**ORIGINAL ARTICLE**

# Molecular identification of the trematode *P. ichunensis* stat. n. from lungs of siberian tigers justified reappraisal of *Paragonimus westermani* species complex

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## Abstract

Flukes from the genus *Paragonimus* Braun, 1899 are medically important foodborne trematodes predominantly occurring throughout Asian countries. Providing molecular genetic characteristics based on ITS2 and partial 28 S rDNA of the paragonimids from the Russian Far East, Northeast, South, and Southeast Asian countries, we performed a partial reappraisal of *Paragonimus westermani* species complex. Members of this complex are genetically distinct worms with different divergence times and explosive expansion during Miocene-Pliocene epochs. We confirm the taxonomic status as valid species for *P. ichunensis* stat. n. (from the Russian Far East and Northern China), and *P. filipinus* (from the Philippines), which were previously considered subspecies of *P. westermani*, and reinstated the species name *P. pulmonalis* (from Japan). We suggest considering the worms from South Korea the Korean variety of *P. ichunensis*, because Korean specimens are sister and genetically closest to *P. ichunensis* from Northeast China and Primorsky region of Russia. Worms from South (India (type 2), Sri Lanka), Southeast (Malaysia, Vietnam, Thailand (types 1 and 2)) and East Asia (Taiwan) were left in the paragonimid systematics as *Paragonimus* sp. We propose to consider Indian worms of type 1 as true *P. westermani*, but in further revisions, due to the lack of holotype and unknown exact type locality, new type specimens (neotype) should be established.

**Keywords** *Paragonimus ichunensis* · *Paragonimus westermani* · Species complex · India · 28S rDNA · BEAST

## Introduction

Paragonimiasis is a parasitic disease caused by the lung flukes from the genus *Paragonimus* Braun, 1899, from them *P. westermani* is one of the most pathogenic infecting millions of people in Asia. *Paragonimus westermani* infection was first described in 1878 by Coenraad Kerbert in a Bengal tiger (*Panthera tigris* (Linnaeus, 1758)) with pneumonia which was brought from India. Subsequently, morphologically similar parasites were found in the human lungs (Miyazaki and Hirose 1976; Miyazaki 1978a). Given the high epidemiological importance of the fluke, a lot of multidisciplinary studies, including ecology, zoology, biochemistry, and molecular genetics were performed Blair 2000; Doanh et al. 2011; Xu et al. 2012; Yoshida et al. 2019), however, the real taxonomic status of *P. westermani* remains unclear. Actually, a number of authors believed that *P. westermani* represents a complex of species (Blair et al. 1997; Blair 2000; Iwagami et al. 2000, 2008; Binchai et al. 2007; Sugiyama et al. 2007; Tandon et al. 2007; Doanh et al. 2009; Devi et al. 2010, 2013). But this complex has many taxonomic and nomenclatural problems. The degree of molecular divergence among members of the *P. westermani*

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complex is as great as the differences between other valid species; their paraphyletic relationships on phylogenetic trees and the significant range of biological characteristics strongly indicate that taxonomic resolution is required. Furthermore, genetic data for the representatives of *Paragonimus* from the Russian Far East, including those confirming their taxonomic status, are unknown to date. It is considered that lung and muscle paragonimiasis in the Russian Far East is caused by *P. westermani ichunensis* Chung et al. 1978, subspecies of *P. westermani* (Bezprozvannykh 1992, 1994). In Primorsky region, only the biology and life cycle of *P. westermani ichunensis* had been well described by the Chinese, Japanese and Russian researchers Chung et al. 1978; Miyazaki and Habe 1976; Miyazaki 1978a, 1982; Habe 1978; Kurochkin and Sukhanova 1979, 1980; Shimazu and Oshima 1983; Besprozvannykh 1994; Esaulova and Seredkin 2012; Belov et al. 2021). For the first time, the addition of samples from the Russian Far East to the phylogenetic analysis will give us a different view on the structure of *westermani* species complex. This study was aimed (i) to determine the taxonomic status of paragonimids found in the lungs of Siberian tigers from the Russian Far East, (ii) to estimate the existing patterns of intra- and interspecific genetic variation, and (iii) provide more thorough coverage of the distribution and diversity of the members of *P. westermani* species complex, performing divergence time estimation from molecular sequence data.

## Materials and methods

### Material examined

Material was obtained from Far Eastern bank of biological materials from specially protected animals and plants (FEBBM), Federal Scientific Centre of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences (Shchelkanov et al. 2017). Siberian tiger (*Panthera tigris altaica* Temminck, 1844) FEBBM-AT103 (male, about 10 years old) was removed from wildlife because of deviant behavior and died over time in one of the Wildlife Rehab Center. Post-mortem and laboratory examinations revealed that death occurred due to acute feline panleukopenia, etiologically associated with Carnivore protoparvovirus 1 (feline panleukopenia virus) (Parvoviridae, Protoparvovirus). During autopsy the whole body of the tiger was examined for worms; a total of 4 adult flukes localized in the lungs (Table 1).

According to the description of Chung et al. (1978) the specimens were identified as *P. westermani ichunensis* based on morphological characteristics as follows: the ventral sucker is much smaller than the oral sucker;

**Table 1** Parasitological material obtained in this study

| Siberian tiger FEBBM-AT103 | *Paragonimus westermani ichunensis* = *Paragonimus ichunensis* stat. n. | | |
|---|---|---|---|
| Lungs | FEBBM Isolate | GenBank accession number | |
| | | ITS2 | 28S rDNA |
| Right | FEBBM-210 | MN069038 | MN069042 |
| Left | FEBBM-211 | MN069039 | MN069043 |
| | FEBBM-212 | MN069040 | MN069044 |
| | FEBBM-213 | MN069041 | MN069045 |

comparatively small testes divided into six branches, situated separately from each other close to intestinal caeca; also there is notable wide free space between lateral fields of vitellarium (Fig. 1). Flukes were recovered, thoroughly rinsed in saline and fixed in 96% ethanol for molecular analysis. Samples were stored at +4 °C. One specimen of *Paragonimus ichunensis* stat. n. from Ussuriysk was deposited to the helminthological collection of the Somov Institute of Epidemiology and Microbiology, Vladivostok, Russia, with accession No. FECEN-7. Contact e-mail: vainutisk@gmail.com. Two subspecies of *P. westermani* were raised to the species rank and the species *P. pulmonalis* was reinstated, all nomenclatural changes were registered in ZooBank as follows: *P. ichunensis* stat. n. (urn:lsid:zoobank.org:act:99 A35A7B-3346-4198-990 A-E2B39C9D59B0), *P. pulmonalis* (urn:lsid:zoobank.org:act:74DA96D3-0972-4B53-93AB-704FE1D80F3D), *P. filipinus* (urn:lsid:zoobank.org:act:D50A32ED-5D2B-49C1-A26D-D82935BF647A).

### DNA isolation and sequencing

We prepared DNA samples from 4 individual flukes using the HotSHOT technique (Truett et al. 2000). Partial 28S and complete internal transcribed spacer 2 (ITS2) rDNA regions were amplified by traditional polymerase chain reaction (PCR) using several sets of primers and Thermo Scientific DreamTaq Green PCR Master Mix (2X) (USA) according to the manufacturer's instructions. The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward) together with BD2 (5'-ATCTAGACCGGACTAGGCTGTG-3') (reverse) (Bowles and McManus 1993) and Dig12 (5'-AAGCATATCACTAAGCGG-3') (forward) together with 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (reverse) (Tkach et al. 2003) to amplify ITS2 and 28 S rDNA, respectively. PCR products were purified using ExoSAP-IT PCR Product Cleanup Reagent from Thermo Scientific and then sequenced on an ABI 3130 Genetic Analyzer using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and internal sequencing primers: 300 F (5'-AGGGTTCGATTCCGGAG-3'), 1200R (5'-GGGCATCACAGACCTG-3'), 900 F (5'-CCGTCTTGAAACACGGACCAAG-3'), 1200 F
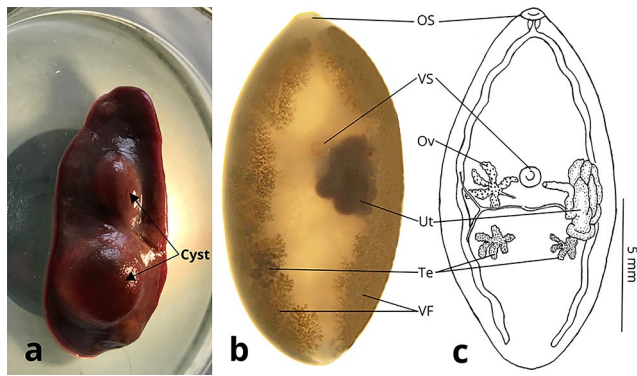
**Fig. 1** Photo of the lung fragment of *Panthera tigris* with the cysts containing adults of *Paragonimus westermani ichunensis* = *Paragonimus ichunensis* (a). Photo of an adult individual of *Paragonimus ichunensis* stat. n. isolated from the lung fragment (b). Schematic line drawing of an adult *Paragonimus ichunensis* stat. n. (c). Abbreviations: OS – oral sucker; VS – ventral sucker; Ov – ovary; Ut – uterus; VF – vitelline follicles; Te – testes

(5′- CCCGAAAGATGGTGAACTATGC-3′) described in Lockyer et al. (2003) in case of 28 S rDNA fragment. Contiguous sequences were assembled using MEGA X (Kumar et al. 2018) and submitted to GenBank (Table 1).

## Phylogenetic analyses and estimation of molecular clock rates

Sequences of *P. westermani ichunensis* obtained in this study and sequences of *Paragonimus* spp. from GenBank were aligned using the Clustal W program with default options (Thompson et al. 1994). The genetic distances of Kimura-2-parameter model were calculated in consideration with all substitutions and with missing and/or gaps as unambiguous changes (Kimura 1980). Phylogenetic analyses using Maximum Likelihood (ML) method with branch support values estimated by 1000 bootstrap replicates were conducted in MEGA X (Kumar et al. 2018). Phylogenetic inferences were also obtained through Bayesian Inference (BI) as implemented in MrBayes 3.1 (Ronquist and Huelsenbeck 2003). We ran two sets of four Markov Chain Monte Carlo (MCMC) chains for 1,000,000 generations, sampled every 1000 generations and added generations until the average standard deviation of split frequencies was less than 0.01; 25% of generations were discarded as burn-in. Node confidence values of the BI trees were presented with Bayesian posterior probabilities (PP). Modeltest 3.7 software (Posada and Crandall 1998) was used to select the nucleotide substitution model; TrN + G and HKY + I for 28 S and ITS2, respectively.

The cross-platform program for Bayesian analysis of molecular sequences BEAST (1.8.2) was used to estimate the evolutionary rates (Drummond et al. 2012). Partial 28 S and complete ITS2 rDNA regions, as previously described, were used to determine an accurate time frame for phylogenetic divergence processes for each *Paragonimus* lineage. We estimated mean node ages and their 95% highest posterior densities (HPDs) under relaxed molecular clock (uncorrelated lognormal (UCLN)) assumption using Bayesian method and the uniform Yule tree prior was chosen, appropriate for hierarchical rather than reticulate relationships. We applied the optimal model HKY + G through the BEAST package BEAUti (1.8.2) (Drummond et al. 2012). In absence of specific information on substitution rates of the gene fragments for our species, we applied lognormal distributions with a mean of 1 and standard deviation of 0.33, allowing for auto-optimizations as the runs progressed. We used two soft constraints: the first detection time of trematode eggs in coprolites of Early - Cretaceous iguanodontian dinosaurs was taken as a deep calibration point (between 100 and 146 Mya (million years ago (Mya)) (Poinar and Boucot 2006); the second calibration point no earlier than 5 Mya was applied to the most recent common ancestor (MRCA) for the *Paragonimus* ingroup as was made by Attwood et al. 2008 for *Schistosoma* species (this time corresponded to the second major Himalayan orogeny, which presumably isolated central Asia (including northwestern part of India) from Orient). BEAST uses MCMC to average over tree space, following a burning of initial 10% cycles, divergence times were sampled once every 1000th generation from 10 million Markov Chain Monte Carlo iterations. TRACER (1.6) is a graphical tool for visualization and diagnostics of MCMC output, was used to check convergence of the chains to the stationary distribution, and to summarize parameter estimates, errors and confidence intervals (Rambaut et al. 2014). The simulation was repeated three times, generating analogous results. The phylograms were visualized and edited using FigTree v.1.4.3.

## Results

### Phylogenetic reconstructions and divergence

On phylogenetic trees based on 28 S rDNA most specimens presumably belonging to *P. westermani* were grouped in Subclade I with reliable statistical support (Fig. 2). This subclade split into seven groups by the geographical distribution of the parasites (Russian Far East together with China (I group), South Korea (II group), Japan (III group), India type 2 (IV group), Malaysia and the Philippines (V group), India type 1 (VI group)), except VII group, which

**Fig. 2** Maximum Likelihood phylogeny (a) and estimated divergence times (b) of *Paragonimus westermani sensu lato* based on 28 S rDNA sequences (1205 bp). Numbers at nodes represent bootstrap support values (ML) or posterior probabilities (Bayesian). Species divergence was estimated by a Bayesian model using MCMCtree with relaxed molecular clock and is given in million years, with 95% confidence intervals. Type I and type II for Indian specimens are given according to Devi et al. (2013). Corresponding humans' silhouettes indicate the presence of paragonimiasis in the country
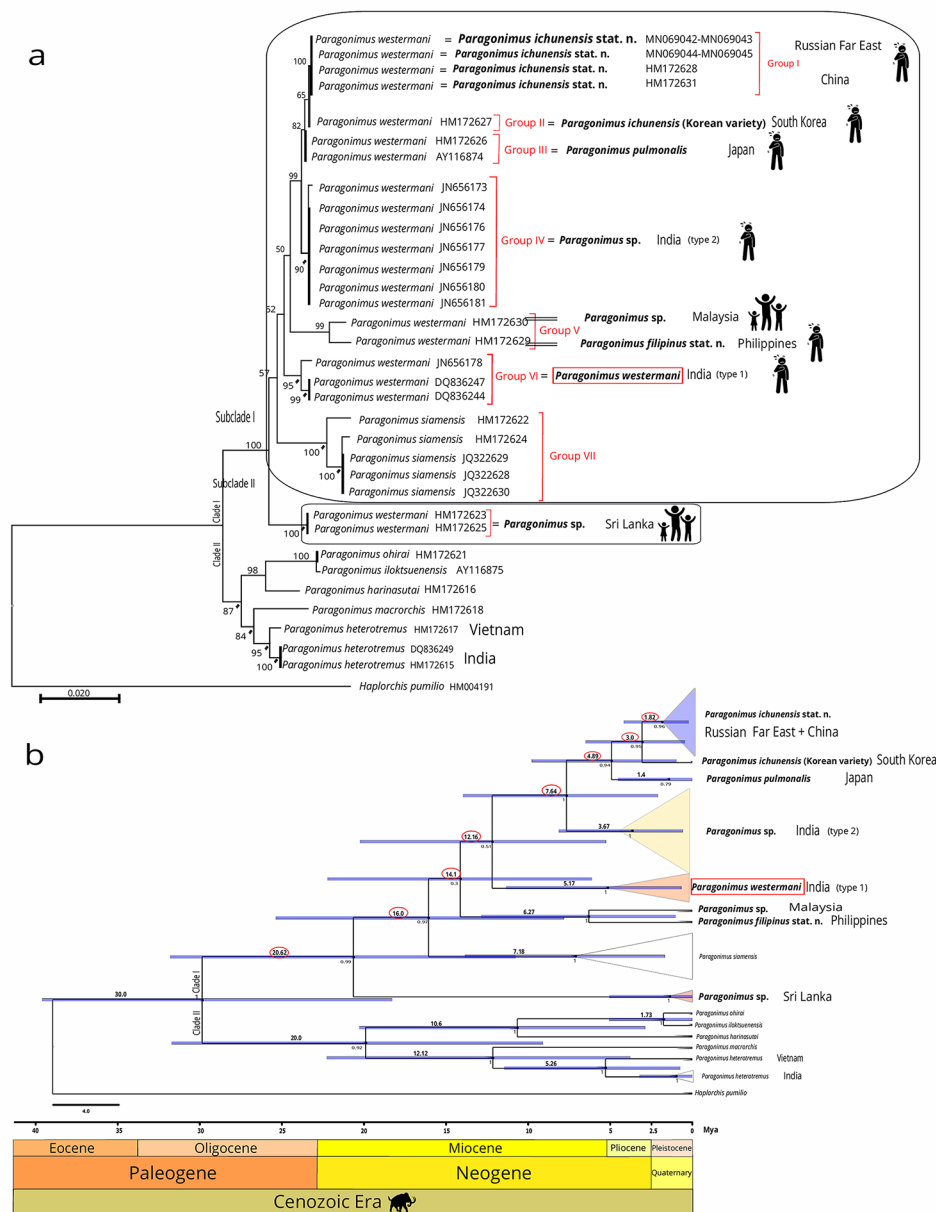
belonged to the sister species *P. siamensis*. Russian specimens diverged from Korean, Japanese and Indian type 2, by 0.1, 0.2 and 0.4%, respectively (SI 1). Strong differentiation was observed when comparing Russian with Southeast and South Asian specimens (p-distances varied from 1.1 to 2%). Malaysian specimens diverged from Philippine by 1.2% and occupied a different branch. We detected notable divergence (1%) within specimens from India. India type 2 (IV group) was evolutionarily much closer (0.2–0.4% of genetic p-distances) to the East Asian species, while Indian type 1 (VI group) was very distant from all studied paragonimids (1.1–1.9% of genetic p-distances). *Paragonimus westermani* from Sri Lanka occupied Subclade II and took a basal

position within Clade I (with p-distances varied from 1.6 to 2%). Clade II formed of the other species from the genus *Paragonimus* (*P. ohirai*, *P. iloktsuenensis*, *P. harinasutai*, *P. macrorchis* and *P. heterotremus*) marked as outgroup.

The overall topologies of the trees based on the ITS2 marker changed due to the additional specimens from Vietnam and Thailand. On the ML tree the first subclade with moderate support split into seven groups (Russian Far East together with China, Taiwan, Japan, and South Korea (I group), Vietnam (II group), India type 2 (III group), Thailand (IV, V groups), Malaysia and the Philippines (VI group)). *Paragonimus siamensis* from Thailand and India together with *P. westermani* from Sri Lanka occupied the

same branch (VII group) (Fig. 3a). Thus, Iwagami et al. (2008) misidentified their specimens, the ITS2 sequences of *westermani* from Sri Lanka (AY240943; AY240942) they provided are in fact the sequences of *P. siamensis*. According to ITS2 marker Russian specimens surprisingly slightly diverged from the rest of the East Asian specimens – 0-0.3%, as well as from Vietnamese (0.7%) and Indian specimens of type 2 (0.3%) (SI 2). More differentiation was observed when compared East Asian with Thai, Malaysian and Philippine specimens: 1.4–2.8%, 1.7, 1.9%, respectively.

Worms from Thailand were divided into two groups. The first group, included Thai worms of type 1, characterized by moderate divergence (genetic p-distances varied from 0.2 to 2.8%) and opposite Thai group, included worms of type 2, characterized by strong divergence (genetic p-distances varied from 2.5 to 3.2%) in comparison with other studied "*westermani*" worms. The specimens from India type 1 clustered separately on a second basal subclade (with genetic p-distances varied from 1.9 to 2.8%) and this division was well supported. The topology of the Bayesian

**Fig. 3** Maximum Likelihood phylogeny (a) and estimated divergence times (b) of *Paragonimus westermani sensu lato* based on ITS2 rDNA sequences (376 bp). Numbers at nodes represent bootstrap support values (ML) or posterior probabilities (Bayesian). Species divergence was estimated by a Bayesian model using MCMC-tree with relaxed molecular clock and is given in million years, with 95% confidence intervals. Type I and type II for Indian and for Thai specimens are given according to Devi et al. (2013) and Sugiyama et al. (2007), respectively. Corresponding humans' silhouettes indicate the presence of paragonimiasis in the country
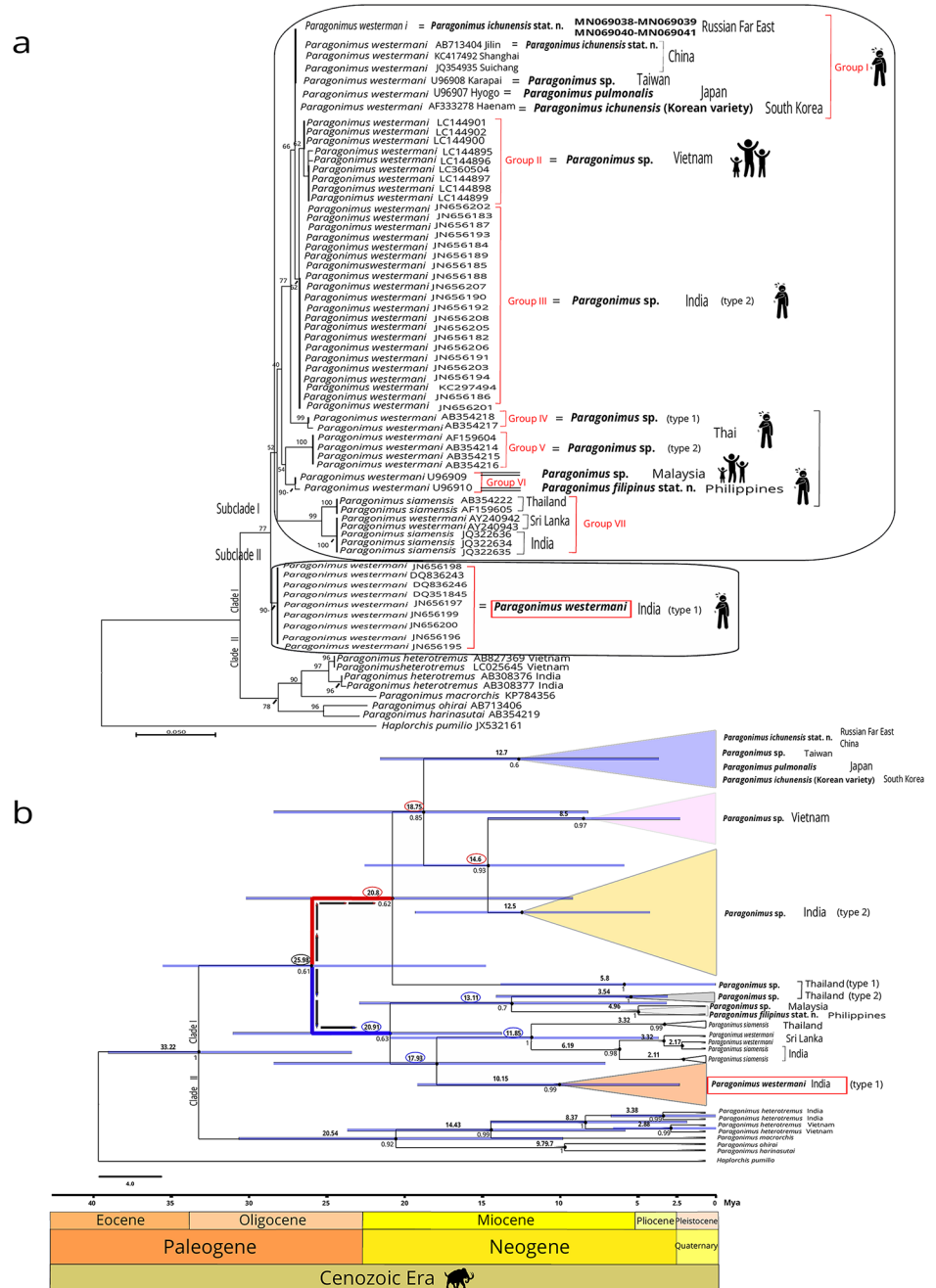
tree with respect to the localization of the worms of type 1 from India was somewhat different from the ML topology described above (Fig. 3b). The worms of type 1 from India on the Bayesian tree did not form an independent subclade, but stayed a basal group strongly separated from the rest of "*westermani*". On both reconstructions, Clade II was represented by different species from the genus *Paragonimus*, marked as outgroup.

## Divergence time estimation

Time tree based on 28 S rRNA gene data clearly demonstrated sequential branching of well-supported monophyletic groups of paragonimus (Fig. 2b). There are eight key divergence events. The ancient branch which separated from a common ancestor in the early Miocene (20 Mya) led to the Sri Lankan specimens. Then in the middle Miocene diverged: *siamensis* (16 Mya), Malaysian-Philippine branch (14 Mya), paragonimids of type 1 from India (12 Mya), followed by Indian paragonimids of type 2 (8 Mya). In the Pliocene worms from Japan (5 Mya) and South Korea (3 Mya) diverged. Speciation of the Russian and Chinese flukes occurred 2 Mya in the Pleistocene.

According to the ITS2 data, the most ancient event (26 Mya in Oligocene) was the divergence of two ancestral lineages of lungworms which gave origin to trematode species inhabit the Russian Far East, Vietnam, India (type 2), Southern Thailand (type 1) and to the species now distributed in central/southern Thailand (type 2), Malaysia, Philippines, Sri Lanka, and India (type 1) (Fig. 3b). The obtained phylogram provides evidence for parallel genetic evolution of these phyletic lineages with the uniform time (the early Miocene (20 Mya)) of the beginning of the divergence events in their internal groups. The splitting of type 1 worms from India and worms from northeast Asia (Russian Far East, China, South Korea, Japan, Taiwan) occurred approximately 18 Mya. Paragonimids from Vietnam were the youngest in comparison with Indian and East Asian worms, and differentiated from the Indian specimens (type 2) – 15 Mya. Worms of type 2 from Thailand together with paragonimids from Malaysia and the Philippines separated 13 Mya, but occupied different branches as a result of specialization (somewhere in the Pliocene (4 Mya). The group of *siamensis* diverged 12 Mya. It is worth mentioning that worms of type 1 from Thailand are about a couple of million years older than worms of type 2 (6 Mya) and belonged to different phyletic lineages.

## Discussion

Due to putative morphological similarity of adults, but despite the geographical, molecular variation, and differences in biological features, since 2000 all "*P. westermani*" flukes have been entered into the *P. westermani* species complex. Phylogenetic reconstructions using posteriori methods of evaluating calibrations in this study incorporated new and all available "*P. westermani*" sequences for proposing a novel reliable evolutionary hypothesis that explains the origin, transformation, and current taxonomy of the species.

First of all, the obtained data have provided strong evidence for the status of Russian worms as independent species *Paragonimus ichunensis* stat. n. Subspecies *P. westermani ichunensis* was first described in the Heilongjiang province in the northeast part of China by Chung et al. (1978). All nuclear sequences of worms from the Russian Far East and Northern China (Beiguho) were identical. But the values of genetic p-distances based on 28 S rDNA sequences between *P. ichunensis* stat. n. and "*westermani*" representatives from Japan, India, Malaysia and Philippines ranged from 0.2 to 2% as between valid species. For example, the molecular differences of 0.13% in the 28 S gene was detected between *Crepidostomum nemachilus* and *C. achmerovi* indicated that these species are at the initial stage of interspecific divergence (Vainutis et al. 2021). When comparing sequences of the 28 S rRNA gene, the divergence between different species of *Nanophyetus* varied 0.1–0.6% (Voronova et al. 2017; Voronova and Chelomina 2018). Pairwise distance between sequences of *P. heterotremus* from Southeast Asia and India was 0.5% which was enough to recognize these flukes as independent species (Voronova et al. 2020).

Specifically, it should be said about taxonomical position of the other two Northeastern populations. It is known that island populations may be points of rapid evolution and speciation. During chromosomal investigations, Miyazaki (1978a) has reinstated the species name *P. pulmonalis* (Baelz, 1880) for the triploid forms, and *P. westermani japonicus* (Miyazaki 1983) was described for the diploid forms from Japan; the species validity of *P. pulmonalis* was later confirmed (Hirai et al. 1985; Prasad et al. 2009; Uabundit et al. 2013). According to Blair et al. (1997), due to the fact that diploids and triploids are not genetically different, the species name *P. pulmonalis*, was suggested to be synonymized with *P. westermani*. Given the 28 S genetic distances and inferred divergence time, the Japanese species can be recognized as independent. Thus, according to the rule of zoological priority, *P. westermani japonicus* previously established by Miyazaki (1983) is a junior synonym of *P. pulmonalis*. The population from Korea is geographically less isolated; 0.1% divergence from Russian and Chinese samples and weakly supported clusterization from the

ancestor node on the phylogenetic trees may indicate that a number of accumulated mutations is insufficient for the final separation of these individuals. Probably the Korean population should be considered *Paragonimus ichunensis* (Korean variety).

Since it is impossible to obtain information about the locality from which the holotype of true *P. westermani* species was described, it seems to be more rational that the species name *westermani* should belong to an older Indian paragonimus of type 1, which divergence was about 12 Mya in the late Miocene. Based on uncertain taxonomic status of Indian paragonimus of type 2, we retain these worms as *sp.* until further reconsideration. Our data in accordance with Devi et al. (2013) clearly demonstrated that lung flukes which had type 1 metacercariae represented a more basal lineage on the phylogenetic trees, and Indian flukes that had type 2 metacercariae were close to populations from East Asia (Japan, Korea, China, and Vietnam). Furthermore, the data obtained on 28 S allows us to confirm that the ancestor of true *P. westermani* could originate in India (as was proposed by Blair 2000; Blair et al. 2001) not Sri Lanka (Iwagami et al. 2008). According to some geological data, there was a land bridge between ancient India and Sri Lanka (Voris 2000) over which ancestral forms of paragonimids could enter the island. BEAST-derived tree demonstrated strong isolation and antiquity (early Miocene) of Sri Lankan species and verified that Indian and Sri Lankan flukes diverged from different ancestors. Now high evolutionary distances (approximately 2%) separating Indian and Sri Lankan populations, indicate that they belong to different species. Importantly that unlike Indian, Sri Lankan worms do not cause paragonimiasis in humans. Human cases of *P. westermani* infection have been found in Russia, China, Korea, Japan, Taiwan, the Philippines, and India, but proven/confirmed cases have never been reported from other countries Sri-Lanka, Malaysia, and Vietnam, where this species is endemic in wild animals (Yoshida et al. 2019).

Species names *Paragonimus filipinus* Miyazaki, 1978 or *P. philippinensis* Ito, Yokogawa, Araki and Kobayashi, 1978 were established for the species occurring in the Philippines (Miyazaki 1978b, 1981). Later it was proposed to refer *P. westermani* populations from the Philippines to one subspecies *P. westermani filipinus* and those from Malaysia and Thailand to another *P. westermani westermani* (Miyazaki 1991). Based on the comparison of the mitochondrial genes, Sato et al. (2003) showed the high divergence level between *Paragonimus* worms from the Philippines and Korea – 11.2%. According to 28 S rDNA marker the detected genetic differences between specimens from the Philippines and Malaysia (1.2%) and in comparison with flukes from East and South East Asia (approximately 2%) were in the interspecific range. The geographical isolation of the Philippines and the inability of Malaysian worms to infect humans also play an important role. Sato et al. (2003) considered the difference in molluscan host specificity sufficient to distinguish the Philippine *P. filipinus* from other *Paragonimus* spp. Thus, we newly propose to restore the validity of the species name for the Philippine *P. filipinus*. *Paragonimus philippinensis* should be reduced to the synonym of *P. filipinus*.

Undoubtedly divergence-time estimation is a complicated, but essential, step for many phylogenetic, evolutionary and comparative analyses. A joint phylogenetic tree for trematodes and cestodes was reconstructed from 104 shared-single copy proteins using the maximum likelihood method, where the split of *P. westermani* was estimated to have occurred somewhere in late Paleocene - early Oligocene (28–58.6 Mya) (Oey et al. 2019), which generally in accordance with the time of splitting of two main clades of the *westermani* ancestors and other paragonimids in our study. By this time, the oldest known carnivoran line mammals Carnivoramorpha was formed (Solé et al., 2016). The first definitive hosts of *Paragonimus* could have been small predators, feeding on crustaceans. Big cats appeared later, the calculated divergence of Pantherinae and Felinae lineages was between 9 and 15 Mya and base of *Panthera* was between 3.37 and 5.84 Mya (Figueiro et al. 2017). Thus, tigers as typical hosts, may have been secondarily infected with *Paragonimus*, proving to be suitable hosts with a long lifespan and a wide range in mainland Asia throughout the Late Pleistocene and Holocene. Unfortunately, only one Indian species from the genus *Paragonimus* was analyzed in the study of Oey et al. (2019). Although the UCLN intervals were large enough that the true age was included, this might be at the cost of lower precision, especially if using a limited sample. Variability in 28 S rDNA is fundamental for the identification of species and reconstruction of divergent events in this study, but unfortunately, 28 S data for the analyzed paragonimus species is incomplete. Therefore, it was decided to use ITS2 region as an auxiliary marker. It is believed that ITS2 region is useful for identification of *Paragonimus* species (Blair 2000; Sugiyama et al. 2007; Fischer et al. 2011; Doanh et al. 2016). The observed percentage of divergence for ITS2 between all studied geographical populations was not high, but within the established for digeneans interspecific ranges (from 0.3 to 2%) (Miller and Cribb 2007; Kasl et al. 2014; Voronova et al. 2017, 2020). The higher percentage of genetic distances between sympatric populations from Thailand and India, and vice versa, the lower percentage of genetic distances between geographically distant populations (Northern India and East Asia) correlates with the mode of their formation in the Miocene (23 – 5 Mya). Hypothetically, according to the BEAST-derived ITS2 tree, studied paragonimids were formed from

two independently diverged ancestors (node 20 Mya). The first ancestor gave the origin of species with distribution over the South and Southeast Asia, and the second one to the species distributed in the South, Southeast and Northeast parts of Asia. Interesting, that according to Himalaya and Tibetan Plateau (HTP) uplift-Asian monsoon intensification hypothesis, approximately 15 – 10 Mya there was a change in temperature on the territory of the modern East Asia. The surface temperature of the Tibetan Plateau (TP) in summer became intolerable and very cold in winter, in addition, TP was covered with extensive snow. The topography of the HTP caused a significant northward migration of the westerly jet during the summer that allowed northwestward penetration of the summer monsoon front into East Asia (Tada et al. 2016). This could contribute to the partial extinction and migration of first (mollusks), second (crustaceans) intermediate and definitive hosts and their parasites to the north, where a warmer climate was gradually established. A northern dispersal of tigers began after the Last Glacial Maximum (LGM) and before the mid-Holocene Climatic Optimum (Cooper et al. 2016). Their dispersal outside the core Late Pleistocene habitat of southern Asia through Southern/ Himalayan Corridor (Cooper et al. 2016) and adaptation to a temperate ecosystem confirms the assumption that tigers could contribute to the spread of *Paragonimus* infection in the Quaternary Period. Indeed, Amur populations of tigers are genetically close to Indochinese (Driscoll et al. 2009), and accordingly we observe the lower percentage of genetic distances between populations of Indian and East Asian Paragonimus flukes. The rise of human civilizations and local extirpation of the tigers further contributed to the division between northern and southern continental host populations and therefore their helminths.

The ITS2 data is rather controversial to make unambiguous conclusions, possibly due to synapomorphies, homogenization and homoplasy typical for this region (thus, the worms from Northeast Asia were not divided into separate species). The Taiwanese paragonimids may represent an independent species - *Paragonimus ringeri* (Cobbold, 1880). Cobbold in 1880 discovered lungworms in a Chinese patient who visited North Formosa (now Taiwan). Probably, we have to recover *Paragonimus ringeri* in future studies, when there would be more representative data. Divergence-time estimation can be complicated by the heterogeneity of rates among lineages and through time. Of all the available, we tried to select markers with minimal conflict, lower root-to-tip variance, and discernible amounts of molecular evolution. Thus, for phylogenetic reconstructions with molecular dating it is preferable to use datasets of neutral markers with a slow rate of substitutions accumulation and length not less than 1000 bp (such as 28 S rDNA) implementing relaxed clock models. Following these adjustments would be more valuable for phylogenies which can be dated with significantly lower error and informatively in regard to rate heterogeneity in datasets. The addition of 28 S sequences for Thai and Vietnamese species is major for determining their taxonomic status and key events in diversification.

## Conclusions

According to the obtained molecular genetic data, flukes in the *P. westermani* complex represent independent species with the difference in the age of formation and explosive expansion during Miocene-Pliocene epochs partially coinciding with a rapid radiation of genus *Panthera* during the Pliocene which is confirmed by evidence from paleogeography and paleoclimate studies (SI 3 and SI 4). We establish the taxonomic status as valid species for *P. ichunensis* stat. n., and *P. filipinus*, which were previously considered subspecies of *P. westermani*, and reinstate the species name *P. pulmonalis*. Worms from South Korea which are sister and genetically closest to *P. ichunensis* from Northeast China and Primorsky region of Russia, could doubtfully belong to *P. westermani*. Thus, we suggest considering the worms from South Korea the Korean variety of *P. ichunensis* until thorough morphological, genetic, and life cycle studies would be performed in order to make final conclusions regarding systematics of these epidemiologically important trematodes. Worms from South (India (type 2), Sri Lanka), Southeast (Malaysia, Vietnam, Thailand (types 1 and 2)) and East Asia (Taiwan) were left in the paragonimid systematics as *Paragonimus* sp. We propose to consider Indian worms of type 1 as true *P. westermani*, but in further revisions, due to the main problems: lack of holotype and unknown exact type locality, new type specimens (neotype) should be established, and Indian *Paragonimus* sp. (type 2) needs to be described as the new species.

## Declarations

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