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Origins, phylogenetic relationships and host-parasite interactions of Troglotrematoidea since the cretaceous

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ABSTRACT

In the current study, we raise the issue concerning origins and historical relationships of the trematodes from the families Troglotrematidae and Paragonimidae using phylogenetic analysis and molecular-clock method for estimating evolutionary rates. For the first time we provided 28S rRNA gene fragment (1764 bp) for the type species *Troglotrema acutum* - zoonotic trematodes that cause cranial lesions (troglotremiasis) in mustelid and canid mammals of the Central Europe, Iberian Peninsula, and North-West Caucasus. Molecular genetic analysis revealed that *T. acutum* belongs to the monophyletic family Troglotrematidae sister with the family Paragonimidae. The family Troglotrematidae includes five genera: *Nanophyetus*, *Troglotrema*, *Skrjabinophyetus*, *Nephrotrema*, and *Macroorchis*; and the family Paragonimidae is monotypic including the only genus *Paragonimus*. We recover the superfamily Troglotrematoidea for these two families. Divergence of the common ancestor of the superfamily Troglotrematoidea (common troglotrematoid ancestor) likely occurred during the Cretaceous period of the Mesozoic Era and potentially originated in the Asiatic region. The lineage of the family Troglotrematidae is much closer to the common troglotrematoid ancestor than the species of the family Paragonimidae. The radiation time of the common troglotrematoid ancestor (126 Ma, the Early Cretaceous), and formation of the families Troglotrematidae and Paragonimidae (96 Ma and 73 Ma respectively, the Late Cretaceous) corresponds to the time of settling in East Asia by many species of mammaliaforms (about 130–70 Ma).

1. Introduction

Parasitic flatworms from the families Paragonimidae Dollfus, 1939 and Troglotrematidae (Odhner, 1914) Braun, 1915 cause dangerous zoonotic diseases of a wide range of mammalian species: paragonimiasis (Blair et al., 1999), troglotremiasis (Koubek et al., 2004; Kierdorf et al., 2006; Ribas et al., 2012; Duscher et al., 2015; Heddergott et al., 2015; Itin and Kravchenko, 2016; Heddergott and Müller, 2020; Heddergott et al., 2021) and nanophyetiasis (Voronova et al., 2017). Despite the great epidemiological importance for more than half a century there have been difficulties concerning the taxonomy of these families. Odhner (1914) included into the family Troglotremidae five genera: *Troglotrema* Odhner, 1914, *Pholeter* Odhner, 1914, *Collyriclum* Kossack, 1911, *Renicola* Cohn, 1904 and *Paragonimus* Braun, 1899. Later the name Troglotremidae was changed to Troglotrematidae. After Odhner (1914), some parasitologists erroneously placed the genus *Paragonimus* in the family Troglotrematidae (Li et al., 2020). Dollfus (1939) criticized the system of Odhner (1914) and subdivided Troglotrematidae in several independent families (Paragonimidae, Nanophyetidae, Pholeteridae, Renicolidae, Collyriclidae, Troglotrematidae, Achillurbainiidae) that was earlier supported by Skrjabin (1958). Dollfus (1939) offered to consider the family Troglotrematidae including the

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subfamilies Troglotrematinae (genus *Troglotrema*) and Nephrotrematinae (genus *Nephrotrema*); the families Paragonimidae (genus *Paragonimus*) and Nanophyetidae (genus *Nanophyetus*) were considered independent in relation to Troglotrematidae. In several studies, researchers used the invalid family name Nanophyetidae (Chai et al., 1996; Won et al., 2016; Tatonova et al., 2018; Nguyen et al., 2021). Blair et al. (1999) did not recognize Nanophyetidae and proposed that families Paragonimidae and Troglotrematidae are independent and later this was supported by phylogenetic reconstructions of Voronova et al. (2017) and Doanh et al. (2020).

The families Troglotrematidae and Paragonimidae were nested in a single clade on the phylogenetic trees of Sokolov and Shchenkov (2017) and Sokolov et al. (2019) where authors questioned the affiliation of these families to the superfamily Gorgoderoidea. However, such reconstructions are unreliable without the type species of the family Troglotrematidae – *T. acutum* (Leuckart, 1842). So, the first aim of our study was to clarify the phylogenetic position of this species in relation to other troglotrematids. The second and important aim of this study was to reconstruct historical relationships with molecular estimates between sister families Troglotrematidae and Paragonimidae. Establishing the exact times of species origin and divergence is essential for understanding the mechanisms of evolutionary adaptation, host-parasite coevolution; for determining the initial sources of parasitic infection, and ways infectious diseases spread.

2. Material and methods

2.1. PCR analysis, sequencing, and alignment

For this study we used DNA sample of *T. acutum* from Austria previously extracted by Duscher et al. (2015). The specimen of *T. acutum* was isolated from the skull of *Mustella putorius* from Hainfeld, Lower Austria. The 28S rRNA gene fragment for *T. acutum* was amplified using forward primer U178 (5′-GCA CCC GCT GAA YTT AAG-3′) and reverse primer L1642 (5′-CCA GCG CCA TCC ATT TTC A-3′) (Lockyer et al., 2003). The amplification protocol was performed under the following conditions: 2 min denaturation hold at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 52 °C, 2 min at 72 °C, and a 7 min extension hold at 72 °C. PCR products were initially purified using ExoSAP-IT™ Express PCR Product Cleanup (Applied Biosystems, USA). Purified PCR products were directly sequenced with an ABI 3130 genetic analyzer using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) (following manufacturer's instructions) and internal sequencing primers: 3S (5'-CGG TGG ATC ACT CGG CTC GTG-3′) (Bowles et al., 1995), 1200F (5′-CCC GAA AGA TGG TGA ACT ATG C-3′), 1200R (5′-GGG CAT CAC AGA CCT G-3′), 900F (5′-CCG TCT TGA AAC ACG GAC CAA G-3′) (Lockyer et al., 2003). Then, partial sequence of the 28S rRNA gene was assembled and aligned using the MEGA X (Kumar et al., 2018) alignment explorer with default options.

2.2. Phylogenetic analysis

Genetic p-distances were calculated for the 28S gene fragment (1255 bp) using Tamura-Nei + G model, including all substitution types with 1000 bootstrap replications. The first four variable domains (D1-D4) of the 28S rRNA gene were identified according to Shylla et al. (2013). Analysis included six species of the family Troglotrematidae (genera *Troglotrema*, *Nanophyetus*, *Skrjabinophyetus* and *Nephrotrema*) and eleven species of the family Paragonimidae (genus *Paragonimus*) with a focus on pathogens of commercial mammals (Table 1). *T. acutum* and *Haplorchis pumilio* (family Heterophyidae) were used as consensus and control outgroup, respectively. The multiple alignment and the inferred phylogenetic tree were used to estimate parasites divergence by a Bayesian framework with relaxed molecular clock (uncorrelated lognormal), Hasegawa-Kishino-Yano (HKY) substitution model + gamma distribution, uniform Yule tree prior, and a chain length of 5,000,000 implemented in BEAST package contains the BEAST program, BEAUti, TreeAnnotator (Drummond et al., 2012). Prior distribution (normal with a standard deviation of 0.5 million years) for key divergences times was specified based on previously published parasitic fossil and biogeographic records. Given the uncertainty inherent in constraints we actually used six calibration points (Table 2 and please see Subsection 4.3 "Main assumptions of this investigation" in Discussion).

Table 1
Molecular data presenting original partial sequences of 28S rRNA gene and molecular data downloaded from GenBank for the phylogenetic analysis.*

Parasite species	28S rRNA	Reference
Troglotrematidae		
Troglotrema acutum	**MW404388**	**This study**
Nanophyetus schikhobalowi	MG966187-MG966188	Voronova and Chelomina, 2018
Nanophyetus japonensis	LT796169-LT796170	
Nanophyetus salmincola	LN871822-LN871823	
Skrjabinophyetus neomidis	AF184252	Tkach et al., 2001
Nephrotrema truncatum	AF151936	Tkach et al., 2000
Paragonimidae		
Paragonimus westermani	JN656173-JN656178	Devi et al., 2011
Paragonimus siamensis	JQ322628	
Paragonimus heterotremus	DQ836249	Narain et al., 2006
Paragonimus heterotremus	KF781294	Tandon, Athokpam, 2013
Paragonimus pseudoheterotremus	HM004189	Thaenkham et al., 2010
Paragonimus macrorchis	HM172618	Devi et al., 2010
Paragonimus miyazakii	HM172620	Devi et al., 2010
Paragonimus mexicanus	HM172619	Devi et al., 2010
Paragonimus ohirai	HM172621	Devi et al., 2010
Paragonimus harinasutai	HM172616	Devi et al., 2010
Paragonimus kellicotti	HQ900670	Curtis et al., 2011
Paragonimus iloktsuenensis	AY116875	Olson et al., 2003
Heterophyidae		
Haplorchis pumilio	KX815125	Le et al., 2017

* The new molecular data obtained during this study are in bold.

3. Results

The families Troglotrematidae and Paragonimidae differed from each other by 25 fixed nucleotide substitutions of which number of transitional substitutions ((Ts) 18 sites) prevailed over the transversional ((Tv) 7 sites): Ts were presented with 11 substitutions G-A and seven – T-C; Tv were presented with four substitutions T-A and three – T-G. When comparing all study sequences, four domains (D1-D4) (1255 bp) contained the following substitution types (Fig. 1): D1 (309–311 bp) contains 22 Ts (G-A and C-T), and nine Tv (A-T, G-T, A-C). D2 (541–549 bp) contains 110 Ts (G-A and C-T) and 28 Tv (A-T, G-T, C-A, and C-G); D3 (184–191 bp) contains 14 Ts (G-A and C-T) and two Tv (A-C); D4 (75 bp) contained the only Ts (A-G) in *H. pumilio* in relation to troglotrematids and paragonimids. Among four domains of 11 *Paragonimus* species, three *Nanophyetus* species, *T. acutum* and *Haplorchis pumilio* D2 (541–549 bp) was the most variable (Supplementary Fig. 1; Fig. 1).

Our phylogenetic analysis based on D1-D4 confirmed the closer relationships of the genera *Troglotrema* (species *T. acutum*) and *Nanophyetus*: both are sister, monophyletic (values of genetic p-distances revealed *T. acutum* is confamiliar to other troglotrematids – 4.788%–6.15%) and belong to the same family Troglotrematidae (Fig. 2, Supplementary Fig. 1). Estimated genetic p-distances within both families were as follows: within the family Troglotrematidae – 0.08 ± 0.078%–7.037 ± 0.8%; within the family Paragonimidae – 0.08 ± 0.078%–5.762 ± 0.762%. Interfamily genetic distances varied from 5.59 ± 0.75 to 9.65 ± 1.06%.

Table 2
Calibrations used in this study.

Node	Calibration		Justification with references
	Maximum bound (Ma)	Minimum bound (Ma)	
	First Cretaceous record of intestinal trematodes in terrestrial vertebrates		Parasitic eggs found inside fossil coprolites provide a particularly valuable source of calibration evidence for small and soft-bodied trematodes (Huntley and De Baets, 2015; De Baets et al., 2020). The first report described parasitic eggs presence in early Cretaceous archosaur coprolites (crocodile or dinosaur) from the feces of the Bernissart locality (Belgium). Since Poinar and Boucot, 2006 did not specify the dates, but justified only the Early Cretaceous period, the first point was the deepest - the beginning of the Cretaceous. The Las Hoyas (Spain) coprolites were the second report of digenean trematodes eggs from the Early Cretaceous and have been assisted as second calibration point in dating the evolution of helminths.
	145	126	
Troglotrematoidea lineage	Beginning of the Cretaceous period	Second report of digenean trematodes eggs	
	The territories of modern Europe and Asia were isolated with Turgai sea[a]		Turgai sea extended north from the present Caspian Sea to the Palearctic region and existed from the Middle Jurassic to the Oligocene (Briggs, 1995). The Turgai Sea was not entire throughout its existence, but it was a stable formation in this region. It divided Southern Europe and Western Asia into many large islands and separated Europe from Asia (Duellman, 1994).
	160	29	
Troglotrematids ingroup	Late Jurassic	Rupelian, or Early Oligocene	
	The Cenozoic Himalayan orogeny[a]		The Cenozoic Himalayan orogeny (the formation and evolution of the Himalayan orogenic belt) isolated central Asia (including northwestern part of India) from Orient. The Himalaya initiated crustal anatexis from middle Eocene after the India–Asia continental collision, which lasted until late Miocene (Ji et al., 2020). Time of high-temperature granite magmatism. Registered that the most leucogranites emplacements associated with orogeny occurred in the Neo-Himalayan period during the (25–14 Ma) (Wu et al., 2020).
	44	7	
Paragonimids ingroup	Middle Eocene	The beginning of the Messinian stage of Late Miocene	

[a] The start and end date of tectonism use as bases to estimate priors on lineage divergence timing.

The origin of the common troglotrematoid ancestor (common ancestor for both families Troglotrematidae and Paragonimidae) is estimated to occur 126 million years ago (Ma) (with a HPD between 128.12 and 123.78 Ma) in Barremian period of Early Cretaceous. The divergence events of the families Troglotrematidae and Paragonimidae began presumably in Late Cretaceous: the family Troglotrematidae – approximately 96 Ma (with a HPD between 124.8 and 67.41 Ma) in Cenomanian period; the family Paragonimidae – approximately 73 Ma (with a HPD between 101.74 and 47.05 Ma) in Campanian period. Within the family Troglotrematidae, the ancestral node of *Nanophyetus* and *Troglotrema* (subfamily Troglotrematinae) is inferred to diverge 63 Ma (with a HPD between 99.96 and 26.63 Ma) in Danian age of Paleogene, and the lineage of *Skrjabinophyetus* and *Nephrotrema* (subfamily Nephrotrematinae) – 51 Ma (with a HPD between 82.23 and 18.43 Ma) in Ypresian age of Paleogene.

The radiation time of the common troglotrematoid ancestor (Early Cretaceous) and of the families Troglotrematidae and Paragonimidae (Late Cretaceous) (Fig. 2) overlaps to the time of settling the East Asia by many species of mammaliaforms between the Late Jurassic and Late Cretaceous periods (Grossnickle and Newham, 2016; Grossnickle et al., 2019) (Supplementary Table 1).

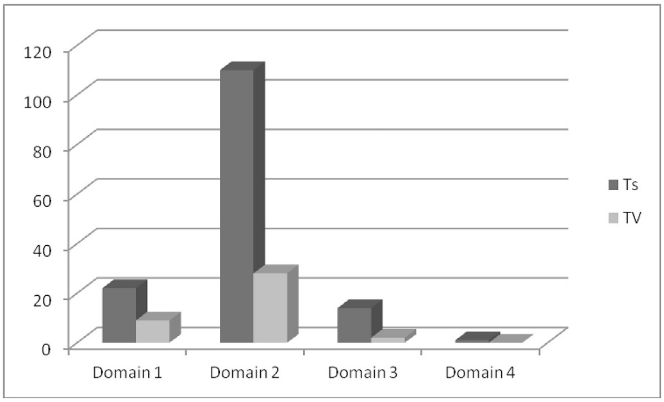

Fig. 1. Histogram showing the content of transitional (G-A and C-T) and transversional (G-T, A-T, G-C, and A-C) substitutions in each of four domains (D1-D4) of the 28S gene rRNA of six species of the family Troglotrematidae, eleven species of the family Paragonimidae, and species *Haplorchis pumilio* (outgroup) from the family Heterophyidae.

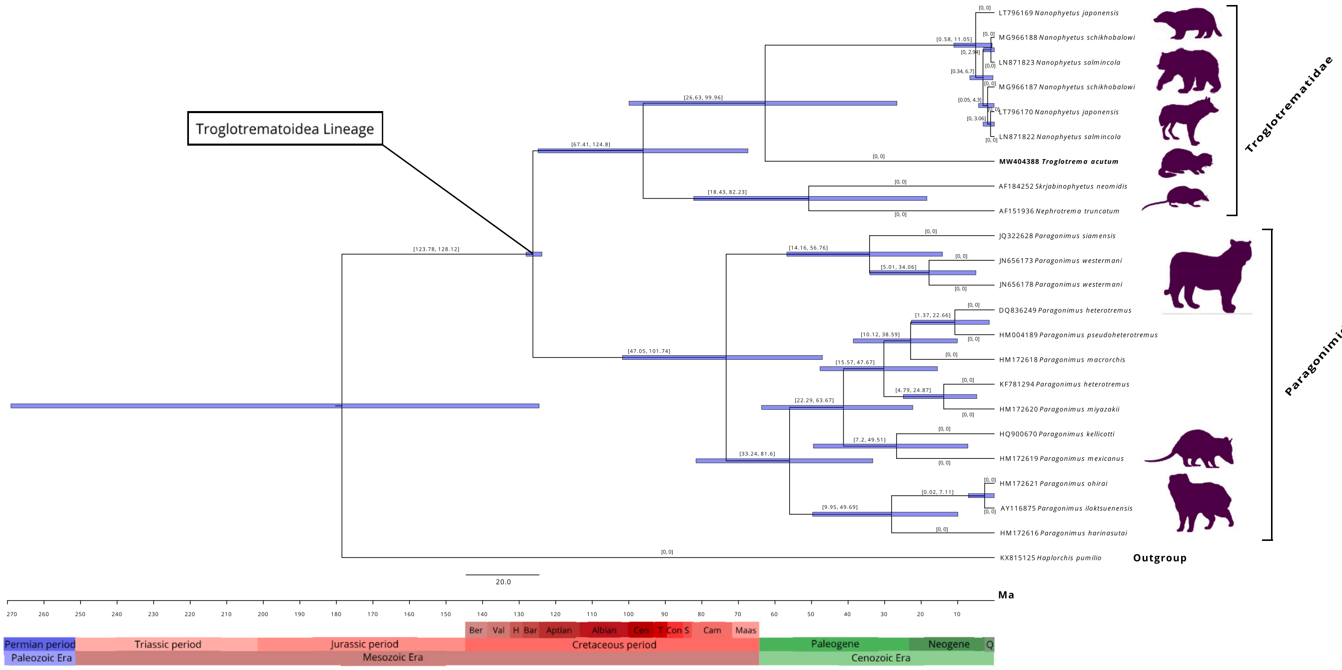

Fig. 2. Phylogenetic tree with estimated divergence times. Species divergence was estimated by a Bayesian model using MCMC-TREE with relaxed molecular clock and is given in million years with 95% highest posterior densities (HPDs) shown in square brackets. Abbreviations on the timescale: ages of the Cretaceous period: Ber – Berriasian; Val – Valanginian; H – Hauterivian; Bar – Barremian; Cen – Cenomanian; T – Turonian; Con – Coniacian; S – Santonian; Cam – Campanian; Maas – Maastrichtian; Q – Quaternary period. The animals on the figure are the type for the corresponding trematodes.

4. Discussion

4.1. Host-parasite interactions

The 28S gene domains (D1-D4) precisely because of neutrality, optimal length, rate of substitutions' accumulation, and mutational saturation together with Bayesian approaches turned out to be useful for molecular dating, estimation of relative timing, and order of genetic differentiation of parasite lineages to understanding the evolutionary history of parasitism. Notwithstanding the scarcity of phylogenetic trees for trematodes with estimating rates and dates, the time of origin of some key nodes in our phylogenetic reconstructions in concordance with those obtained using protein-coding sequences (Oey et al., 2019). The analysis using an advanced platform BEAST 2 estimated the split of a single *P. westermani* branch to have occurred 31.5 Ma the same data was demonstrated here.

According to our investigation, the divergence of the common troglotrematoid ancestor (126 Ma) occurred during the Cretaceous period of the Mesozoic Era (from 145 to 65 Ma) (Fig. 2). Exactly at this time (126–129 Ma upper Barremian) in the Southwest of Europe, almost in the center of Laurasia, the abundant vertebrate coprolites were found in the fossil site of Las Hoyas, Spain. These trace showed that the Las Hoyas still-water ecosystem documents the early connection between basal ray-finned fish and digenetic trematodes that can be easily extrapolated to the other freshwater ecosystems (Barrios-de Pedro et al., 2020). In such ecosystems, trematodes can infect almost all piscivorous/omnivorous vertebrate animals. In the Cretaceous, there was a great variety of the Mammalia species: Laurasiatheria (ungulates, insectivores (order Eulipotyphla), predators (order Carnivora)) and Euarchontoglires (order Primates and order Rodentia) (Tarver et al., 2016). At the end of Cretaceous (66 Ma), the Cretaceous/Palaeogene mass extinction occurred (Goswami et al., 2016) and has been linked with major climatic and geological changes on the Earth: oscillation in the nitrogen composition, growing of ice sheets, falling of sea level, and narrowing of continental shelves to name but a few (Kast et al., 2019). Subsequently, all these events could result in adaptive radiation of paleo-mammals and their parasites respectively.

At present times, species from the families Troglotrematidae and Paragonimidae infect numerous species from the orders Carnivora, Eulipotyphla, Rodentia, and Primates (human) (Ribas and Casanova, 2005; Voronova and Chelomina, 2018). But, considering the emergence of humans (genus *Homo*) nearly 5–6 Ma (Wildman and Goodman, 2004) and carnivorous mammals in Cretaceous-Paleocene 62.3–67.5 Ma (Nyakatura and Bininda-Emonds, 2012), we suggest that the relatively small mammals (probably rodents or insectivores), widely distributed on Earth's surface in Cretaceous, could serve as ancestral definitive hosts of both troglotrematid and paragonimid trematodes. For successful realization of the whole life cycle, rodent-like mammals had to feed on crustaceans and fish (second intermediate hosts), or insectivores could play the role of definitive hosts if metacercariae of troglotrematoids infected insects.

Herewith the species of the family Troglotrematidae are much closer to the common troglotrematoid ancestor than the species of the family Paragonimidae and therefore earlier took niches of mammals as definitive hosts. Probably, the ancestor, like contemporary species of *Troglotrema*, parasitized in the nasal cavities of their vertebrate hosts. Because the transmission through the nasopharynx is much easier for the parasite to enter the body of the definitive host, and from there the worm could also easily get into the intestines (*Nanophyetus*, *Macroorchis*, and *Skrjabinophyetus*). The transition to the lungs and other internal organs in the case of paragonimids can be regarded as a secondary acquisition or complication. The evolution of parasitic species likely constantly changes the neighboring adaptive zones and, accordingly, the fitness landscape. If the population is not ready to move at the rate of displacement of the adaptive peak, it may extinct. Troglotrematids were highly specialized to host tissues. At present, we have evidence that, due to the secondary acquisition, paragonimids turned out to be more resistant, successful in dispersal among hosts, and ecologically flexible than troglotrematids. The fossil record evidences the discovery of *Paragonimus* eggs in human coprolites from northern Chile dated to 5900 BCE. Exoskeletal fragments of freshwater crayfish or shrimp were found in the same coprolites (Horne, 1985). It is surprising that paleoosteopathologies in fossils of vertebrate hosts attributable to *Troglotrema* have so far not been reported, although this is not difficult to do, given the visible lesion they leave on cranial remains.

To the date, in Latin America, six species of *Paragonimus* were considered valid, thereafter, of them, five species were regarded as the synonyms of *Paragonimus mexicanus* (Tongu, 2001). Currently it is known that *Paragonimus caliensis* is a separate species in relation to *P. mexicanus* in Costa Rica (Hernández-Chea et al., 2017). Only *Paragonimus kellicotti* is known from North America. Total, three species of *Paragonimus* inhabit both Americas that are clearly indicating the fact of divergence events of *Paragonimus* spp. in North America with further distribution through the Central to the South America (will be discussed further). Considering the phylogenetic tree, major divergent events of *Paragonimus* spp. began in Paleogene period, particularly the divergence period of the American species *P. kellicotti* and *P. mexicanus* lasted between 40 and 25 Ma (Fig. 2). According to the observation of Asher et al. (2019), fossil rodents from the genus *Ischyromys* inhabited the territory of the present USA 42–38 Ma. The periods of divergence of American *Paragonimus* species and existence of *Ischyromys* spp. coincided, so that we suggest the latter species, or probably other representatives of the family Ischyromyidae, could be the definitive hosts of the American *Paragonimus* species in Eocene-Oligocene epochs.

The lack of representative genetic data (for the 28S gene) for troglotrematids from the genus *Macroorchis* will not allow us to fully evaluate the phylogenetic relationships of the troglotrematid lineage and clarify the ways of their distribution. Species of *Macroorchis* are intestinal trematodes of different mammals (moles, shrews, raccoons etc.) from East Asia; their molecular data are presented only with ITS1 rRNA (Won et al., 2016). Considering the divergence times within Troglotrematidae, Asiatic distribution of *Macroorchis* and *Nanophyetus* (except for *N. salmincola* from North America), species diversity within Paragonimidae in the Asiatic region (20 of 50 nominal species) the common troglotrematoid ancestor probably originated in the Asiatic region, particularly in the Pacific regions of the Far East. The later colonization of the European part of Laurasia by troglotrematids is also confirmed by the later divergence of the European genera *Skrjabinophyetus* and *Nephrotrema* in relation to *Nanophyetus* and *Troglotrema* (Fig. 2). There are already known many mammalian species that could possibly be the definitive hosts of troglotrematoid ancestor in the Asiatic region.

4.2. *Expected hosts and phylogeography*

In the Early Cretaceous, the largest species diversity (10 nominal species) was observed for the carnivorous genus *Gobiconodon* (Fig. 3a, silhouette on the map) (see Supplementary Table 1). Remarkably most of *Gobiconodon* (7 species) were found in the modern East Asia (Mongolia, China). Only four species inhabited western part of Laurasia: Belgium (*G. haizhouensis*), Great Britain (*G. bathoniensis*), Morocco (*G. palaios*), USA (*G. ostromi*). Considering these facts possible way of distribution was from Mongolia (*G. borissiaki*, *G. hoburensis*, *G. hopsoni*) to Eastern/Southeastern China (*G. haizhouensis*, *G. luoianus*, *G. tomidai*, *G. zofiae*) and through Western Siberia (*G. borissiaki*) to western Laurasia. Common troglotrematoid ancestor could have moved across Laurasia in western direction and to North America infecting species of *Gobiconodon*.

Mammaliaforms *Khorotherium yakutensis* and *Sangarotherium aquilonium* inhabited Western Yakutia (Russia) (Averianov et al., 2018) and possibly could transfer troglotrematoid ancestor in western direction to Europe, in southern direction to South East Asia, and in eastern direction – to Beringia and further to North America (Fig. 3a).

In the Late Cretaceous, insectivorous *Zalambdalestes* (2 nominal species) and omnivorous *Alphadon* (7 nominal species) could have maintained the persistence of troglotrematids and paragonimids in Asia and North America respectively (Fig. 3b). The first carnivoramorphan mammals (for example family Miacidae) is considered to originate in Paleocene in North America (after the period of Cretaceous-Paleogene extinction) and then diverged to species-rich group with further distribution to Europe and Asia (Spaulding et al., 2010). Probably this group of mammals provided the beginning of radiation to first carnivorans, direct ancestors of the present order Carnivora – definitive hosts of the

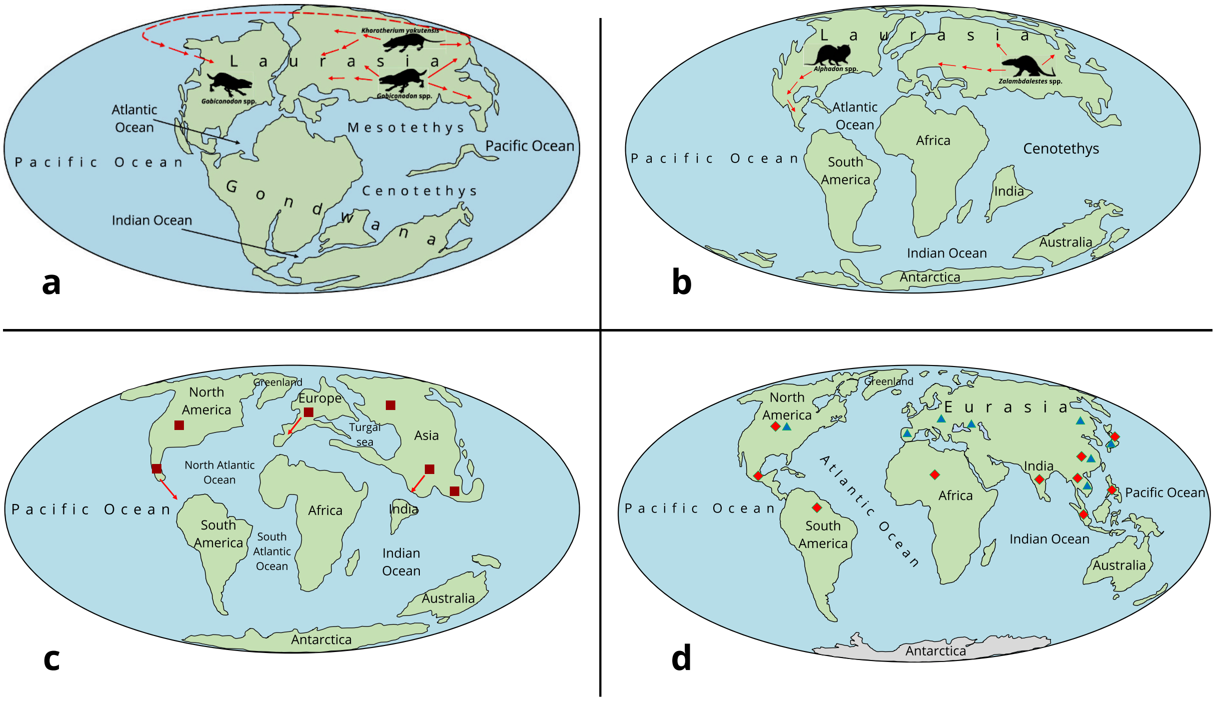

Fig. 3. Hypothetical scheme of distribution of troglotrematids in the period from Cretaceous to present days. (a) Lower Cretaceous (map was prepared sensu Fyodorov, 2006); (b) Upper Cretaceous (sensu Fyodorov, 2006); (c) Earth surface 50 Ma, Middle Eocene (sensu Scotese, 1997). Symbols: red squares – supposed habitats and ways of distribution of the ancestral troglotrematids and paragonimids; (d) Current continents' topology. Symbols: blue triangles – troglotrematids; red rhombus – paragonimids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

most current troglotrematids and paragonimids.

Nearly 50 Ma (Eocene epoch), Turgai Sea and Turgai strait divided Eurasia into separate areas corresponding to modern Europe and Asia (Fig. 3c) (Duellman, 1994), which led to the formation of a specific fauna of hosts and parasites in both regions. In the same time (50 Ma), India was settled with *Paragonimus* after colliding with South Asia, most likely by several species of this genus, since at least two species are currently known in India – *P. heterotremus* and *P. westermani*. Currently, only Europe is inhabited with the following genera: *Troglotrema*, *Skrjabiophyetus*, and *Nephrotrema*.

4.3. Main assumptions of this investigation

In order to calibrate rates and timescales into units of absolute time commonly used various forms of temporal information, such as the ages of fossils, geological events or ancient samples (Ho et al., 2015). Any type of calibrations has their own issues and peculiarities which need to be considered, but increasing the number of calibrations in an analysis can have beneficial effects on the resulting estimates of divergence times, because the inclusion of multiple calibrations can (1) reduce the influence of erroneous calibrations, (2) reduce the average distance of nodes from calibrations, (3) improve the robustness of estimates to clock-model misspecification (Duchene et al., 2014). Notwithstanding imperfections, first and foremost, we implemented calibrations extracted from the parasites' fossil records (Table 2), because fossil evidence was traditionally provided the timescale for evolutionary history and remains the principal means by which the molecular clock is calibrated to time (De Baets et al., 2016, 2020) For instance, calibrations based on host fossils may be inaccurate in this study due to high frequency of host shifting events among ancient zoonotic troglotrematids and paragonimids. The use of biogeographic calibrations should be accompanied by careful consideration of the assumptions made in their implementation (Ho et al., 2015). While, it can be challenging to determine geological/tectonic/climatic events responsible for speciation (Warnock and Engelstädter, 2021), we assumed that the main driver of the parasitic lineage split was genetic isolation that resulted from the geological events having strong impact on the studied organisms. This means that a specific geological event is causal to the biogeographic event that underpins the cladogenesis or distribution of descendent species (De Baets et al., 2016). To avoid the invalid use of biogeographic calibrations, we based on independent geological evidence causing a lineage divergence and tried to escape potential circularity. Nevertheless, in view of acknowledgement of biogeographic calibrations' uncertainty, discussed in depth by Ho et al. (2015) and De Baets et al. (2016), we have taken one of the most flexible Bayesian approaches with the form of a uniform prior distribution well agree with such type of calibrations, providing means of incorporating uncertainty in the timing of geological events and their correspondence to biogeographic signals.

4.4. Remarks on the systematics of Troglotrematoidea

In the different phylogenetic studies accumulating data on the morphology, physiological features, life cycles, and molecular data, researchers considered the sister families Troglotrematidae and Paragonimidae as a single (Troglotrematidae) or several independent (Nanophyetidae, Troglotrematidae, Paragonimidae).

On the phylogenetic tree of Olson et al. (2003), the families Troglotrematidae and Paragonimidae took the position within the one clade of the superfamily Gorgoderoidea along with the clade including the families Haploporidae and Atractotrematidae (superfamily Haploporoidea); then Gorgoderoidea was included into the new suborder Xiphidiata (Olson et al., 2003) later Littlewood et al. (2015) confirmed that fact, also showing the presence of other five superfamilies (Haploporoidea, Plagiorchioidea, Microphalloidea, Opecoeloidea, Brachycladioidea) in Xiphidiata. Blair et al. (1999) were first who showed the separate taxonomic position of Troglotrematidae and Paragonimidae. Both families and Gorgoderoidea representatives were divided with the species currently forming superfamily Haploporoidea. In the phylogenetic reconstructions of Sokolov and Shchenkov (2017) and Sokolov et al. (2019), researchers questioned the affiliation of these families to the superfamily Gorgoderoidea. The clade of the families Troglotrematidae and Paragonimidae was closely related to the clade of the superfamily Brachycladioidea (Sokolov et al., 2019). Considering the phylogenetic reconstruction of Sokolov et al. (2019), the families Paragonimidae and Troglotrematidae formed an independent clade, they were not grouped with other families from the superfamily Gorgoderoidea: Callodistomidae, Gorgoderidae, Dicrocoeliidae, Orchipedidae, and Encyclometridae. Pérez-Ponce de León and Hernández-Mena (2019) have tested the phylogenetic relationships between digenean trematodes and showed it is clearly seen that the cluster including Troglotrematidae and Paragonimidae is absolutely independent in relation to Gorgoderoidea and any other superfamily of the suborder Xiphidiata. More frequently on different reconstructions, both families Troglotrematidae and Paragonimidae had close relationship to Brachycladioidea (2 trees) or Haploporoidea (2 trees) clades but as an independent superfamily.

According to the values of molecular clocks in our investigation (Fig. 2) and phylogenetic reconstructions from previously published studies (Olson et al., 2003; Blair et al., 1999; Littlewood et al., 2015; Sokolov et al., 2019; Pérez-Ponce de León and Hernández-Mena, 2019), we exclude the families Troglotrematidae and Paragonimidae from the superfamily Gorgoderoidea and recover the superfamily Troglotrematoidea for these families with key troglotrematids (*Skrjabinophyetus*, *Nephrotrema*, *Troglotrema*, *Nanophyetus*, and *Macroorchis*). Thus, according to the international code of zoological nomenclature the family Troglotrematidae maintains the type status in the superfamily Troglotrematoidea. Blair et al. (1999) proposed that families Paragonimidae and Troglotrematidae are independent and later this suggestion was supported by phylogenetic reconstructions (this study; Voronova et al., 2017; Doanh et al., 2020). Then both families are monophyletic, and the name Nanophyetidae is the junior synonym of the family Troglotrematidae. Suborder Troglotremata earlier established by Schell (1982) for the families Paragonimidae, Troglotrematidae, Nanophyetidae, and Collyriclidae is a senior synonym of Xiphidiata.

5. Conclusions

The major events of microevolution within the genera of the families Troglotrematidae and Paragonimidae were in the Cenozoic Era, Oligocene-Miocene and upper epochs. And the major events of macroevolution of the superfamily Troglotrematoidea were in the Mesozoic Era, Cretaceous period (Fig. 2). The first troglotrematids and paragonimids appeared in the Early and Late Cretaceous, respectively. There were no restrictions for their distribution on the territory of the single continent Laurasia, and later they could be isolated after the division of Laurasia into North America and Eurasia, and after the division of Eurasia into Europe and Asia by the Turgai Strait. According to genetic data obtained and those from the literature (Voronova et al., 2017; Doanh et al., 2020) the families Troglotrematidae and Paragonimidae are monophyletic in the superfamily Troglotrematoidea and sister in relation to each other; the family Troglotrematidae includes five genera: *Nanophyetus*, *Troglotrema*, *Skrjabinophyetus*, *Nephrotrema*, and *Macroorchis*; and the family Paragonimidae is monotypic including the only genus *Paragonimus*.

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CRediT authorship contribution statement

Konstantin S. Vainutis: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Anastasia N. Voronova:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. **Georg G. Duscher:** Conceptualization, Resources, Writing – original draft. **Egor M. Shchelkanov:** Visualization, Investigation. **Mikhail Yu. Shchelkanov:** Conceptualization, Resources, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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