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The complete mitogenome of *Podonevadne trigona* (Sars, 1897) (Cladocera: Onychopoda: Podonidae) sheds light on the age of podonid differentiation

Полный митогеном *Podonevadne trigona* (Sars, 1897) (Cladocera: Onychopoda: Podonidae) прояснил возраст дифференциации подонид

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КЛЮЧЕВЫЕ СЛОВА: Евразия, Понто-Каспийский регион, филогеография, Crustacea.

ABSTRACT. In this study, we have sequenced and annotated the complete mitogenome of *Podonevadne trigona* (Sars, 1897) (Cladocera: Onychopoda: Podonidae) with the aim of to estimate the differentiation age of the podonid genera. The complete mitogenome of *P. trigona* (NCBI GenBank accession no. OR799522) has a length of 19222 bp, and includes 13 protein coding genes (PCG), 22 t-RNA genes, 2 rRNA genes, and a control region containing tandem repeats of 1409 bp. Due to a weak coverage and ambiguous results of some previous assemblages, our phylogenetic reconstruction was based on protein-coding loci only. Our molecular clock analysis included several approaches: (1) relaxed molecular clock with two calibration points; (2) strict molecular clock with a single calibration point; (3) strict molecular clock based on mutation rate of 1.4% per 1 MYR; (4) Optimised Relaxed Clock model with two calibration points. In all cases, even with maximally younger clades, the molecular clocks suggest a very old, Late Mesozoic to Early Cenozoic, differentiation of the Podonidae and even the genera within this family in contrast to opinion of Cristescu & Hebert [2002] about the Late Miocene podonid differentiation in the Pontian Sea-Lake existed just 6–7 MYA. We can roughly hypothesise that Podonidae was originated from a freshwater ancestor and differentiated as a coastal (maybe, an estuarine?) group already in Tethys, during Late Mesozoic, but then all genera (1st scenario) or a part of the genera (2nd scenario) survived in the Paratethys (then in the Sarmatian Sea, and then in the recent Ponto-Caspian basin).

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РЕЗЮМЕ. В данной работе мы секвенировали и аннотировали полный митогеном *Podonevadne trigona* (Sars, 1897) (Cladocera: Onychopoda: Podonidae) с целью оценки возраста дифференциации родов подонид. Полный митохондриальный геном *P. trigona* (NCBI GenBank OR799522) имеет длину 19222 п.н. и включает 13 белок-кодирующих генов (PCG), 22 гена т-РНК, 2 гена рРНК и контрольный регион с тандемными повторами длиной 1409 п.н. Из-за слабого покрытия и неоднозначных результатов некоторых предыдущих сборок наша филогенетическая реконструкция была основана только на белок-кодирующих локусах. Анализ времени расхождения клад, основанный на «молекулярных часах» включал несколько подходов: (1) расслабленные молекулярные часы с лвумя точками калибровки; (2) строгие молекулярные часы с единственной точкой калибровки; (3) строгие молекулярные часы, основанные на скорости мутаций 1,4% на 1 миллион лет; (4) модель Оптимизированных Расслабленных Часов с двумя точками калибровки. Во всех случаях, даже для самых молодых клад, молекулярные часы свидетельствуют об очень древней (поздней мезозойской – ранней кайнозойской) дифференциации Podonidae и даже родов внутри этого семейства, что противоречит мнению Кристеску и Хеберта [Cristescu, Hebert, 2002] о поздней Миоценовой дифференциации подонид в Понтийском море-озере, существовавшем всего 6–7 млн. лет назад. Можно предположить, что Podonidae произошли от

пресноводного предка и дифференцировались как прибрежная (может быть, эстуарная?) группа еще в Тетисе, в позднем мезозое, но затем все роды (1-й сценарий) или часть родов (2-й сценарий) выжила в Паратетисе (затем в Сарматском море, затем в современном Понто-Каспийском бассейне).

Introduction

Foundation of phylogeography at the end of the 20th century [Avise, 2000] has significantly improved our understanding on the history of biogeographic pattern formation in different terrestrial and freshwater animals [Hewitt, 2001]. To date, a set of different methods has been proposed to reveal the dispersion centers and directions, to estimate age of the phylogroup differentiation, etc., mainly based on data on the mitochondrial gene variability and mitochondrial haplotype distribution. Such recent efforts involve different freshwater invertebrates and cover different regions of the planet [Bernatchez, Wilson, 1998; Santamaria, 2013; Bolotov *et al.*, 2017; Tomilova *et al.*, 2020], although any publications on a global scale still are relatively rare both for terrestrial and aquatic animals [Hewitt, 2000; Durbin *et al.*, 2008].

Cladocera (Crustacea: Branchiopoda) is an important model group for phylogeographic studies since pioneer works of P. Hebert's group [Taylor *et al.*, 1998; Weider *et al.*, 1999; Cox, Hebert, 2001]. In the Holarctic, significant progress is achieved in our understanding of Late Pleistocene/Holocene evolutionary history of different cladoceran genera, mainly *Daphnia* O.F. Müller, 1785 [Petrusek *et al.*, 2007; Fields *et al.*, 2018; Zuykova *et al.*, 2019], while references to much older scenarios are less common [Adamowicz *et al.*, 2009; Xu *et al.*, 2011; Kotov, Taylor, 2011; Kotov *et al.*, 2021; Hamza *et al.*, 2022].

Genomics, as a "style" and rapidly growing direction of genetic studies, has opened a new page in phylogeography due to a huge increase in the volume of information available for conducting any mathematical analyses and modelling for accurate reconstructions of paleo-events. Reconstructions are usually based on a single or few mitochondrial genes, while complete mitochondrial genomes (mitogenomes) provide much more information for a multilocus mitochondrial phylogeny, even if we know that a mitogenome is a single linkage group [Rubinoff, Holland 2005].

First tests of the cladoceran mitogenome-based phylogeography were very promising [Fields *et al.*, 2018], but any global phylogeographic studies on this group based on mitogenomes are absent. It is important to note that studies of mitogenomes provide us with information of events on different time scales, from very recent to ancient. If our ideas on the Pleistocene history of cladocerans based on mitochondrial phylogeography are more or less obvious [Taylor *et al.*, 1998; Ishida, Taylor, 2007; Faustova *et al.*, 2011; Zuykova *et al.*, 2019; Karabanov *et al.*, 2021], deeper evolutionary history of many taxa requires further studies [Cornetti *et al.*, 2019; Van Damme *et al.*, 2022].

Using a genomic technology, we can try to resolve a question concerning age and region of the differentiation of the family Podonidae Mordukhai-Boltovskoi, 1968 (Cladocera: Onychopoda), a remarkable group of predatory cladocerans mainly distributed in the Caspian Sea, Black Sea, and coastal seas of the World Ocean [Mordukhai-Boltovskoi, Rivier, 1987] in contrast to the majority of water fleas inhabiting continental water bodies. Recently several podonid taxa are expanding their distribution range, moving from the Ponto-Caspian basin north through the large rivers of this basin, and even were occasionally introduced to the Baltic Sea [Mordukhai-Boltovskoi, Rivier, 1987], where they have strongly expanded their population size and sometimes modified the local ecosystems [Kotov *et al.*, 2022].

Note that the Ponto-Caspian basin played an important role in the radiation of other euryhaline crustaceans [Dumont, 1998] as strong salinity fluctuations in this region are well-known for different epochs [Dumont, 2000; Esin *et al.*, 2018]. Sars [1902] proposed a doubled origin of the Caspian onychopod fauna from freshwaters and the World Ocean. Mordukhai-Boltovskoi & Rivier [1987] agreed with him; they also assumed a much earlier differentiation of Podonidae and Polyphemidae (and Cercopagididae, as the closest relative of the latter) and an ancient penetration of the marine onychopods to the World Ocean from freshwaters. Rivier [1998] emphasized that onychopods penetrated the seas from freshwaters, and then podonids descend from a pure marine ancestor. The Caspian onychopod fauna originated "both from freshwater and from oceanic ancestors", and "repeated penetration of the oceanic species to Caspian basin, or *vice versa*" took place [Rivier, 1998: 101].

Cristescu & Hebert [2002] have performed a phylogeographic study covering most genera of the Onychopoda and proposed an alternative scheme of a Middle Miocene differentiation (from a freshwater ancestor) of the families in the Sarmatian Sea (a remnant of Paratethys) and Late Miocene differentiation of the podonid genera in the Pontian Sea-Lake located in recent Ponto-Caspian basin. In their opinion, only in the Pliocene some podonids, being to that time endemics of the Ponto-Caspian basin, penetrated the World Ocean during the periods when the former was interconnected with the latter. This reconstruction was based on the NJ linearized trees from three mtDNA genes (COI, 12S, 16S) and a single nuclear gene (18S) and molecular clock estimations based on previous ideas on the rate of sequence divergence per million years. Such a scenario seemed to be nice and logical, but pioneer mitogenomic studies have caused doubts in so young an age of onychopod differentiation. Xu *et al.* [2021] have proposed a Triassic, and Van Damme *et al.* [2022], based on nuclear genes, even a Permian differentiation that is almost ten times older, compared to the estimation of Cristescu & Hebert [2002].

In this study, we have sequenced and annotated the mitogenome of *Podonevadne trigona* (Sars, 1897) with the aim to estimate the differentiation age of the podonid genera. Note that partial mitogenomes of *Podon* Lilljeborg, 1853 and *Evadne* Lovén, 1836 have already been studied previously [Xu *et al.*, 2021].

Material and Methods

DNA Sequencing. A single adult parthenogenetic female of *P. trigona* from a sample collected in the Volgograd Water Reservoir (51.6662° N, 46.1781° E) by D.P. Karabanov and R.Z. Sabitova and fixed in 96% EtOH (sample AAK M-6091 in the working collection of A.A. Kotov at SIEE RAS, Moscow) was used for this study. DNA from the specimen was isolated using the QiAmp Micro Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was checked with Qubit 3.0. Library preparation and sequencing (Illumina NovaSeq6000, paired reads of 150bp, 77 mln reads in total) were performed by Novogene Co., Ltd. (https://www.novogene.com/).

Assembly and annotation of the mitogenome. Raw reads were processed and filtered by fastp v0.23.3 [Chen, 2023] with default parameters. After that, get_organelle_from_reads.py script from GetOrganelle v1.7.7.0 [Camacho *et al.*, 2009; Bankevich *et al.*, 2012; Langmead, Salzberg, 2012; Jin *et al.*, 2020] was used with several custom flags (--max-reads 3E10 --reduce-reads-for-coverage inf -R 10 -k 45,65,85,105,127 -F animal_mt) and custom seed database based on mitogenome of *Daphnia magna* (GenBank RefSeq NC_026914.1). The obtained sequence was annotated by the "annotate" command from Mitoz v3.6 [Birney *et al.*, 2004; Gertz *et al.*, 2006; Krzywinski *et al.*, 2009; Li, Durbin, 2009; Nawrocki, Eddy, 2013; Li *et al.*, 2009; Juhling *et al.*, 2013; Huerta-Cepas *et al.*, 2016; Meng *et al.*, 2019]. Subsequently, automatically produced annotation was corrected manually.

The tRNA genes were verified by predicted secondary structures with tRNAScan-SE 2.0 web-service (http://lowelab.ucsc.edu/tRNAscan-SE/ [Chan, Lowe, 2019], in two cases, when the latter failed, ARWEN web-service (http://130.235.244.92/ARWEN/ [Laslett, Canback, 2008] was used. The secondary structures were visualised in 'forna' web-service (http://rna.tbi.univie.ac.at/forna/ [Kerpedjiev *et al.*, 2015]). The rRNA genes, where it was possible, were delineated by the boundaries of neighboring tRNA genes. Boundaries of PCGs were deducted by analysis of their alignments in the corresponding genes of other cladocerans, for whom the mitogenomes were available, using MAFFT v7.520 [Katoh, Standley, 2013], respecting several conditions: 1) the genes start with a start codon; 2) the genes end with a stop codon, "T" or "TA"; 3) there are no stop codons inside; 4) in accordance with "tRNA Punctuation Model" [D'Souza, Minczuk, 2018], there are no overlaps with tRNA genes on the same strand. The NOVOPlasty v4.3.3 [Dierckxsens *et al.*, 2017] with assembled sequence as a bait, using the base config file and the raw reads, circularised the mitogenome. Boundaries of the control region were delineated by the boundaries of the flanking genes, and the control region was included into annotated assembly with UGENE v.48.1 [Okonechnikov *et al.*, 2012]. Using UGENE a new sequence origin was set at the start of cox1, and plus-strand was made co-directional with it. Nucleotide and codon analyses were performed in UGENE and MEGA-11 [Tamura *et al.*, 2021]. Gene map visualisation was created using the Proksee web-service [Grant *et al.*, 2023].

Phylogenetic analysis. For our phylogenetic study we formed a matrix of 36 previously published and de-novo assembled mitogenomes [Xu *et al.*, 2021]. Due to a weak coverage and ambiguous results of some assemblages, only protein-coding loci were analysed (Suppl. Table 1). Their sequences were extracted from the genomes to individual files in the UGENE editor [Okonechnikov *et al.*, 2012]. A global alignment was performed for each locus using the MUSCLE v.5 [Edgar, 2022] algorithm taking into consideration a triplet translation. Then all files were composed into a single NEXUS file with partition block using SequenceMatrix v.1.9 [Vaidya *et al.*, 2011].

Phylogenetic reconstruction was performed in BEAST2 v.2.7 package [Bouckaert *et al.*, 2019]. We used BEAST2 add-on bModelTest v.1.3 [Bouckaert, Drummond, 2017] to search for best-fitting models of the nucleotide substitutions for each locus taking into consideration the position of each codon in the triplet. According to the identified models, Bayesian phylogenetic reconstruction for the whole unlinked dataset was performed by four independent runs (10M generations, with selection of each 10k generations) for each tree. We used Tracer v.1.7 [Rambaut *et al.*, 2018] to evaluate MCMC chain convergence based on ESS>200. Trees were combined in LogCombiner v.2.7, a consensus tree based on the maximum clade credibility (MCC) was obtained in TreeAnnotator v.2.7 with burn-in of the first 20% trees according to recommendations of Drummond & Bouckaert [2015]. A Yule process model [Yule, 1924] was selected as a prior to the speciation process as the most general for most datasets [Steel, McKenzie, 2001]. Posterior probabilities from BEAST2 were used to estimate branch support [Drummond, Bouckaert, 2015].

Our molecular clock analysis included several approaches:

1) relaxed molecular clock [Drummond *et al.*, 2006], calibration points: Notostraca/Diplostraca — 250 MYA, *Daphnia*/other Daphniidae — 145 MYA [Kotov, Taylor, 2011];

2) strict molecular clock [Ferreira, Suchard, 2008] calibration point: Notostraca/Diplostraca — 365 MYA [Gueriau *et al.,* 2016];

3) strict molecular clock [Ferreira, Suchard, 2008], mutation rate 1.4% per 1 MYR for crustaceans [Schwentner *et al.* 2013];

4) Optimised Relaxed Clock model [Douglas *et al.,* 2021], calibration points: Notostraca/Diplostraca — 250 MYA, *Daphnia*/other Daphniidae — 145 MYA [Kotov, Taylor, 2011].

Results

Structure and composition of the mitogenome. The complete mitogenome of *Podonevadne trigona* (GenBank accession no. OR799522; Fig. 1 and Suppl. Table 2) has a length of 19222 bp, and includes 13 protein coding genes (PCG), 22 t-RNA genes, 2 rRNA genes, and a control region containing tandem repeats of 1409 bp (NOVOPlasty reconstructed 3 units, though it is impossible to resolve their number accurately using only short reads). The plus strand is a majority strand with 23 genes (including 9 PCG and 14 tRNA), 14 genes (4 PCG, 8 tRNA, 2 rRNA) belong to the minus strand. Furthermore, the plus strand is a light strand with T+G = 48% excluding CR (48.2% including CR with 3 repeats, 49.2% — repeat unit), or, in terms of AT and GC skews, AT-skew = (A – T) / (A + T) = 0.01 excluding CR (0.01 including CR with 3 repeats, 0.00 — repeat unit), GC-skew = (G – C) / (G + C) = –0.09 excluding CR (–0.08 including CR with 3 repeats, –0.03 — repeat unit). The GC content is 36.4% excluding CR (37.6% including CR with 3 repeats, 41.7% — repeat unit).

All 22 tRNA genes typical of the invertebrates were found in the mitogenome of *P. trigona,* (Fig. 2, Table 2). Lengths of these genes vary from 63 bp (trnG and trnC) to 71 bp (trnV), total length equals 1471 bp. All tRNA genes could be folded into the typical cloverleaf secondary structure, excluding trnS1, which lack the DHU arm, similarly to other animals [Juhling *et al.*, 2012]. There are rRNA genes for both large and small subunits of the mitochondrial ribosome, their total length is 2181 bp.

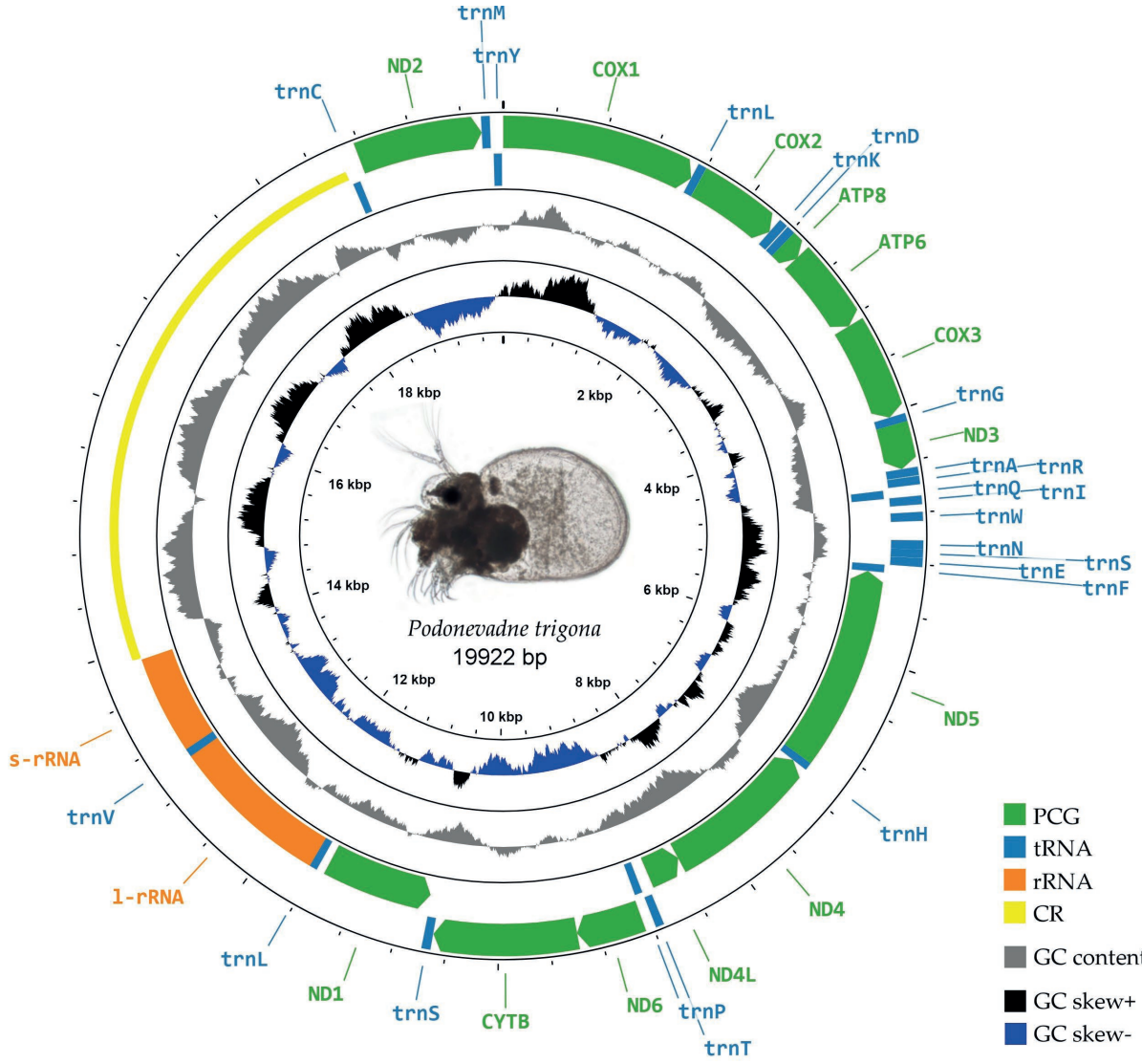

Fig. 1. Gene map of the mitogenome of *Podonevadne trigona*. Starting from the outermost ring: Ring 1 — DNA (+) strand; Ring 2 — DNA (–) strand; Ring 3 — GC content; Ring 4 — GC skew.

Рис. 1. Генная карта митохондриального генома *Podonevadne trigona*. Начиная с внешнего кольца: Кольцо 1 — нить ДНК (+); Кольцо 2 — нить ДНК (–); Кольцо 3 — содержание GC; Кольцо 4 — перекос GC.

The mitogenome of *P. trigona* uses the invertebrate mitochondrial genetic code (NCBI translation table no. 5). Total length of PCG is 11073 bp. Generalised sequence for the start codons is "RTB", the vast majority of them is "RTG" (11/13), and "ATG" (9/13) is the most common one. "ATC" and "ATT" are found only in nad6 and nad4l, respectively. More than a half (7/13) of the stop codons are truncated ("TA": 2, "T": 5), full ones in all but one case ("TAG" in nad1) are "TAA". The most frequent amino acids are leucine (16%) and serine (13.4%), while glutamine (2.1%) and aspartic acid (2.4%) are the rarest ones. In all positions of the codons, "T" is the most frequent (37–39%), "G" is the rarest (17–18%). There are 3678 codons in toto, excluding the stop codons. Relative synonymous codon usage is shown at Fig. 3.

If we compare the gene order of *P. trigona* and the Pancrustacea gene order (Fig. 4), a hypothetical ancestral state [Castellucci *et al.*, 2022] of Pancrustacea, Cladocera, and "Olygopoda" (Anomopoda + Onychopoda), it is clear that changes in *Podonevadne* affect only tRNA genes, moreover, the affected genes were located in a compact area between CR and trnY. Firstly, trnM, trnC (and, hypothetically, trnW) were transposed relatively to nad2. Secondly, several tRNA genes were relocated into the tRNA gene cluster between nad3 and nad5, specifically between trnR and trnN. Remarkably, all involved genes preserved their strand orientation. The affected regions appear as rearrangement hotspots in the mitogenomes of the Branchiopoda [Castellucci *et al.*, 2022].

Molecular clock estimations. Different approaches lead to very different ideas on the age of major clades differentiation. We ignore here questions on the cladoceran origin, and discuss only the order Onychopoda and the family Podonidae within the latter. Relaxed molecular

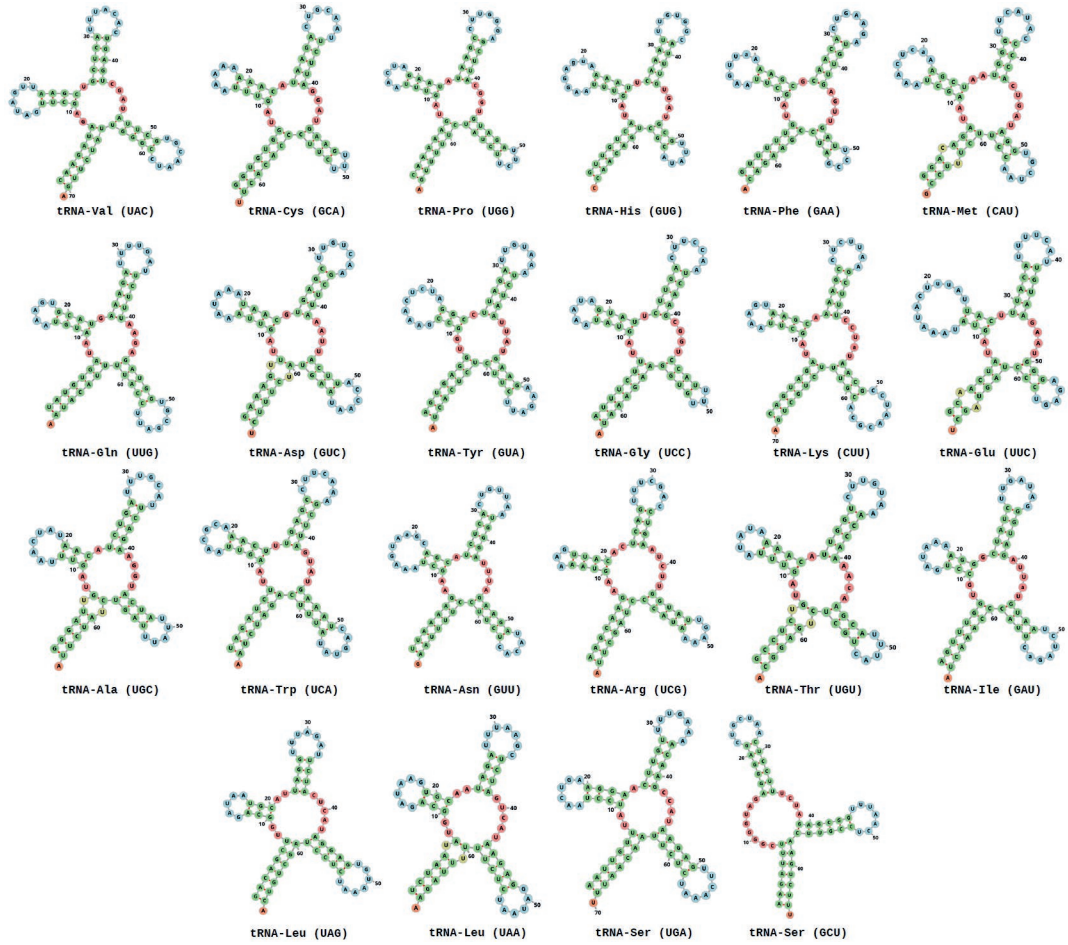

Fig. 2. Inferred secondary structure of tRNA genes in the mitogenome of *Podonevadne trigona*.
Рис. 2. Предполагаемая вторичная структура генов тРНК в митогеноме *Podonevadne trigona*.

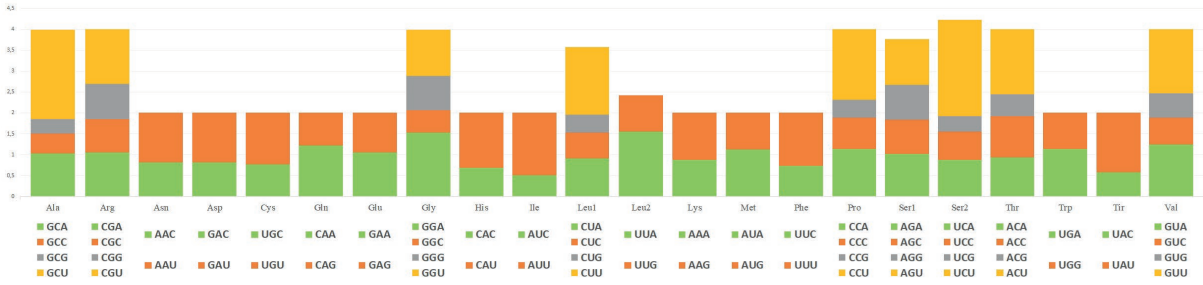

Fig. 3. Relative synonymous codon usage (RSCU) of the mitogenome of *Podonevadne trigona*.
Рис. 3. Относительное использование синонимичных кодонов (RSCU) в митохондриальном геноме *Podonevadne trigona*.

clock estimates the onychopod differentiation time as the Upper Triassic-Lower Jurassic and the podonid differentiation time as the Upper Jurassic-Lower Cretaceous (Fig. 5). Strict molecular clock with fossil calibration estimates the onychopod differentiation time as the Silurian-Devonian and the podonid differentiation time as the Carboniferous-Permian (Fig. 6). The strict molecular clock based on mutation rate estimates the onychopod differentiation time as the Upper Cretaceous and the podonid differentiation time as the Eocene (Fig. 7). The optimised relaxed clock model demonstrates wide ranges of estimated ages both for Onychopoda and Podonidae (Jurassic to Palaeogene (up to Oligocene?)), with medians of ca. 130–120MYA for Onychopoda (Lower Cretaceous) and ca. 70–60 MYA (Upper Cretaceous) for Podonidae (Fig. 8).

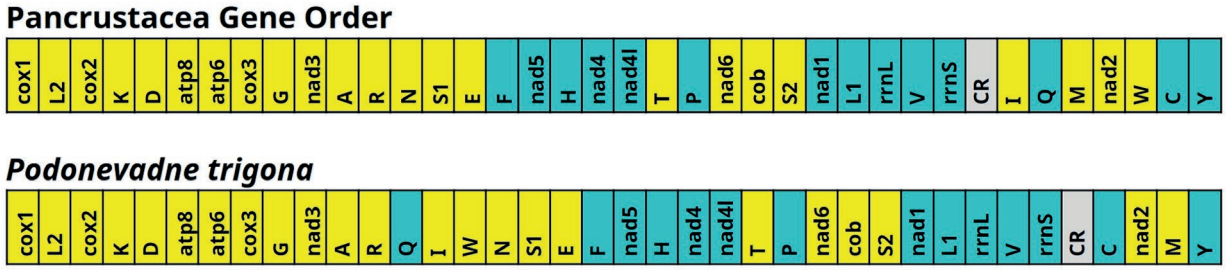

Fig. 4. Gene order in the mitogenome of *P. trigona* (bottom) compared with a hypothetical ancestral Pancrustacea Gene Order (top). Yellow and cyan colours indicate different strands.

Рис. 4. Порядок генов в митохондриальном геноме *P. trigona* (внизу) в сравнении с гипотетическим порядком генов предков Pancrustacea (вверху). Желтым и голубым цветами обозначены разные цепи ДНК.

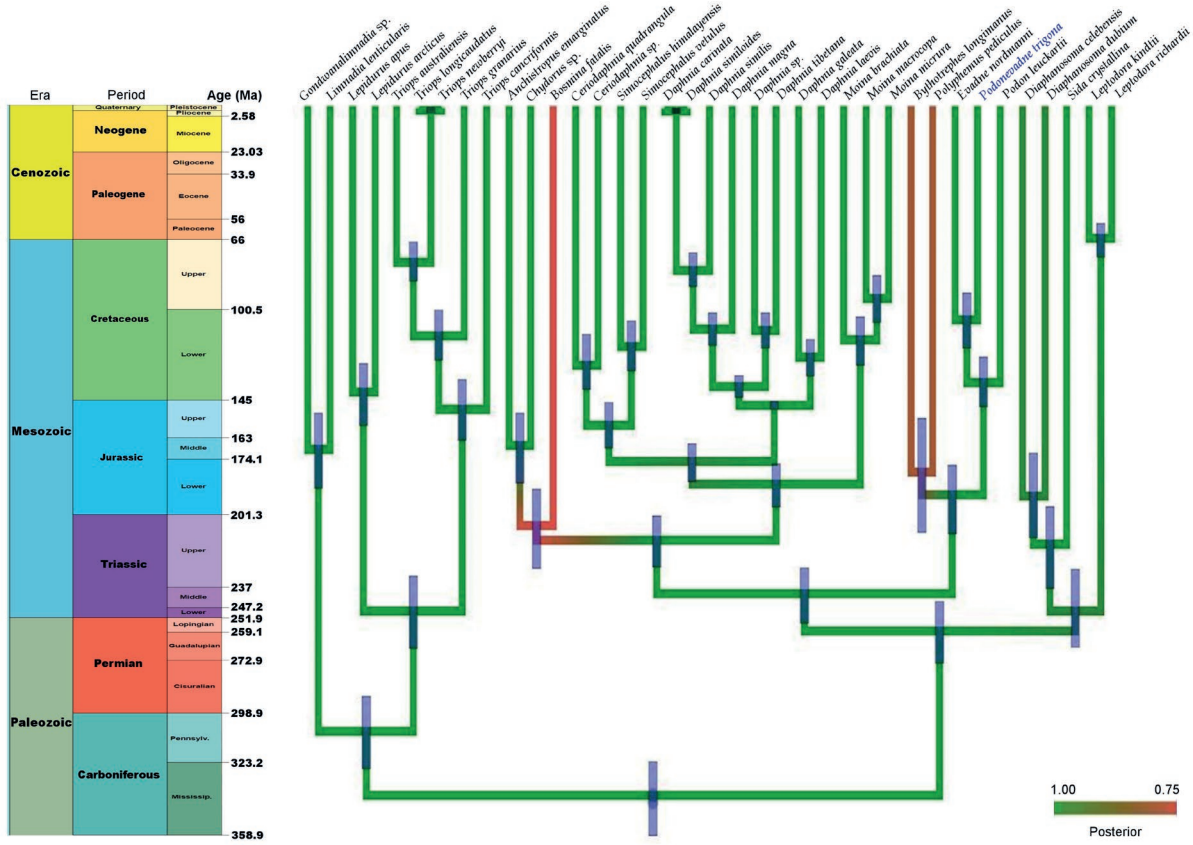

Fig. 5. Phylogenetic tree based on protein-coding mitochondrial genes for Cladocera and Notostraca with relaxed molecular clock based on two calibration points.

Рис. 5. Филогенетическое дерево на основе белок-кодирующих митохондриальных генов для Cladocera и Notostraca с оценкой времени расхождения клад по расслабленным молекулярным часам на основе двух точек калибровки.

Discussion

Above we represent four different variants of the molecular clocks, with estimated age of the main branch differentiation several times different. A phylogenetic tree with relaxed molecular clock based on two calibration points (Fig. 5) gives results similar to those of previous authors (e.g. Cornetti *et al.* [2019]). But for molecular clocks based on fossil calibration points, just choise of the latter is critical [Luo, Ho, 2018], while other priors do not affect significantly the results of such analysis [Sarver *et al.* 2019]. Comparing the trees based on relaxed (Fig. 5) and strict (Fig. 6) molecular clock, we can see doubled difference in the branch length.

Strict molecular clock based on the Notostraca/Diplostraca calibration point (Fig. 6) seems to be minimally realistic among others. It suggests that Branchiopoda differentiation took place in the Neo-Proterozoic what seems to be a ridiculous idea. Probably, in this case we see the result of mutation saturation at such large time intervals,

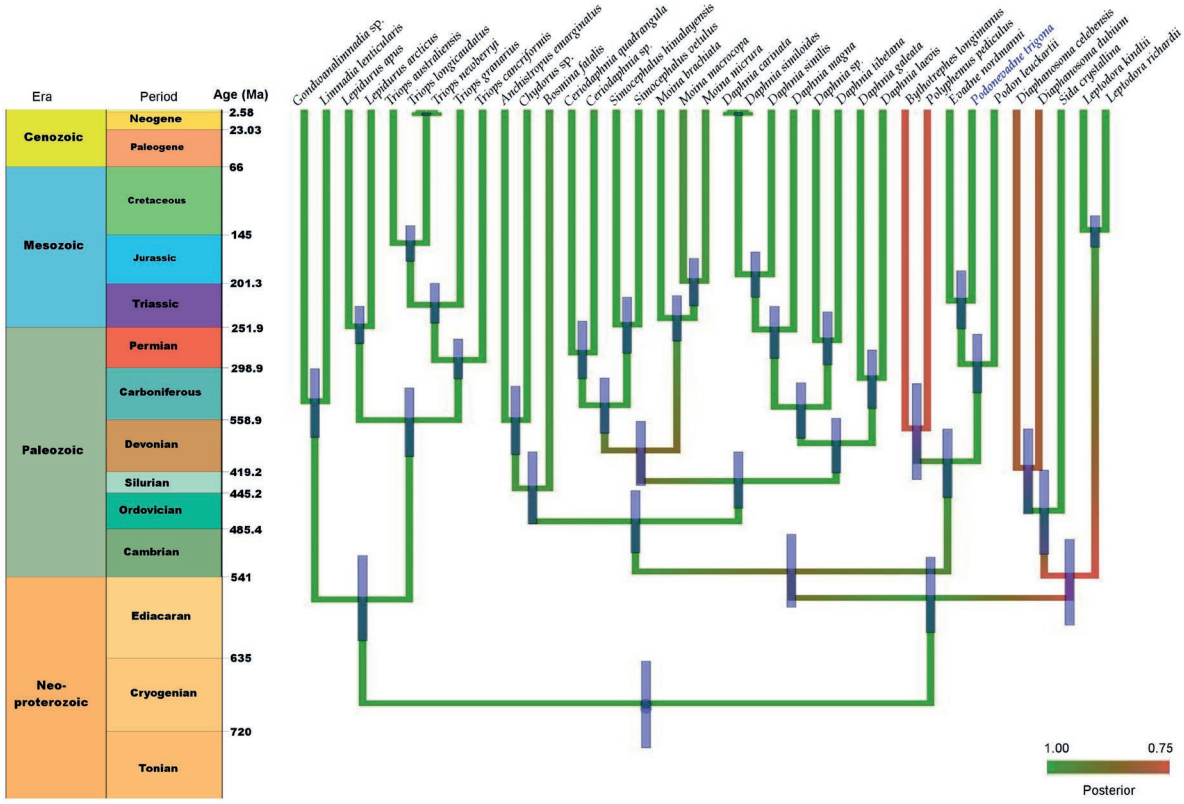

Fig. 6. Phylogenetic tree based on protein-coding mitochondrial genes for Cladocera and Notostraca with strict molecular clock estimates based on a single calibration point.

Рис. 6. Филогенетическое дерево на основе белок-кодирующих митохондриальных генов для Cladocera и Notostraca с оценками времени расхождения клад по строгим молекулярным часамв на основе одной калибровочной точки.

and the idea of molecular clock does not work on such a time scale. Such limitations are overcome by using of the relaxed molecular clock model [Drummond *et al.*, 2006], allowing to vary mutation rates in different tree portions. This modern approach has all advantages of traditional relaxed molecular clock, but it is more productive and correct as compared to the latter [Douglas *et al.*, 2021].

The approach with strict molecular clock estimates based on mutation rate 1.4% per 1 MYr gives also adequate results, i.e. the time of *Daphnia* (*Daphnia*) / *Daphnia* (*Ctenodaphnia*) divergence is estimated as the Upper Cretaceous event, which is not far from the time of first record of these two subgenera, 145MYA [Kotov, Taylor, 2011]. Also, the age is similar to that from calibrated trees by Cornetti *et al.* [2019] and Garibian *et al.* [2021], but strongly differs from their estimations based on the mutation (substitution) rate. This fact can be explained using of different mutation rates. It seems that Cornetti *et al.* [2019] mutation rate is at least 2% per 1 MYR, i.e. homoplasies are accumulated much faster than in our model.

The tree based on the Optimised Relaxed Clock model (Fig. 8) has a similar topology with one with the relaxed clock with two calibration points, but the former demonstrates huge ranges of divergence times for any clades. Such ranges reflect more adequately the divergence times as different molecular clocks give 20 times different dating of the same evolutionary events [Pulquerio, Nichols 2007]. We need to conclude that all our molecular clocks are rough, and further search for new calibration points is urgently needed.

In all cases, even with the youngest clades, the molecular clocks indicate a very old, from the Late Mesozoic to Early Caenozoic, differentiation of the Podonidae and even the genera within this family. Even most younger scenarios in this paper disagree with one of Cristescu & Hebert [2002]: podonids, most probably, have differentiated at the time when the Pontian Sea-Lake (existed 6–7 MYA [Esin *et al.*, 2018]) and even Paratethys was not completely formed yet, at least a wide strait (to the Indian Ocean) existed between Africa and Proto-Eurasia. Most probably, the maritime regions of future Paratethys or Peri-Tethys (see Palcu & Krijsman [2021]) at that time had an oceanic salinity and did not differ from the Tethys Ocean in any abiotic variabilities. Therefore, the Pontian Sea-Lake young (Late Miocene) scenario of the podonid differentiation, which is quite attractive and logical, is not supported by our newly obtained genomic data.

At the same time, the family Podonidae is a monophyletic group, and their multi-time independent penetration

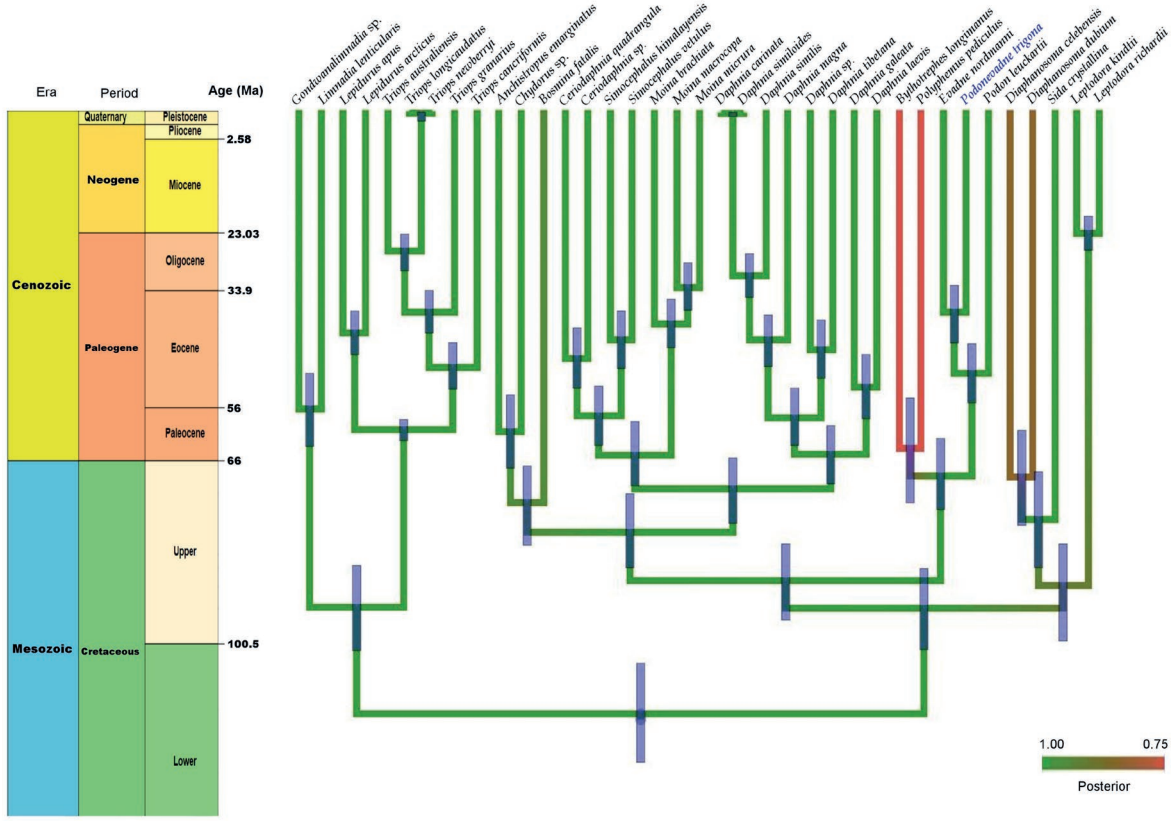

Fig. 7. Phylogenetic tree based on protein-coding mitochondrial genes for Cladocera and Notostraca with strict molecular clock estimates based on mutation rate 1.4% per 1 MYR for crustaceans.

Рис. 7. Филогенетическое дерево на основе белок-кодирующих митохондриальных генов для Cladocera и Notostraca с оценкой времени расхождения клад по строгим молекулярным часам на основе скорости мутаций 1,4% на 1 миллион лет для ракообразных.

to the World Ocean from freshwaters is not the parsimonious version. Most probably, they penetrated the World Ocean only once. The appearance of several World Ocean sub-clades (*Podon*, *Evadne* + *Pseudevadne*, *Pleopis*) within the Ponto-Caspian major clade [Crustescu, Hebert, 2002] must have another explanation.

In our opinion, Paratethys and then the Ponto-Caspian region served as a refugium of already differentiated group (1st scenario) or a part of the group (2nd scenario) rather than a center of its origin. We can roughly hypothesise that Podonidae was differentiated as a coastal (maybe, an estuarine?) group already in Tethys, during the Late Mesozoic, but then all genera (1st scenario) or a part of genera (2nd scenario) survived in the Paratethys (then in the Sarmatian Sea, and then in the recent Ponto-Caspian basin). In the case of the second scenario, just from the latter they had a chance to re-colonize the World Ocean secondarily. Recent information does not allow us to choose the first or the second scenario; further studies of the marine podonids are needed. The COI locus demonstrates a relatively shallow phylogeographic structure which is consistent with a Quaternary (1–4 MYA) Pacific-Atlantic split. But we do not expect to find any consequences of the Mesozoic and Early–Mid Cenozoic events in recent haplotype distribution, i.e. due to ocean faunal mixing, by global currents.

Thus, our scenario is a synthesis of ideas of Sars [1902] on a very old age of the onychopods and their origin from a freshwater ancestor, and ideas of Crustescu & Hebert [2002] about especial role of the Ponto-Caspian basin in their evolutionary history. But such studies must be continued, i.e. genomes of other podonids need to be studied.

The most striking feature of our mitogenome assembly is the repeat of 1409 bp. Although NOVOPlasty has reconstructed three units, it is impossible to accurately resolve their number using only short reads. An assembly based on long reads would be helpful to decisively solve this problem. But our short-read assembly will allow us to design primers for the monitoring of *P. trigona* as invasive species in the Volga-Kama basin (and, potentially, in other basins). Since many onychopods, including *P. trigona,* are notorious invasive species, it is necessary to develop methods of their monitoring. Such methods can be based on PCR tests with species-specific primers, as well as on metabarcoding with group-specific primers. Complete mitogenomes of onychopods will facilitate development of both approaches. As far as we know, this work pres-

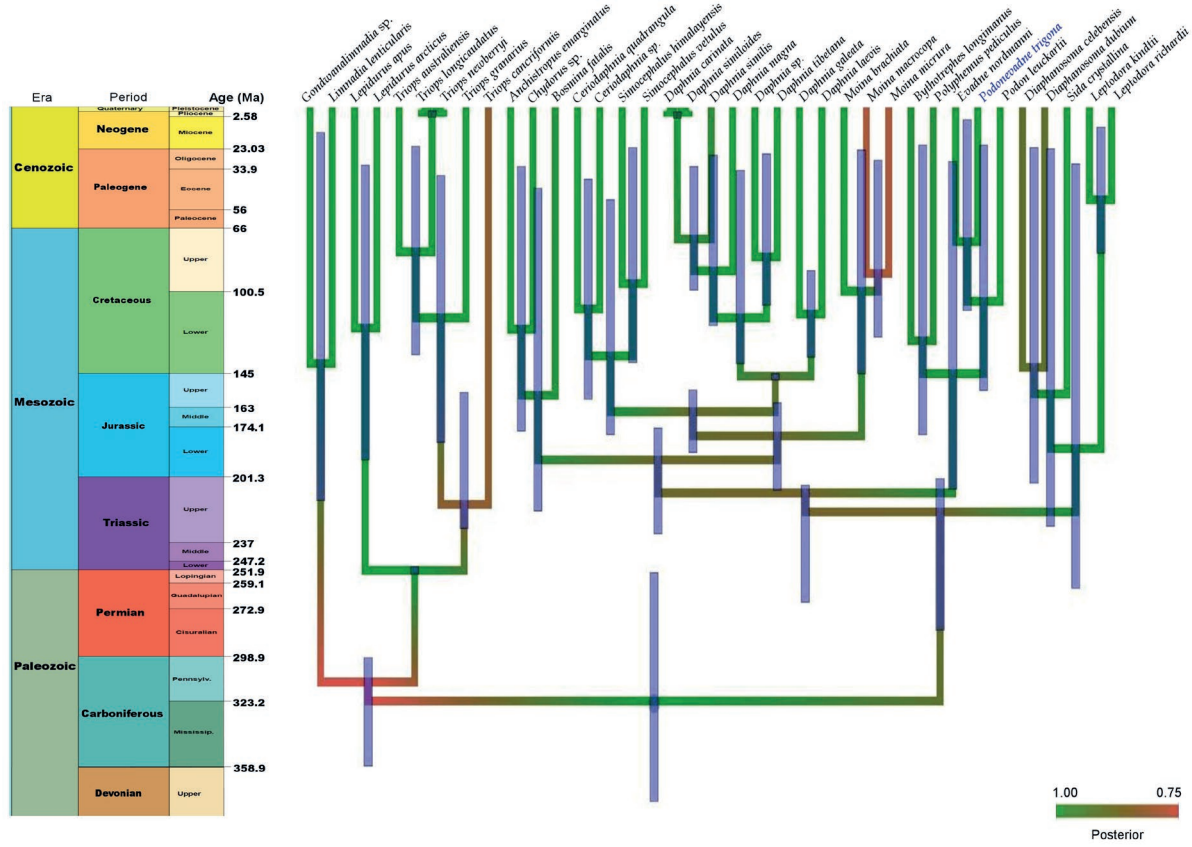

Fig. 8. Phylogenetic tree based on protein-coding mitochondrial genes for Cladocera and Notostraca with the Optimised Relaxed Clock model estimates based on two calibration points.

Рис. 8. Филогенетическое дерево на основе белок-кодирующих митохондриальных генов для Cladocera и Notostraca с оценками времени расхождения клад по модели Optimised Relaxed Clock с использованием двух калибровочных точек.

ents the first complete and annotated mitogenome of the Onychopoda.

Compliance with ethical standards

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

Ethical approval: No ethical issues were raised during our research.

Supplementary Issues

Supplementary Table 1. Mitogenomes incorporated to our analysis (author's taxon names are represented, without a discussion of an identification accurateness).

Supplementary Table 2. Characteristics of the mitogenome of *Podonevadne trigona*.

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