order taxa is an overlap zone of phylogenetic analysis and population genetics usually referred to as phylogeography (AVISE 2000). Such cases concern characteristic times measured by thousands to hundreds of thousand years, too short for accumulation of sufficient variation in most molecular markers.

Mitochondrial DNA in animals is on average more variable than nuclear DNA, yet popular mitochondrial marker cytochrome oxydase I (*COI*), widely used for barcoding of animals (BALLARD & WHITLOCK 2004; AVISE 2009), is at the limit of usability for revealing within-species evolution

and phylogeography. The phylogeny reconstructed from mitochondrial markers often contradicts to both phylogenies reconstructed from nuclear markers and traditional taxonomy based on morphology. In some groups of organisms (CHENG et al., 2023), including the family Coenagrionidae in Odonata (DOW et al. 2019, DENG et al. 2021; GALIMBERTI et al. 2021, GEIGER et al. 2021), mitochondria seem as if to 'live the life of their own'. Most drastic patterns were revealed in *Coenagrion* Kirby, 1890, where mitochondrial happlotypes cross species barriers but seem unable to cross the Hybraltar Strait (FERREIRA et al. 2016; GALIMBERTI et al. 2021, GEIGER et al. 2021), and in *Ischnura elegans* (Vander Linden, 1820), where Japanese population appeared strongly divergent from the continental ones (DENG et al. 2021). Such drastic patterns can hardly be explained by incomplete lineage sorting or introgressive hybridisation, the phenomena usually supposed to explain the discordance of phylogenetic reconstructions based on the nuclear and mitochondrial markers.The reason could be co-selection of mitochondria with strains of the intra-cellular bacterial endosymbiont *Wolbachia* Hertig, 1826 (DENG et al. 2021). Horizontal transfer of mitochondria via an unknown agent is also postulated but not proved (GURDON et al. 2016). Another problem of using markers based on mitochondrial DNA are sporadic occurrence of mtDNA fragments adopted by the nuclear genome (NUMT) (for examples in Odonata see OŽANA et al. (2022) or LORENSO-CARBALLA et al. (2022)), which are variably divergent from the actual mtDNA and may result in false phylogenetic results, although they are rarely co-amplified with the true mtDNA from DNA isolate as having a much less template concentration. For the purpose of revealing evolutionary history at short time distances, the microsatellite or SSR markers were for a long time popular and applied also to Odonata (e.g., LOWE et al. 2008). These are tracts of very short (one to few nucleotides) repeats in which the number of copies frequently changes because of slippage mispairinig and unequal crossing over,. Such markers suffer from high rate of homoplasy, when different events of copy number change result in the same alleles, here understood as particular numbers of repeat copies but still are the marker of choice for population genetic studies. The modern next generation high throughput sequencing and genomic approach offer ample phylogenetic data (for examples in Odonata see e.g. FUTAHASHI ET AL., 2015; BYBEE ET AL., 2021; KOHLI ET AL., 2021), potentially useful for analysis even at short evolutionary distances, but are expensive and more demanding to sample preparation. Although the prices are getting lower, these technologies still remain unnafordable for many researchers in those countries which harbour the richest biodiversity. Therefore, there is still a need of easily analysed and cheap nuclear markers based on Sanger sequencing which provide good resolution at the species level and could be useful at least for fast preliminary revealing the evolutionary history of populations, subspecies and closely related species.

Non-coding sequences, whose variability is determined only by physical properties of DNA replication, are useful candidates for variable phylogenetic markers. Their use is limited by possibility to work out universal primers that could be achieved by involvement of bordering conserved sequences. Of such markers, the so-called ITS region including internal spacers between the conserved ribosomal RNA genes, *ITS1* and *ITS2*, is most popular among nuclear markers (for its use in Odonata see, e.g., HOVMÖLLER & JOHANSSON 2004; DUMONT et al. 2010; KARUBE et al. 2011; SCHNEIDER et al. 2023). The highly repetitive nature of the nucleolus organiser provides an advantage of high concentration of the template in preparations of genomic DNA and a disadvantage of possible heterozygosity as well as cis-heterogeneity within individual copies of the

nucleolus organiser (HOVMÖLLER & JOHANSSON 2004). Recently a useful approach has been proposed, focusing on introns of nuclear genes (FERREIRA et al., 2014). The primary structure of introns has scarce adaptive constraints excluding mutations affecting splicing. At the same time, the bordering exons are usually conservative enough to allow for universal primers design. These markers have low concentration of template genomic DNA, since the genes involved are unique and so present in the genome only in two copies, so they may be less readily amplifiable from specimens with somewhat degraded DNA as compared to the highly repeated sequences of the ITS region.A common drawback of non-coding sequences as phylogenetic markers are frequent indels (for an examle in one of the EPIC (exon-primed intron-crossing) markers, *PRMT*, see e. g. Lorenzo-Carballa et al. (2019), for that in the ITSregion see e. g. Lorenzo-Carballa et al. (2021)), which usually cannot be resolved by sequencing DNA amplified directly from genomic DNA preparation without cloning and exert difficulties of alignment.

In the present work we propose and test usability of a new phylogenetic marker, resembling the spacer between ribosomal RNA genes, as being a spacer between coding genes and a part of a tandem repeat. This is a spacer between the genes of the conservative core histones H3 and H4 and partial coding sequences of these histones, which we designate as*the histone H3-H4 region*. In animals, the genes for five histones (H1, H2A, H2B, H3, H4) are included into the so-called histone repeat, tandemly repeated copies of which form the histone cluster (EIRÍN-LÓPEZ et al., 2009). Several important circumstances should be noted in this respect:

(i) Histones H3 and H4 are among the most conserved proteins in eukaryotes (STEIN et al. 1984; DOENECKE et al. 1997; Eirín-López et al. 2009), so their coding sequences allow primers of very broad applicability across taxonomic groups.

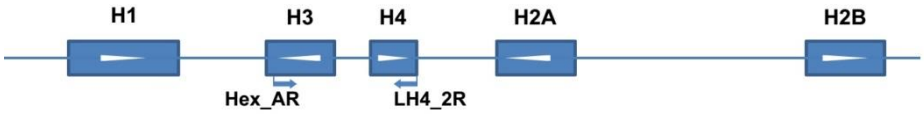

Figure 1. Positions of the primers used to amplify the histone H3-H4 region in the histone repeat as exemplified by a fragment of assembly of *Ischnura elegans* genome (NW_025791746). White arrowheads indicate direction of transcription.

(ii) In insects, genes of these two histones are disposed in the histone repeat relatively close to each other. The order of histone genes (e.g. H3, H4, H2A, H2B, H1 in *Drosophila melanogaster* and in all Odonata species (Fig. 1) tested in this paper) and orientations of their reading frames is variable among different insect orders. Although this variation may take place at lower taxonomical levels, and is sometimes observed even in different copies of the histone repeat in the same chromosome, in all species of Odonata tested by us (see below) we obtained a PCR product using the same primer pair matching histones H3 and H4. So the primers we suggest are useful at least across dragonflies and damselflies.

(iii) The spacer between H3 and H4 histone genes is non-coding and therefore is expected to undergo neutral evolution, hence being a kind of molecular clock.

(iv). Insects have hundreds of copies of the histone repeat (SOLOVYEV et al. 2022), that facilitates amplification from total genomic DNA, but may also bring about problems related to cis- (within-cluster) and trans- (allelic between homologous chromosomes) heterogeneity when amplified from genomic DNA preparations.

(v) Most insects have only one histone cluster, while in some of them and in other animal groups there are a number of paralogous clusters (EIRÍN-LÓPEZ et al. 2009). A single histone cluster is an advantage since this includes intra-individual trans-cluster heterogeneity which would strongly complicate an analysis.

To develop this marker we designed original primers which worked in all tested species of Odonata.

To test the marker we chose the order Odonata (mainly its suborder Anisoptera). We tested its resolution at different taxonomical levels, by sequencing amplicons from representatives of different families (Calpterygidae, Coenagrionidae, Aeshnidae, Gomphidae, Corduliidae and Libellulidae), as well as several species of the genera *Sympetrum* Newman, 1833 and *Somatochlora* Selys, 1871, and individuals belonging to different species of *Sympetrum* , including those collected in the same population

Materials and methods

Material

The specimens used for sequencing were preserved either in 96% ethanol (*Sympetrum croceolum* (Selys, 1883), *S. danae* (Sulzer, 1776), *S. flaveolum* (Linnaeus, 1758), *S. uniforme* (Selys, 1883)) or treated overnight with acetone and then dried out (the rest of the specimens). The following species and specimens were taken for analysis (all collected by O.E. Kosterin unless otherwise indicated; the GenBank accession numbers of the sequences of the histone H3-H4 region obtained from each specimen are provided in the list below in parentheses.

Calopterygidae

Atrocalopteryx atrata (Selys, 1853) – 1 ♂ (PQ498535), Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki, 48.19° N, 134.68° E, 15.07.2014.

Coenagrionidae

Coenagrion johanssoni (Wallengren, 1894) -1 ♂ (PQ498539), Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki, 48.19° N, 134.68° E, 15.07.2014.

Aeshnidae

Aeshna juncea (Linnaeus, 1758) – 1 ♂ (PQ498536), Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.30° N, 134.83° E, 9.07.2014.

Gomphidae

Nihonogomphus ruptus (Selys in Hagen, 1858) – 1 ♂ (PQ498542), Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki, 48.19° N, 134.68° E, 15.07.2014.

Corduliidae

Cordulia aenea (Linnaeus, 1758) – 1 ♂ (PQ498537), Russia, Novosibirsk Academy Town, 54.8473° N, 83.1072° E, 1.07.2015.

Somatochlora arctica (Zetterstedt, 1840) – 1 ♀ (PQ498546), Russia, Novosibirsk Academy Town, Zvezda Beach, 54.8314° N, 83.0538° E, 30.06.2015.

Somatochlora graeseri Selys, 1887 – 1 ♀ (PQ498561), Russia, Primorskiy Kray, Khanka District, Lake Khanka W bank 2 km N of Platono-Aleksandrovskoe village, 45.062° N, 131.995° E, 4.07.2014.

Macromiidae

Macromia amphigena fraenata Martin, 1906 – 1 ♂ (PQ498540), Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki, 48.19° N, 134.68° E, 15.07.2014.

Synthemistidae s.l.

Macromidia rapida Martin, 1907 – 1 ♂ (PQ498541), Cambodia, Mondulkiri Province, a left tributary of the Monorom Waterfall river, 3.5 km SE of Sen Monorom, 12.441 N, 107.159 , 13.06.2014.

Cordulegastridae

Cordulegaster picta Selys, 1854 – 1 ♀ (PQ498538), Russia, Krasnodarskiy Kray, Kabardinka village, the Krasnaya Shchel' Valley, 44.671 N, 37.918 E, 30.07.2015.

Libellulidae

Orthetrum albistylum (Selys, 1848) - 1 ♀ (PQ498543), Russia, Primorskiy Kray, Khanka District, Lake Khanka W bank 2 km N of Platono-Aleksandrovskoe village, 45.062° N, 131.995° E, 4.07.2014.

Orthetrum glaucum (Brauer, 1865) – 1 ♂ (PQ498544), Cambodia, Mondulkiri Province, a brook downstream Buu Sraa Waterfall, 12.570 N, 107.418 E, 10.06.2014.

Rhyothemis phyllis (Sulzer, 1776) – 1 ♀ (PQ498545), Cambodia, Kampot Province, a left oxbow of the Kampot River downstream of Tek Chhou Rapids, 10.6719 N, 104.1367 E, 21.08.2011.

Sympetrum arenicolor Jödicke, 1994 – 1 ♂ (PQ498547), Iran, Lorestan, Khorramabad County, 3.2 km NNW Pasil village, 33.33° N, 48.88° E, 26.05.2017.

Sympetrum cordulegaster – 1 ♂ (PQ498588), Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, a pond S of Bychikha village, 48.292° N, 134.829° E, 21-22.08.2016.

Sympetrum croceolum (Selys, 1883) – 10 ♂♂ (Sc-1 – Sc-10; PQ498574–PQ498582), Russia, Amursk District, Bolon'skiy Nature Reserve, Kirpu ranger stationt, 49.5061° N, 136.028° E, 15.09.2016, V.V. Dubatolov leg.

– 2 ♀♀ (Sc-31, Sc-32; Q498583, PQ498586), Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.292° N, 134.829° E, 21-22.08.2016, V.V. Dubatolov leg.

– 2 ♂♂ (Sc-41, Sc-42; PQ498584, PQ498585), Russia, Altai Republic, Mayma District, Lake Manzherok SE bank, 51.82° N, 85.81° E, 16.09.2016, O.E Kosterin & P.Y. Malkov leg.

Sympetrum danae (Sulzer, 1776) – 12 ♂♂ (Sd-1, Sd-3 – Sd-12; PQ498549–PQ498557), Russia, Novosibirsk, Academy Town, botanical garden, 54.8252° N, 83.1137° E, 8.10.2015.

Sympetrum flaveolum (Linnaeus, 1758) – 1 ♂ (Sf-1; PQ498558), 1 ♀ (Sf-2; PQ498559), Russia, Novosibirsk, Academy Town, botanical garden, 54.8252° N, 83.1137° E, 8.10.2015.

– 1 ♀ (Sf-3; PQ498560), Russia, Primorskiy Kray, Lake Khanka W bank just N of Platono-Aleksandrovskoe village env., 45.06° N, 131.99° E, 4.07.2014

Sympetrum fonscolombii 1 ♂ (No. 1), Iran, Esfahan Province, Golpayegan City, the Ghomrood (Anaarbar) River, 33.46° N, 50.26° E, 21.05.2017.

– 1 ♂ (No. 2; PQ498589), Russia, Krasnodarskiy Kray, Bol'shoy Utrish village env., 44.76° N, 37.39° E, 5.08.2015.

Sympetrum pedemontanum – 1 ♂ (PQ498591), Russia, Novosibirsk, a glade in a pine forest between Nizhnyaya El'tsovka and Pravye Chyomu estates, 54.8640° N, 83.0510° E, 4.08.2018.

Sympetrum sanguineum – 1 ♂ (Ss-1, PQ498590), Russia, Novosibirsk Province, Kyshtovka District, a roadside swamp in the Kyshtovka village NW suburbs, 56.579° N, 76.603° E, 14.07.2015.

– 1 ♂ (Ss-2), Krasnodarskiy Kray, Lake Krugloe, 44.677° N, 37.594° E, 10.06.2016.

Sympetrum striolatum – 1 ♂ (PQ498592), Iran, Lorestan, Borujerd County, Chenar Chashmah, 33.798° N, 40.905° E, 25.05.2017.

Sympetrum vulgatum – 1 ♂ (PQ498593), Russia, Krasnodarskiy Kray, Kabardinka village env., Krasnaya Shchel' Valley, 44.672° N, 37.922° E, 6.07.2016.

Sympetrum uniforme (Selys, 1883) – 5 ♂♂, 4 ♀♀ (Su-21–Su-29; PQ498565–PQ498573), Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.292° N, 134.829° E, 21-22.08.2016, V.V. Dubatolov leg.

– 1 ♂ (Su-1; PQ498563), 1 ♀ (Su-2; PQ498564), Russia, Khabarovskiy Kray, Amursk District, Bolon'skiy Nature Reserve, Kirpu ranger station, 49.5061° N, 136.028° E, 15.09.2016, V.V. Dubatolov leg.

Sequences from public databases

To expand our sample by sequences obtained by others, we downloaded from the Whole Genome Sequence datasets available in public databases the sequences of the histone H3-H4 region of 14 more Odonata species (*Hetaerina americana* (Fabricius, 1798), *H. titia* (Drury, 1773), *Ischnura elegans*, *Pseudagrion microcephalum* (Rambur, 1842), *Argia fumipennis* (Burmeister, 1839), *Pyrrhosoma nymphula* (Sulzer, 1776), *Platycnemis pennipes* (Pallas, 1771), *Prodasineura notostigma* (Selys, 1860), *Tanypteryx hageni* (Selys, 1879), *T. thoreyi* (Selys, 1889), *Uropetala carovei* (White in Dieffenbach, 1843), *Brachytron pratense* (Müller, 1764), *Pantala flavescens* (Fabricius, 1798), *Pachydiplax longipennis* (Burmeister, 1839)). Besides, one sample (*Ladona fulva* (Müller, 1764)) was assembled from SRA archive with the use of MIRA software (CHEVREUX et al. 1999). Their accession numbers are indicated at the species names in Figs 2-5.

Primer design

We designed some 14 primers to match different parts of insect histone genes coding for H1, H2B, H3, H4 comprising the histone gene cluster. At the start of the present work we did not know the precise order and orientation of the histone genes in the cluster, so we tested different primer combinations to select primer pair(s) which would produce an amplification product containing the fragments of the coding sequences of the genes of histone H3 and H4 and a spacer between them. We found out that the pair of original primers Hex_AR matching the 3' portion of the H3 gene (in reverse orientation) and LH4_2R matching the 3' portion of the H4 gene (in reverse orientation) (Fig. 1) produced the needed product, indicating at the tail-to-tail orientation of the H3 and H4 genes with respect to their reading frames (Fig. 1). The primer sequences are as follows:

LH4_2R: 5' ttaaccgccgaaaccgtacagggt

Hex_AR: 5' atatccttgggcatgatggtgac

The primer LH4_2R matching the coding region of the histone H4 of the moth *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) was worked out in the course of our previous study of the variation of the histone H1 gene in some Lepidoptera (SOLOVYEV ET AL. 2015). The linker histone H1 protein has variable C- and N- terminal domains but conserved central domain; correspondingly, its gene has variable 5'- and 3' regions and conserved central region (EIRÍN-LÓPEZ et al. 2009). So, to sequence the histone H1 gene in Lepidoptera we amplified sequences expanding from the conserved central region of the histone H1 gene to the conserved genes neighbouring genes of core histones (for details see SOLOVYEV ET AL. 2015). For this purpose a number of primers was worked out to match the histone H3. H2A and and H4 genes, including the LH4_2R primer used in this paper, although this particular primer was not mentioned in the cited work (SOLOVYEV ET AL. 2015) and is published here for the first time. It turned out that this primer LH4_2R, worked out for Lepidoptera, matched the homologous histone H4 gene for all species of Odonata we tested.

The Hex_AR primer was worked out to match the coding sequences of the histone H3 gene of *Ophiogomphus severus* (Hagen, 1874) (Odonata: Gomphidae) taken from GenBank (accession AY125228).

The conding sequences of histones H3 and H4 are so conserved (STEIN et al. 1984; DOENECKE et al. 1997; EIRÍN-LÓPEZ et al. 2009) that the primers worked out to match a single sequence each, one of which was from Odonata and the other from Lepidoptera, appeared to work well for all tested species of Odonata.

DNA isolation, sequencing and analysis

Dragonfly legs were homogenized in a mortar in 0.2 ml of isolation buffer (0.1 Tris-HCl, pH 8.0; 0.05 M EDTA; 1.25% SDS; 0.5 M NaCl) with Al_2O_3 as grinding particles, then mixed with 0.8 ml of the same buffer. The mixture was incubated for 1 hr at 55°C, then added with 350 µl of 5 M potassium acetate, incubated for 30 min on ice and centrifuged at 16.1 g for 10 min. The supernatant was transferred to fresh tubes, mixed with 0.6 ml of isopropanol, incubated at room temperature for 1 hr and centrifuged at 12.2 g for 10 min. The precipitate was washed twice with 0.1 ml 70% ethanol with subsequent centrifugation at 12.2 g for 5 min, dried at 50°C for 5 min and dissolved in 50µl of deionized water.

PCR reaction was carried out in a volume of 20µl with 2 µl of 10x ammonium sulphate buffer, 2 µl of 25 mM $MgCl_2$, 0.3 µl of the Hot Start Taq polymerase produced by SIBENZYME company, Novosibirsk (5 U/µl), 0.15 µl BSA (10 mg/ ml), 1 µl of forward and reverse primers (10 pM) each, 2 µl of 2 mM dNTPs, 2 µl of diluted DNA (20-60 ng) and 10.55 µl of deoinised water. For PCR, BIO-RAD MyCycler thermal cycler was used, with the reaction parameters as follows: denaturation at 95 °C for 3 min followed by 32 cycles including denaturation at 94 °C for 30 s, annealing at 55 °C for 25 s, elongation at 72 °C for 45 s. PCR-products were purified with Invisorb® Spin Filter PCRapid Kit and Sanger sequenced using Big Dye Terminators version 3.0 or 1.1 at SB RAS Genomic Core Facility.

Raw trace files were visualized and translated into nucleotide sequences with the use of the Gap4 software (STADEN ET AL. 2003).

The sequences were aligned with ClustalW (LARKIN et al., 2007) using MEGA 6.0 software package (Tamura et al. 2013) with default parameters and adjusted manually.

The phylogenetic relationships were reconstructed with the Maximum Likelihood method using MEGA 6.0, with Kimura 2-parameter substitution model, as default in the package, rate among sites: gamma-distributed with invariant sites. The sequences of the histone H3-H4 region of two species of Diptera, *Anopheles funestus* Giles, 1900 and *A. marshalli* Theobald, 1903) from GenBank, were used as the outgroup for the order Odonata-wide phylogenetic reconstruction (Figs 2-4), because in Diptera we found the same order and orientation of the genes of histones H3 and H4.

The uncorrected p-distances between different alleles of the within species of *Sympetrum* were calculated with the MEGA 6.0 software (the entire matrices are not shown).

Results

The histone H3-H4 region was successfully amplified with the above suggested primer pair and sequenced from DNA samples of all above enumerated specimens of Odonata which were 59 individuals of 24 species. The sequences were sent to GenBank with the following accession numbers: (pending, to be filled in later). Together with the 15 sequences adopted from public databases, this comprised a sample of 74 sequences of 39 species.

The sequences of the histone H3-H4 region contained parts of the conservative coding sequences of histones H3 (351 bp) and H4 (282 bp) and the spacer between them of a variable length about 250 bp. All substitutions revealed in the coding sequence fragments were synonymous except for the substitution t->a in the first position of the second codon of the histone H4 gene, which changes threonine to serine, in both sequences of *S. fonscolombii* (not shown). As expected, the sequences of bordering coding sequences were unambiguously aligned. At the same time the spacer expectedly appeared hyper-variable so its alignment was much less certain and retained some ambiguity.

One specimen of *S. sanguineum* (Ss-2, from Krasnodarskiy Kray), one specimen of *S. fonscolombii* (No. 1, from Iran), and one specimen of *S. uniforme* (Su-23) appeared heterogeneous containing reads with and without a deletion of a number of nucleotides in the spacer. One of those indels found in *S. sanguineum* (Ss-2) concerned just one base pair, however, we were able to infer both sequence variants from the chromatogram but used for further analysis only one of them, chosen randomly. Indels found in the other two species were longer, about 5 and 10 bp. Although the sequences beyond the deleted region could be reconstructed, we preferred to exclude these specimens from further analyses.

In some positions, the chromatograms revealed two peaks of comparable height suggesting within-specimen heterogeneity for nucleotides occupying these positions (reflected by the relevant IUPAC codes in the sequences). Those positions reflected either heterozygosity for different alleles or cis-heterogeneity for the histone repeat along a histone cluster, quite expectable in the case of repeated units. Such positions made uncertain the exact number of unique alleles found in a species. Some chromatograms did not resolve nucleotids in a number of positions adjacent to the primers, we nevertheless involved the shortened, well resolved sequences into phylogenetic reconstructions.

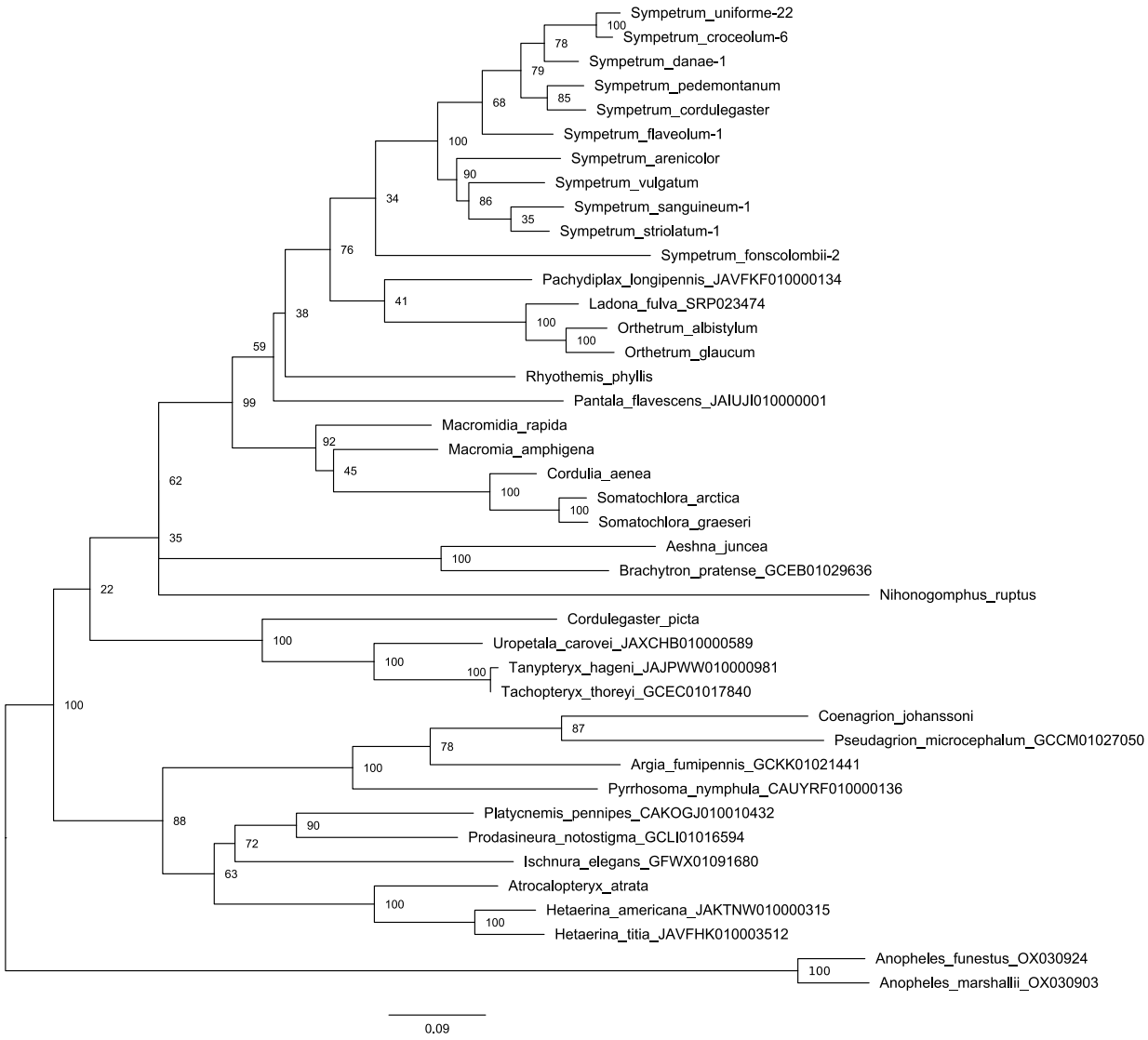

Figure 2. Phylogenetic tree of the studied species of Odonata reconstructed with Maximum Likelihood method from the histone H3-H4 region sequences from .

First, we reconstructed a phylogenetic tree based on the sequence of the H3-H4 region from one representative of each involved species, both newly sequenced and available from public databases (Fig. 2). We see that three genera with two species involved, *Orthetrum* Newmann, 1833, *Somatochlora* Selys, 1871 and *Hetaerina* Selys, 1853, appeared to cluster with the very high bootstrap support values of 98 (the former) – 100 (the two latter). The genus *Sympetrum* Burmeister, 1839, with nine species analysed, clustered with a weak support, butif to exclude the divergent species *S. fonscolombii*, the remained eight species clustered together with the maximum boostrap support of 100. The overall tree topology corresponded to the family system of Odonata rather well, except for the odd position of *I. elegans*, which belongs to Coenagrionidae but clustered with Platycnemididae, although with a weak bootstrap support of 73. All other involved families formed monophyletic clusters but with variable support, from 68 of Libelluliade to the maximum support of 100 for Corduliidae, Aeshnidae, Petaluridae and Calopterygidae (but too few species of those

families were included in the analyses). Petaluridae and Neopetalidae together also formed a cluster with the maximum support. Even representatives of the three families (Corduliidae, Macromiidae and Synthemistidae sensu lato), previously considered in the family Corduliidae in the broad sense, also grouped in a well-supported cluster (with a bootstrap support value of 96). Both suborders formed monophyletic clades but that of Anisoptera was of a negligible support of 23 and the support of Zygoptera of 88 was also rather poor. We may conclude that the phylogenetic information provided by the proposed marker is at the border of sufficiency to reliably reconstruct the overall phylogenetic relationships of Odonata.

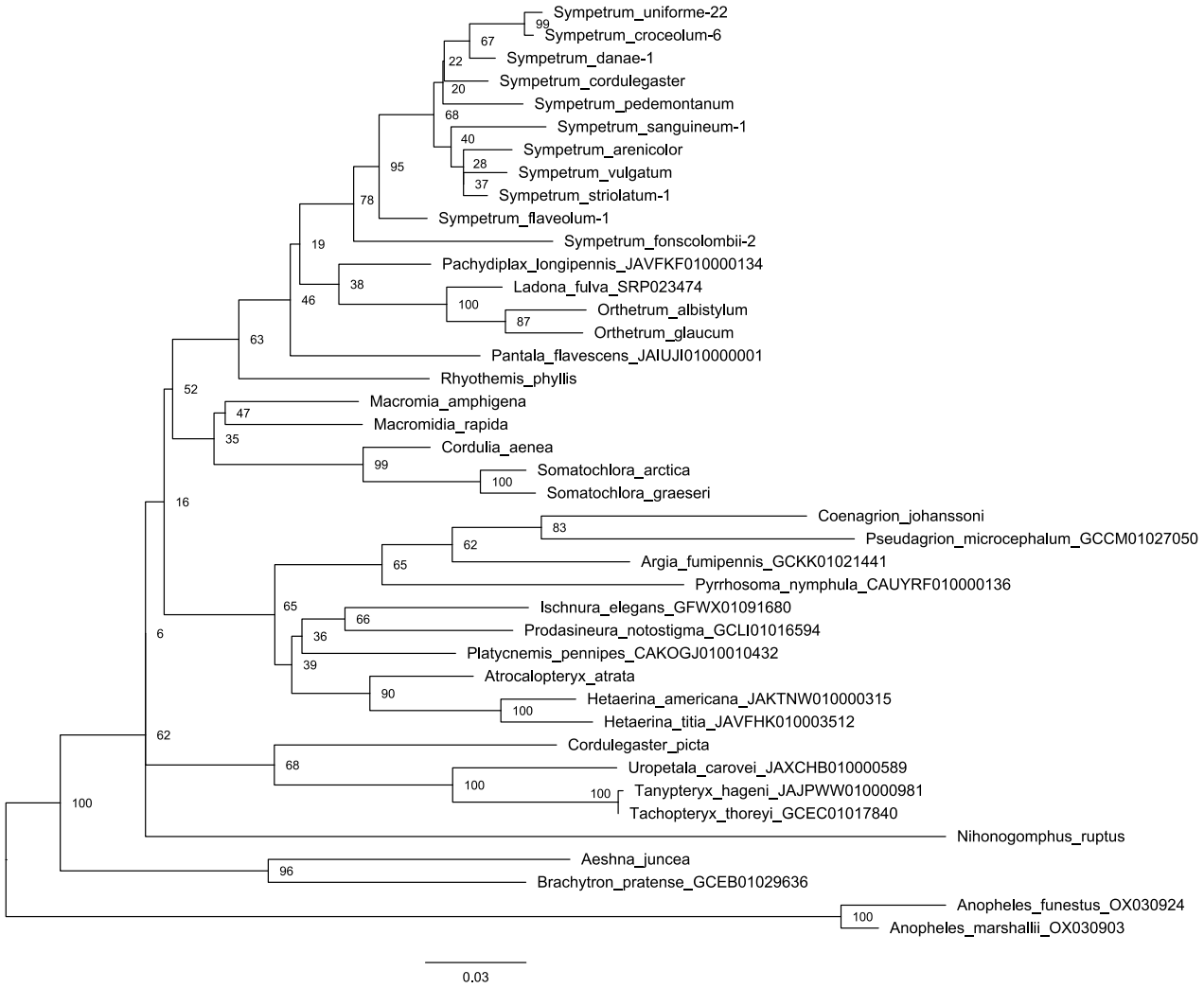

Figure 3. Phylogenetic tree of the studied Odonata species reconstructed with Maximum Likelihood method from the s fragments of coding sequences of histone H3 and histone H4 genes involved into the proposed ‘histone H3-H4 region’ marker.

It was interesting to evaluate separate inputs into this phylogenetic resolution of the histone coding sequences and spacer, so we reconstructed phylogenetic trees based on these two components separately (Figs 3 and 4, respectively). We see that the histone coding sequences taken separately (Fig. 3) in general resolved the same topology but with slightly weaker supports. The supports of families with few species remained high, although lowered a bit.

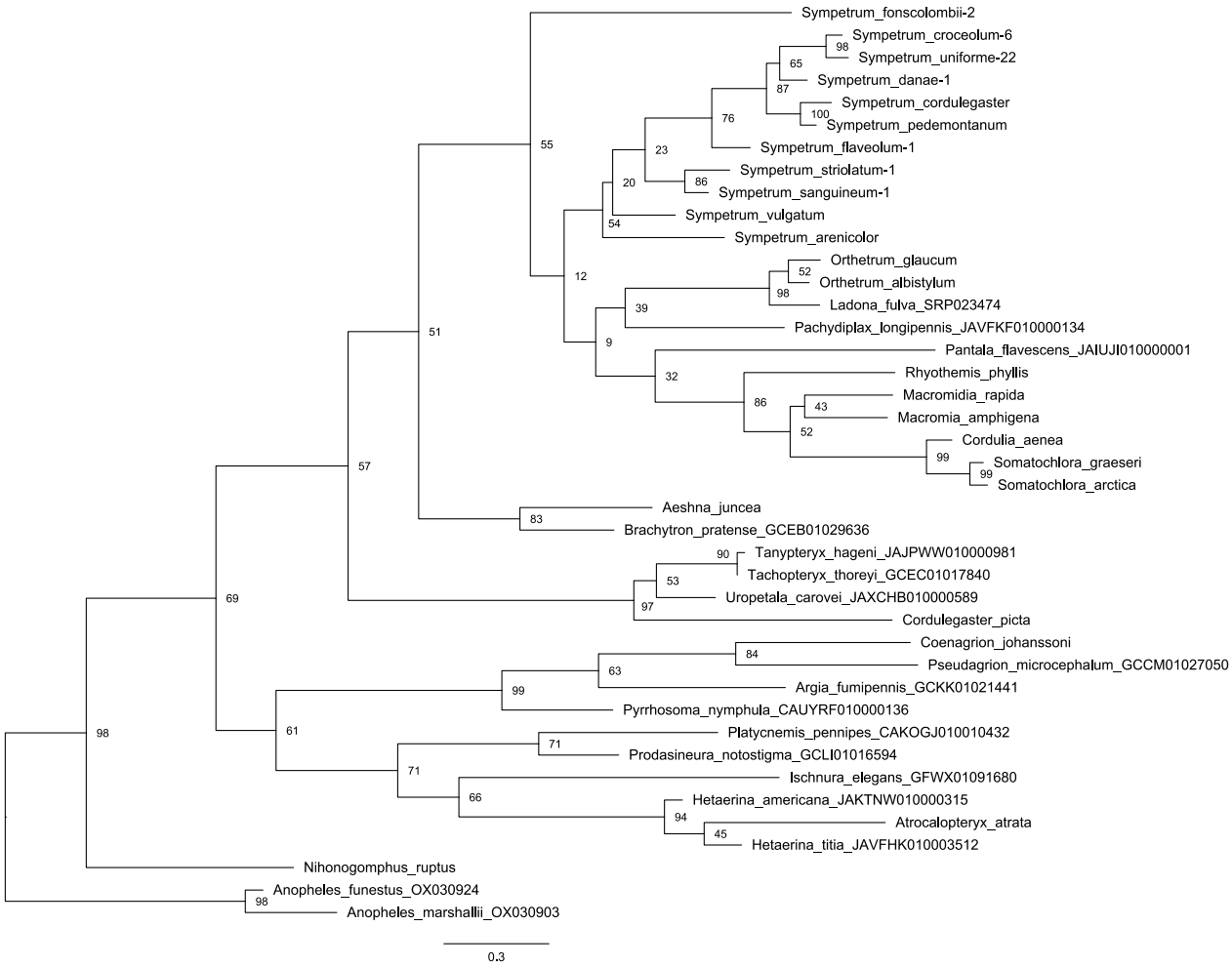

Figure 4. Phylogenetic tree of the studied Odonata species reconstructed with Maximum Likelihood method from the intergenic spacer between histone H3 and histone H4 genes.

The overall topology of the phylogenetic tree based on the non-coding spacer sequences only (Fig. 4) is completely non-supported and has scarce biological sense, but the terminal branches of the *Sympetrum* cluster are mainly well supported. We may conclude that the non-coding spacer is too variable to resolve the phylogeny at the family level but well resolves species inside.

Anyway, we see that both parts of the histone H3-H4 region have their input into its resolving power.

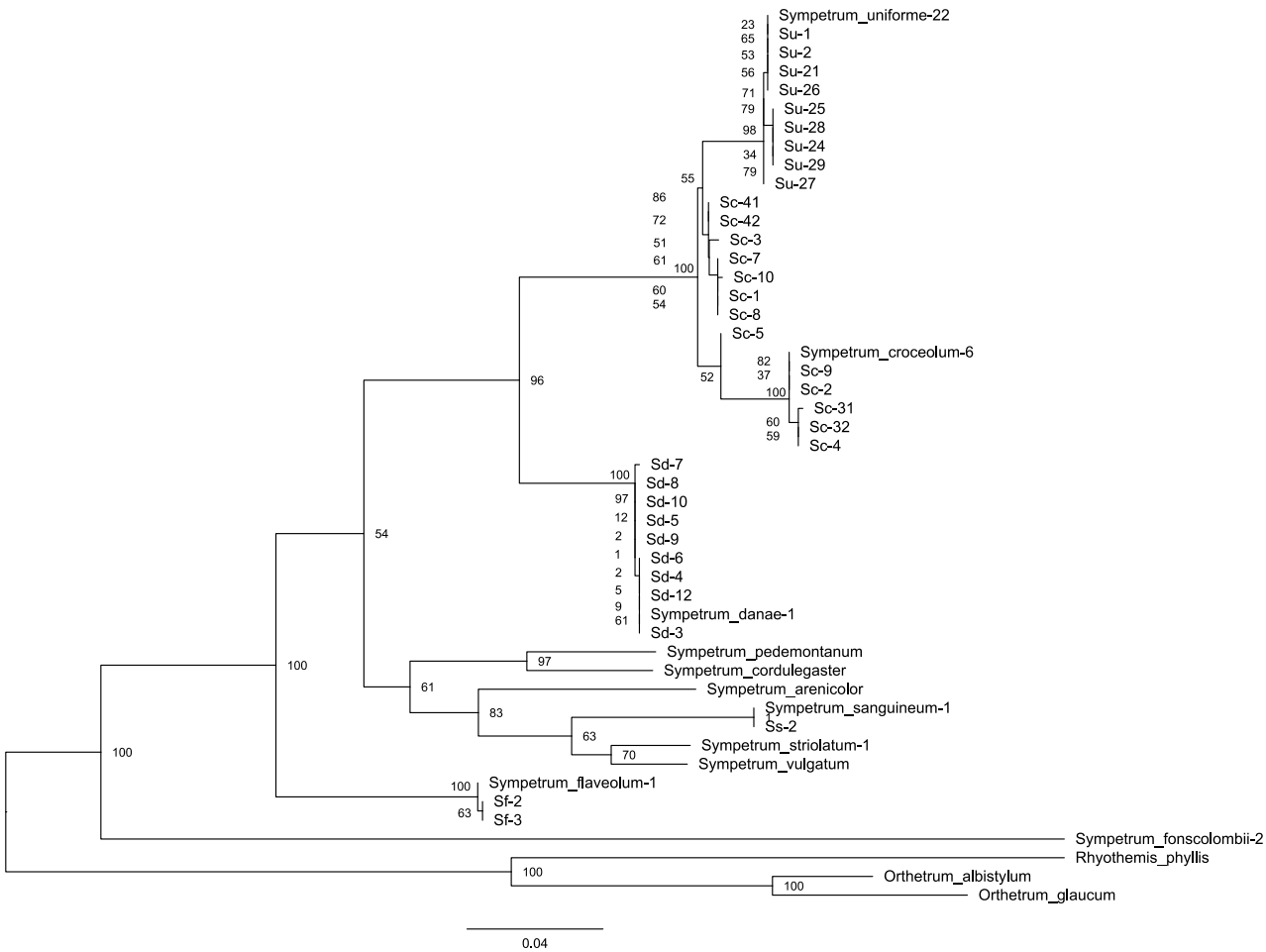

Figure 5. Phylogenetic tree of the studied *Sympetrum* species reconstructed with Maximum Likelihood method from the histone H3-H4 region sequences *Orthetrum albistylum* , *O. glaucum* and *Rhyothemis phyllis* serve as an outgroup.

To test applicability of the proposed marker to evaluating intra-generic and intra-species variation we estimated its variation and reconstructed a phylogenetic tree for all specimens of the genus *Sympetrum* involved, using the sequences of *R. phyllis*, *O. albistylum* and *O. glaucum* as the outgroup (Fig. 5). The magnitude of intra-species variation of the histone H3-H4 region sequence appeared quite substantial. For the three species represented by quite a number of specimens, *S. croceolum*, *S. uniforme* and *S. danae*, the maximum uncorrected p-distances (that is the share of positions occupied by different nucleotides among all positions) between different alleles within a species appeared to be respectively 0.0216, 0.0037 and 0.0011, that is ca 2.1, 0.4 and 0.1%. The averaged difference between any two sequences within S. *croceolum*, *S. uniforme* and *S. danae* were 0.0165, 0.0009 and 0.0003, respectively.

In the reconstructed phylogenetic tree (Fig. 5), ten sequences of *S. uniforme*, ten sequences of *S. danae* and three sequences of *S. flaveolum* expectedly clustered with the maximum bootstrap support of 100. Strikingly, the cluster of *S. uniforme* appeared to be nested inside that of *S. croceolum*, with the united cluster of these two species also having the support of 100. *S. pedemontanum* and *S. cordulegaster* clustered with a very high support of 76. At the same time, *S.*

fonscolombii showed a very deep divergence from the rest of *Sympetrum*. We may conclude that sequences of any species cluster together with the maximum support or nearly so, while cases of tight clustering of different species corresponded to the notion of their relatedness based on morphology.

It is noteworthy that the sequences of two specimens of *S. croceolum* from its West Siberian isolate (specimens Sc-41 and Sc-42 from Lake Manzherok) (SEE KOSTERIN 2002) did not show divergence from those from the main Far Eastern range of the species (the rest specimens) but nested among them.

Discussion

The marker proposed

The high conservation of the histone H3 and H4 proteins is reflected by the high homology of their coding sequences, the variation of which is nearly confined to synonymous substitutions only (STEIN et al. 1984; DOENECKE et al. 1997; EIRÍN-LÓPEZ et al. 2009). This allowed us to design primers highly specific to these particular genes but of a very broad specificity as to the biological objects. It is noteworthy that the substitutions in the histone coding sequence, the overwhelming majority of which are synonymous, in the histone H3-H4 region provide enough variation for satisfactory resolving phylogenetic relations between the studied specimens (Fig. 3). Because of this, the histone H3 gene coding sequence has been broadly used as a phylogenetic marker for short evolutionary distances, in Odonata, e.g., by CARLE et al (2015), with conserved positions permitting universal primers whereas the phylogenetic signal was mostly provided by synonymous substitutions.

The here proposed marker, the histone H3-H4 region, has an advantage of possibility to design highly universal primers matching the most conserved eukaryotic coding regions, those of histones H3 and H4. Note, that we used LH4_2R primer designed to match the sequence of a lepidopteran, *Bombyx mori*. The amplified fragment contains most of the coding region of histone H4 and about a half of that of histone H3, and ca 250 b.p. long spacer between them. No adaptive constraint is expected for variation of the spacer, which hence has a neutral regime of evolution, while variation for the coding regions is almost restricted to synonymous substitutions only, as we also observed in the present study.

The histone H3-H4 region involving both highly conserved sequences and a non-coding spacer plus being tandemly repeated makes the proposed phylogenetic marker similar in biological and technical respects to such popular nuclear marker as the ITS region of the nucleolus organiser (for its use in Odonata see HOVMÖLLER & JOHANSSON 2004; DUMONT et al. 2010; Schneider et al. 2023) including the internal spacers *ITS1* and *ITS2*. The length of ca 250 b.p. of the spacer in the here proposed marker is comparable to ca 200 b.p. of *ITS1* and ca 160 b.p. of *ITS2* (these figures are for Odonata). Both markers, the ITS and histone H3-H4 region, have comparable lengths (ca 900 b.p.) and are encoded by the nuclear genome but functionally unrelated. Hence the histone H3-H4 region can be used for the same purposes as the ITS region. Hence it can update the traditional analysis of the ITS region with about the same amount of independent phylogenetic information of the same nature. A joint analysis of both similar but unrelated nuclear markers, ITS and H3-H4

regions (by their concatenation or, better, involving software specially designed for simultaneous analysis of different markers, such as StarBeast), is expected to provide a more robust phylogenetic inference than the analysis of ITS alone. Judging by the phylogenetic trees obtained (Figs 2, 5), the use of the histone H3-H4 region as a phylogenetic marker is highly recommendable at the levels of species and genera. Since it correctly resolves the family structure of the order but with variable and often insufficient support, it could be also useful at the level of families as well but only as an additional marker analysed together with other phylogenetic markers.

Because of conservativeness of the histone H3 and H4 genes, the new marker can be used with the primers provided herein for any Odonata and those insects with the same order and orientation of the histone H3 and H4 genes in the histone repeat, e.g. Diptera exemplified in the present study by such genera as *Anopheles* Meigen, 1818 used for outgroup in the phylogenetic trees of Figs 2-4. For the use of the here proposed marker in insects with other order or orientation of these genes, relevant other primers have to be worked out. For example, in Lepidoptera, the same LH4-2R primer can be used in combination with a primer matching the same H3 histone gene region as Hex-AR but in the reverse orientation. In this case, the portion of the H3 gene will be smaller, 37 bp, and the spacer will be somewhat longer - about 900 - 1400 bp.

***Sympetrum* spp.**

Two rather unexpected results concerned the species *S. croceolum* and *S. uniforme*. First, *S. uniforme* appeared to be an inner branch nested inside *S. croceolum*. This result appeared robust regardless of the methods and models of phylogenetic reconstructions (not shown). These species are obviously related but well distinguishable by the morphology of the male genitalia and female vulvar scale, the wing coloration (dull, complete but gradually changing in intensity in *S. uniforme* versus bright but with gaps in *S. croceolum*) and size (the former is somewhat larger). In East Asia, they usually co-exist in a wide range of lentic habitats (ONISHKO & KOSTERIN 2022), while *S. croceolum* also has an isolated range fragment in the southern West Siberia (KOSTERIN 1987; 2002; POPOVA & HARITONOV 2020). It should be stressed that specimens of *S. uniforme*, identified by external characters, formed a highly supported cluster (Fig. 5). There is no doubt that *S. croceolum* and *S. uniforme* are *bona species*. The phylogenetic pattern obtained, where the sequences of *S. uniforme* are nested inside those of *S. croceolum*, suggests *S. uniforme* being a phyletic descendant of *S. croceolum*, which hence appeared paraphyletic. This contradicts the cladistic approach in systematics and the phylogenetic concept of species. At the same time, this pattern fits well the so-called punctuated equilibria mode of speciation (ELDREDGE & GOULD 1972), suggesting that speciation takes place for short time periods in evolutionary scale (tens to hundreds of thousand years) in small, isolated populations in the periphery of parental species' ranges, while species exist almost unchanged for millions of years (evolutionary stasis). This concept better fits basics of evolutionary genetics (MAYR 1963; BERDNIKOV 1999) than the earlier prevailing model of gradual divergent evolution. In the punctualistic point of view, species 'propagate' as if being individuals, with younger species often co-existing with their parental species.

The lack of divergence of the analysed sequence of two specimens of *S. croceolum* from its West Siberian isolate (specimens Sc-41 and Sc-42 from Lake Manzherok) from specimens from the main Far Eastern range of the species (the rest specimens) is remarkable. Specimens from the West

Siberian isolate (KOSTERIN 2002) differ from the Far Eastern specimens by much more developed wing amber colour and appearance of a brown enfumation in the wing apical parts and so were for a long time supposed to represent a separate subspecies (KOSTERIN 1987; POPOVA & HARITONOV 2020), which, however, has not got a name yet. This lack of divergence suggests the West Siberian isolate to be very young in evolutionary time scale and well fits its hypothetic Holocene age, implying the range split after Atlantic time (KOSTERIN 2002), as well as in some nemoral species of Lepidoptera (DUBATOLOV & KOSTERIN 2000; SOLOVYEV et al. 2015; 2022). This, however, does not exclude a subspecies rank of the West Siberian population(s), since subspecies are entities of well-defined geographical variation for some phenotype characters and so imply specific divergence for the genes determining these characters rather than for any genes of the genome.

In all phylogenetic reconstructions (Figs 2-5), *S. fonscolombii* is strongly diverged from the rest of the genus *Sympetrum*. The same result was earlier obtained by PILGRIM & VON DOHLEN (2012) who undertook a molecular phylogenetic study of *Sympetrum* and related genera based on the joint analysis of the nuclear marker *EF- 1α* and ITS2, and the mitochondrial genes *16S, tRNAvaline, 12S*, and *CO*I. This fact has long ago been recognised at the level of phenotype, resulting in a suggestion to move *S. fonscolombii* to the genus *Tarnetrum* Needham & Fischer, 1936 (SCHMIDT 1987). This genus was erected for the Nearctic species *Mesothemis corrupta* Hagen, 1861, and *M. illota* Hagen, 1861, mentioned therein as well as presently considered in combinations *Sympetrum illotum* and *S. corruptum*, with *M. illota* (sub. *S. illotum*) indicated as the type species (NEEDHAM & FISCHER 1936). Of them, *S. corruptum* is quite closely related to *S. fonscolombii* (together with *S. villosum* Ris, 1911 and *Nesogonia blackburni* (McLachlan, 1883)) while *S. illotum* is not (PILGRIM & VON DOHLEN 2012). Since the type species of the genus *Tarnetrum* is not closely related to *S. fonscolombii*, this genus is not suitable for the latter species (DIJKSTRA & KALKMAN 2015). Therefore, the genus *Sympetrum* in the current sense deserves further reconsideration, maybe with erection of a new genus at least for the *fonscolombii*-group *sensu* PILGRIM et al. (2012).

Acknowledgements

The work was supported by the project FWNR-2022-0019 of the Institute of Cytology and Genetics SB RAS. DNA sequencing was performed at the SB RAS Genomics Core Facility, Novosibirsk. Assembly of sequences from SRA archave was performed at Siberian SuperComputer Computational Facility, Novosibirsk.

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