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Hidden cases of tRNA gene duplication and remolding in mitochondrial genomes of amphipods

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ABSTRACT

The evolution of tRNA genes in mitochondrial (mt) genomes is a complex process that includes duplications, degenerations, and transpositions, as well as a specific process of identity change through mutations in the anticodon (tRNA gene remolding or tRNA gene recruitment). Using amphipod-specific tRNA models for annotation, we show that tRNA duplications are more common in the mt genomes of amphipods than what was revealed by previous annotations. Seventeen cases of tRNA gene duplications were detected in the mt genomes of amphipods, and ten of them were tRNA genes that underwent remolding. The additional tRNA gene findings were verified using phylogenetic analysis and genetic distance analysis. The majority of remolded tRNA genes (seven out of ten cases) were found in the mt genomes of endemic amphipod species from Lake Baikal. All additional mt tRNA genes arose independently in the Baikalian amphipods, indicating the unusual plasticity of tRNA gene evolution in these species assemblages. The possible reasons for the unusual abundance of additional tRNA genes in the mt genomes of Baikalian amphipods are discussed.

The amphipod-specific tRNA models developed for MiTFi refine existing predictions of tRNA genes in amphipods and reveal additional cases of duplicated tRNA genes overlooked by using less specific Metazoa-wide models. The application of these models for mt tRNA gene prediction will be useful for the correct annotation of mt genomes of amphipods and probably other crustaceans.

1. Introduction

Mitochondrial (mt) genomes in the majority of animals possess a set of 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (rRNAs) and 22 transfer RNA genes (tRNAs). The set of 22 mt tRNAs is sufficient for the translation of all proteins encoded in the mt genome (Boore, 1999) due to the wobble base pair interaction in the third codon position (Barrell et al., 1980). Each amino acid corresponds to a tRNA gene, and two tRNA variants are used for serine and leucine.

The mt genomes of animals show both evolutionary conservation and peculiarities in the architecture (Lavrov and Pett, 2016). In invertebrates, the mt genomes can vary significantly in length and in gene order (Gissi et al., 2008). One particular feature that shows variation is the presence of additional tRNA gene copies.

Additional tRNA genes as well as other types of genes appear through duplication events, which are not infrequent in mt genomes (Jühling et al., 2012). In some lineages, additional tRNA gene copies may be maintained for relatively long periods of time. As an example, two trnM genes were found in the mt genomes of some mantellid and dicroglossid frogs (Kurabayashi et al., 2008; Zhang et al., 2018), Scaridae fishes (Mabuchi et al., 2004), bivalve mollusc lineages (Wu et al., 2012, 2014), terebelliform annelids (Zhong et al., 2008), and kinorhynch worms (Popova et al., 2016); duplicated trnT genes were found in most lineages of ardeid birds (Zhou et al., 2014); and additional trnM and trnR genes were found in almost all Porifera lineages (Lavrov et al., 2012; Maikova et al., 2015; Wang and Lavrov, 2011; Sahyoun et al., 2015). However, a more frequent scenario of duplicated tRNA gene evolution is that in which one of the copies gradually accumulates substitutions and turns into a pseudogene (reviewed in Jühling et al., 2012). Such degenerated copies may be identified by their higher evolutionary rate in comparison to that of their functional paralogs and impaired secondary structure (Liu et al., 2013). Additional

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tRNA genes copies that seem to undergo degeneration were found in the mt genomes of many vertebrate and invertebrate animals, e.g., *Sinochlora* insects (Liu et al., 2013), *Paphia* clams (Xu et al., 2012), the decapoda *Stenopus hispidus* (Shi et al., 2012), the sea scallop *Placopecten magellanicus* (Smith and Snyder, 2007), and *Babina* frogs (Kakehashi et al., 2013). As any of the duplicated gene copies may degenerate, tandem duplication-random loss (TDRL) events are acknowledged as the main mechanism of gene order rearrangement (Jühling et al., 2012).

It was noted that some of the additional tRNA genes possess high sequence similarity with tRNA genes of another aminoacylation type. This can happen when one of the tRNA gene copies changes its identity through a mutation in its anticodon sequence. This process is called tRNA gene remolding (Cantatore et al., 1987) or tRNA gene recruitment (Saks et al., 1998). The identity of such tRNA gene sequences is defined based on the anticodon sequence, and it often leads to annotation that does not reflect their true evolutionary origin. Identification of such tRNA remolding cases as well as ancestral remolding events is possible by means of phylogenetic analyses, genetic distance analyses (Belinky et al., 2008; Lavrov and Lang, 2005; Rawlings et al., 2003; Wang and Lavrov, 2011; Wu et al., 2012, 2014; Sahyoun et al., 2015), and methods that consider both the sequence and structural information of tRNAs to evaluate similarities (Sahyoun et al., 2015).

For most mt tRNAs, the anticodon sequence is one of the main identification elements for aminoacyl-tRNA synthetase (aaRS) recognition (Salinas-Giegé et al., 2015), and changes in the anticodon sequence might potentially affect its aminoacylation specificity. Experimental studies confirm the ability of aaRS to charge remolded tRNAs in accordance with the anticodon. For example, *Escherichia coli* knockout experiments demonstrated that the alloaceptor trnR(UCU) with a point mutation in the anticodon (UCU to UGU) can successfully substitute a non-functional trnT(UGU) (Saks et al., 1998). It was also shown that both duplicated trnRs in most Demospongiae species, trnR (UCG) and trnR(UCU), which originated from trnT(UGU) remolding events (Wang and Lavrov, 2011; Sahyoun et al., 2015), are arginylated *in vitro* by a single arginyl-tRNA synthetase (Igloi and Leisinger, 2014).

In some cases, a duplicated tRNA with an altered identity may be charged with another amino acid and then gradually replace the initial cognate tRNA (codon reassignment). This mechanism shows the possibility of tRNA gene evolution unconstrained by the genetic code (reviewed in Kollmar and Mühlhausen, 2017; Moura et al., 2010; Watanabe and Yokobori, 2011). Indeed, bioinformatic and experimental approaches revealed episodes of mt genetic code changes during the evolutionary history of molluscs (Rawlings et al., 2003; Sahyoun et al., 2015), arthropods (Abascal et al., 2012), Protostomia, Ambulacraria and Porifera (Sahyoun et al., 2015), and yeasts (Su et al., 2011) and nuclear genome code changes in some mammals (Tang et al., 2009; Velandia-Huerto et al., 2016), arthropods (Rogers et al., 2010; Velandia-Huerto et al., 2016), etc.

Although it is accepted that the main role of tRNAs is translation decoding, recent studies discovered many other processes in which tRNAs and tRNA-like sequences (pseudo-tRNAs, poorly acetylated tRNAs, tRNA fragments) participate (reviewed in De Lay and Garsin, 2016; Hamashima and Kanai, 2013). For instance, uncharged, poorly charged tRNAs and 5′ tRNA fragments regulate the level of translation in eukaryotic cells and in bacteria through interaction with translation factors and DNA fragments (Henkin, 2008; Hinnebusch, 2005; Ivanov et al., 2011; Wendrich et al., 2002). Experiments in human cells showed the participation of different tRNA fragments in silencing (Haussecker et al., 2010; Lee et al., 2009; Pederson, 2010). Misacylated tRNAs are involved in mechanisms of cell protection against oxidative stress (Jones et al., 2011; Netzer et al., 2009; Wiltrout et al., 2012), antibiotic resistance in prokaryotes (Rogers et al., 2012; Schwartz et al., 2016), and adaptation to new environmental conditions (Yona et al., 2013). Transfer RNAs, tDNAs and tRNA-like structures also participate in the biosynthesis and transport of amino acids, posttranscriptional regulation of gene expression, modulation of chromatin organization, initiation of replication and in other processes (De Lay and Garsin, 2016; Hamashima and Kanai, 2013; Seligmann and Labra, 2014). Taking into account the variety of tRNA biological functions and the complex character of tRNA gene evolution, further studies of tRNA genes in different genomes are needed.

Previous studies have revealed several tRNA gene remolding events in the evolutionary history of Amphipoda (Sahyoun et al., 2015). Amphipoda is the most numerous order of the Crustacea, containing about ten thousand species (Lowry and Myers, 2017). Amphipods are very diverse, both morphologically and ecologically. They are distributed worldwide. Most species are marine, but approximately 19% occupy varied freshwater habitats. Generally, they are benthic, epibenthic, or subterranean dwellers; however, 3% of amphipods are supralittoral or terrestrial. Amphipods have different feeding strategies, including detrital-feeding, scavenging, predation, and algae-feeding; small portions of both marine and freshwater amphipods are parasites (Lowry and Myers, 2017; Väinölä et al., 2007). Amphipods are an indispensable component of many aquatic ecosystems as a nutrition source for predatory fish and birds.

The genetic diversity of amphipods is higher in cool and temperate waters. One of the most spectacular examples of amphipod species diversity is found in the Siberian Lake Baikal (Bazikalova, 1945; Kamaltynov, 2009). Baikal is the most ancient and deepest (approximately 1600 m.) contemporary lake, which has existed as a continuous water body for approximately 28 myr (Mats et al., 2011). Long-term isolation and abundance of various ecological niches in the lake led to the origination of many species flocks (Brooks, 1950) (amphipods, molluscs, turbellarians, ostracods, cottoid fishes, sponges) (Maikova et al., 2015; Martens, 1994; Schon et al., 2000; Sherbakov, 1999), among which amphipods are the most ancient, ecologically diverse and numerous (more than 350 species) (Bazikalova, 1945; Kamaltynov, 2009; Sherbakov, 1999). Baikalian amphipods inhabit all biotopes of the lake (including pelagic zone) along the whole range of depths and possess very diverse morphological features and feeding habits (Bazikalova, 1945; Kamaltynov, 2009). These species are a good model for studying the mechanisms of adaptive radiation.

In our recent work (Romanova et al., 2016a), we investigated the structure and evolution of mt genomes of ten Baikalian amphipod species. The study revealed profound variability in mt genome lengths and different extents of gene rearrangements in comparison to each other and to the Pancrustacean ground pattern (PGP). Moreover, five out of ten studied species were found to possess additional copies of tRNA genes. The low degree of similarity between several duplicated tRNA genes and their respective isoacceptor families indicated multiple tRNA gene identity changes (i.e., tRNA remolding) in the evolution of these mt genomes (Romanova et al., 2016a). In this paper, we examined more carefully the tRNA gene content in the mt genome sequences of both Baikalian amphipods and all available mt genomes of other amphipods. Using amphipod-specific models and covariance model scoring, phylogenetic inference, and genetic distance analysis, we discovered and characterized new cases of tRNA gene duplication and remolding in mt genomes of amphipods, which have been overlooked in previous analyses. The findings show complex tRNA gene evolution in the mt genomes of amphipods. Such underestimated tRNA gene abundance in amphipod mt genomes implies the possibility of similar findings in other groups of organisms, which can be identified using the bioinformatics approaches described in this paper.

2. Materials and methods

2.1. tRNA genes dataset

We predicted tRNA genes in 88 available (by January 2019) mt genomes of amphipod species using MiTFi (Jühling et al., 2012). Complete and nearly complete (> 10 K bp) sequences of mt genomes

were considered in the analysis. The list of amphipod species used for this study is given in Table S1 of the Supplement.

2.2. Covariance model (cm) score evaluation. Amphipod-specific model construction

Similarity scores of amphipod tRNAs to the covariance models of tRNA families were evaluated using cmsearch of Infernal software (Nawrocki and Eddy, 2013). The initial search for remolding candidates was performed with the metazoan mt tRNA models implemented in MiTFi (Jühling et al., 2012). Remolding candidates were identified by higher bit score values to the tRNA models other than their isoacceptor family model. The amphipod-specific models for tRNA families were constructed with cmbuild from the alignments of amphipod sequences and calibrated with cmcalibrate of Infernal software. Potential remolding candidates identified previously using the metazoan models were excluded from the alignments. Selection of sequences for the alignments of trnG, trnY, and trnL1 families relied exclusively on the annotation of amphipod tRNA genes. The alignments for each tRNA family were constructed with MAFFT (Katoh and Standley, 2013) using the Needleman-Wunsch algorithm (globalpair or G-INS-i option). Prior to the generation of models, the alignments were reduced to 30 sequences using the clusters option of the trimAl program (Capella-Gutierrez et al., 2009) to lower the sequence redundancy and refined manually to ensure compliance with the tRNA secondary structure. The secondary structures for amphipod tRNAs were predicted with MiTFi.

The amphipod-specific models were used for the detection of tRNA genes in the set of available 88 amphipod mt genomes. The new tRNA genes predicted by amphipod-specific models were used for the evaluation of similarity scores. All tRNA genes found using both metazoan and amphipod-specific mt tRNA models were taken for further analysis with the exception of findings with very low bit scores (< 20 bits), which were defined as erroneously predicted. A tRNA alignment with a score of 20 bits will have an e-value of approximately 1e-03 for a search done in a typical mt genome of arthropods (15 K bp).

2.3. Phylogenetic inference

A total set of tRNA genes from the mt genomes of amphipods was separated into five groups according to the phylogenetic relationships of amphipod species. The following groups were defined: i. Baikalian species, ii. gen. *Metacrangonyx* species, iii. gen. *Pseudoniphargus* species, iv. amphipod species group 1 and v. amphipod species group 2. The list of amphipod species in every group is shown in Table S1 of the Supplement.

For every tRNA gene group, we used six modes of sequence alignment. We used ClustalW (Thompson et al., 1994), MAFFT (Katoh and Standley, 2013), and T-coffee (Notredame et al., 2000) to align both the entire tRNA gene sequences and the sequences partitioned by 14 charsets (Supplementary Fig. S1) according to their secondary tRNA structure predicted by MiTFi (Jühling et al., 2012).

To define the best mode of alignment, we evaluated each dataset using the algorithm comprising three stages. The first stage included the selection of the nucleotide substitution model for the sequence alignment by ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE version 1.6.9 (Nguyen et al., 2014). The second stage included inference of the maximum likelihood (ML) tree in IQ-TREE (Hoang et al., 2017). In the third stage, every aligned nucleotide set, its appropriate phylogenetic tree and the selected substitution model were used to estimate likelihood values, the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). For the third stage, performance was summarized by a custom R script (Supplementary file 1) using the "phangorn" package (Schliep, 2011). The best mode of alignment was determined as the one yielding the minimal BIC value with this method. ML trees for the best alignment were rooted using either the midpoint or with any clade to obtain a suitable topology for visualizing the clades of interest. The trees were visualized using FigTree v.1.4.3 (Rambaut, 2014).

To relate the evolution of tRNA genes with the phylogeny of amphipod mt genomes, we built an ML phylogenetic tree based on amino acid sequences of 13 mt PCGs. Amino acid sequences were used in the inferences in order to eliminate the effect of mutational saturation in the 3rd codon positions. Crustaceans *Eophreatoicus* sp. 14 FK-2009 (FJ790313), *Ligia oceanica* (DQ442914), and *Neomysis japonica* (KR006340) were used as an outgroup. Orthologous sequences of every protein were aligned separately and then concatenated into a single alignment. The mtMet + F + R5 model, selected as best-fit, was used for tree inference with IQ-TREE software (Kalyaanamoorthy et al., 2017; Nguyen et al., 2014). The node support values were assessed using 3000 ultrafast bootstrap replicates (Hoang et al., 2017). The resultant tree was rooted with the outgroup species and visualized using FigTree v.1.4.3 (Rambaut, 2014).

2.4. Pairwise identity analysis

The evolutionary origins of duplicated tRNA genes and single tRNA genes predicted only by metazoan- or amphipod-specific models were additionally tested using pairwise identity analysis. We analysed the distributions of pairwise genetic distances for each tRNA gene of interest to the sets of different isoacceptor tRNAs. Each tRNA gene of interest was tested with groups of isoacceptor tRNAs defined in this study for phylogenetic analysis (see Table S1 of Supplement). tRNA genes of each isoacceptor group were aligned (d1), and then each group was complemented with the tRNA tested (d2), where the tRNA gene sequence of the same species as the one that was examined was removed. The alignment of these tRNA groups was made according to the best mode found for the appropriate species set in phylogenetic analysis. We calculated pairwise p-distance values for sequences in each isoacceptor group pair (d1 and d2). Genetic distance values for sets d1 and d2 were used to build histograms of distributions. We utilized Sturges' formula (Sturges, 1926) to define the number of bins for every histogram and Fisher's chi-square test for the comparison of two histograms (Gagunashvili, 2006). The tRNA type that had the maximum *p*-value for distributions of genetic distances between the d1 and d2 groups was acknowledged as the progenitor for the examined tRNA gene. If this tRNA type did not match the anticodon type of the tRNA under examination, we concluded that a tRNA gene remolding event occurred. We also calculated the mean distance (m1) for the d1 group and the mean distance (m2) between the sequence under examination with the last position in the alignment and the remaining sequences in d2. Then, we estimated the ratio between m1 and m2 using the following formula: 1-m1/m2. The tRNA group with the minimum value of this ratio was identified as the progenitor for the tRNA gene under examination. The calculations of distribution plots and genetic distances were made in R (Supplementary file 2), and the scheme of the analysis is illustrated in Supplementary Fig. S2.

R scripts developed in this study with examples of input and output files, files of amphipod-specific models for tRNA gene prediction for use with MiTFi, the resulting files of tRNA gene annotation in mt genomes of amphipods using metazoan and amphipod-specific models, and the results of cm analysis were deposited into GitHub (https://github.com/barnsys/trna_data).

3. Results

3.1. Duplicated tRNA genes and remolding candidates found by the metazoan models of MiTFi

The search for tRNA genes in 88 available amphipod mt genomes revealed 18 to 22 tRNA genes in incomplete genome sequences and 21 to 24 tRNA genes in complete mt genomes (Supplementary Table S2). In total, 1933 tRNA genes were predicted using the metazoan models of

Table 1
The duplicated tRNA genes found in mt genomes of amphipods based on metazoan and amphipod-specific models and tRNA genes discovered only by metazoan or amphipod-specific models.

Species	tRNA genes	Coordinates of tRNA genes in mt genomes	Cm model predicted tRNA genes
Acanthogammarus victorii	*trnD1(GUC)*	16,280–16,340	metazoan- and amphipod-specific
	trnD2(GUC)	9568–9627	metazoan- and amphipod-specific
	trnA1(UGC)	7245–7303	metazoan- and amphipod-specific
	trnA2(UGC)	1671–1730	amphipod-specific
	trnY1(GUA)	1443–1503	metazoan- and amphipod-specific
	trnY2(GUA)	5232–5287	amphipod-specific
Brachyuropus grewingkii	*trnF1(GAA)*	4645–4704	metazoan- and amphipod-specific
	trnF2(GAA)	16,730–16,795	metazoan
Crypturopus tuberculatus	*trnX(G-U)*	1320–1380	amphipod-specific
Eulimnogammarus vittatus	*trnP1(UGG)*	8136–8197	metazoan- and amphipod-specific
	trnP2(UGG)	11,308–11,368	metazoan- and amphipod-specific
Garjajewia cabanisii	*trnL1-1(UAG)*	3178–3237	metazoan- and amphipod-specific
	trnL1-2(UAG)	248–307	metazoan- and amphipod-specific
	trnL1-3(UAG)	17,315–17,372	amphipod-specific
Gmelinoides fasciatus	*trnQ1(UUG)*	12,205–12,261	metazoan- and amphipod-specific
	trnQ2(UUG)	13,355–13,414	metazoan- and amphipod-specific
	trnV(UAC)	7620–7678	amphipod-specific
	trnN1(GUU)	12,469–12,529	metazoan- and amphipod-specific
	trnN2(GUU)	16,919–16,975	amphipod-specific
Pallaseopsis kessleri	*trnF/I(GAAU)*	13,706–13,766	metazoan- and amphipod-specific
	trnV1(UAC)	13,150–13,201	metazoan- and amphipod-specific
	trnV2(UAC)	11,187–11,236	amphipod-specific
Metacrangonyx spinicaudatus	*trnM1(CAU)*	1205–1263	metazoan- and amphipod-specific
	trnM2(CAU)	2260–2319	amphipod-specific
	trnA1(UGC)	7043–7111	metazoan- and amphipod-specific
	trnA2(UGC)	14,734–14,793	amphipod-specific
Pseudoniphargus cupicola	*trnH1(GUG)*	9027–9086	metazoan- and amphipod-specific
	trnH2(GUG)	708–765	metazoan- and amphipod-specific
	trnQ1(UUG)	763–821	metazoan- and amphipod-specific
	trnQ2(UUG)	1396–1454	metazoan- and amphipod-specific
	trnI1(GAU)	640–702	metazoan- and amphipod-specific
	trnI2(GAU)	1273–1334	amphipod-specific
	trnC1(GCA)	825–882	metazoan- and amphipod-specific
	trnC2(GCA)	1457–1511	amphipod-specific
Pseudoniphargus sorbasiensis	*trnV(UAC)*	14,518–14,568	amphipod-specific
Gondogeneia antarctica	*trnX1(UUA)*	7668–7730	metazoan- and amphipod-specific
	trnX2(UUA)	8127–8191	metazoan- and amphipod-specific
Haploginglymus sp. JP-2016	*trnN2(AUU)*	68–128	metazoan
	trnI(GAU)	68–128	amphipod-specific
Hyalella lucifugax	*trnC1 (GCA)*	224–280	metazoan- and amphipod-specific
	trnC2 (GCA)	2–41	amphipod-specific
Onisimus nanseni	*trnV(UAC)*	12,134–12,187	amphipod-specific
	trnI(GAU)	13,364–13,422	amphipod-specific
Platorchestia japonica	*trnQ1(UUG)*	14,660–14,718	metazoan- and amphipod-specific
	trnQ2(UUG)	7619–7683	amphipod-specific
Pseudocrangonyx daejeonensis	*trnL2-1(UAA)*	1541–1598	metazoan- and amphipod-specific
	trnL2-2(UAA)	6164–6215	amphipod-specific
Stygobromus foliatus	*trnW1(UCA)*	15,369–15,434	metazoan- and amphipod-specific
	trnW2(UCA)	7579–7643	amphipod-specific
Stygobromus indentatus	*trnS1(UCU)*	4435–4484	amphipod-specific

MiTFi.

The inspection of Baikalian amphipod mt genomes showed the presence of additional tRNA gene copies, as described previously (Romanova et al., 2016a) using the MITOS annotation pipeline (Bernt et al., 2013). The mt genome of *Acanthogammarus victorii* has two trnD genes (annotated as *trnD1* and *trnD2* respectively), *Eulimnogammarus vittatus* has two trnP genes (*trnP1* and *trnP2*), *Garjajewia cabanisii* has two trnL1 genes (*trnL1-1* and *trnL1-2*), and *Gmelinoides fasciatus* has two trnQ genes (*trnQ1* and *trnQ2*). *Pallaseopsis kessleri* also has an additional tRNA gene, which possesses four bases at the anticodon site. The nucleotides in the middle of the anticodon loop of the *P. kessleri* tRNA gene may correspond to either phenylalanine or isoleucine depending on the frame (annotated as *trnF/I*) (Romanova et al., 2016a). However, in the current analysis, an additional copy of the trnF gene was found in the Baikalian species *Brachyuropus grewingkii* (*trnF2*). The examination of mt genomes of non-baikalian amphipod species revealed additional tRNA gene copies in three species. The mt genome of *Pseudoniphargus cupicola* possesses two trnQ genes (*trnQ1* and *trnQ2*) and two trnH genes (*trnH1* and *trnH2*). *Haploginglymus* sp. JP-2016 has two copies of the trnN gene (*trnN1* and *trnN2*) but lacks a trnI gene at the same time. The mt genome of *Gondogeneia antarctica* has two additional tRNA genes, which were identified by MiTFi as *trnX* due to a tRNA sequence (UUA) that is unusual for invertebrates in their anticodons.

The tRNA genes identified by this search generally match those from the appropriate GenBank annotations. Several of the detected differences correspond to the additional gene copies listed above. Another set of discrepancies involves newly predicted tRNA genes (mainly *trnV*) that are lacking in GenBank annotations. Most of the latter cases were subsequently discarded during further examination as false positive predictions (see further, Supplementary Table S5). There is also a discrepancy in the coordinates of the trnS2 gene in the mt genome annotation of *Metacrangonyx longipes* (AM944817), the trnC gene of *Pseudoniphargus cupicola* and the trnW, trnC, and trnG genes of *Parhyale hawaiiensis* (AY639937). These differences may be explained by the use of different tRNA prediction software programmes (ARWEN, MITOS, tRNAscan-SE) for these annotations (Bernt et al., 2013; Laslett and

Table 2
Remolding candidates identified by the covariance model (cm) scoring using amphipod-specific tRNA profiles.

Species	tRNA genes	isoacceptor family cm bit score	highest scoring cm for tRNA	highest bit score for tRNA	bit score Δ with second best scoring cm
Acanthogammarus victorii	*trnD2(GUC)*	22.03	trnH	41.57	19.51
	trnA2(UGC)	14.8	trnI	25.9	3.37
	trnY2(GUA)	16.42	trnD	35.22	18.92
Brachyuropus grewingkii	*trnF2(GAA)*	19.4	trnY	20.24	0.84
Eulimnogammarus vittatus	*trnP2(UGG)*	14.78	trnL1	44.26	19.37
Gmelinoides fasciatus	*trnQ2(UUG)*	0	trnH	37.68	6.96
	trnN2(AUU)	0	trnI	24.9	14.04
Pallaseopsis kessleri	*trnF/I(GAAU)*	–	trnG	24.2	5.34
Crypturopus tuberculatus	*trnX(G-U)*	–	trnM	43.27	26.23
Gondogeneia antarctica	*trnX1(UUA)*	–	trnW	50.52	6.43
	trnX2(UUA)	–	trnW	56.14	6.79
Metacrangonyx spinicaudatus	*trnA2(UGC)*	19.54	trnR	20.28	0.74
Metacrangonyx sp. 4 MDMBR-2012	*trnI(GAU)*	30.54	trnF	32.75	2.06
Metacrangonyx remyi	*trnI(GAU)*	32.11	trnF	32.8	0.69
Platorchestia japonica	*trnQ2(UUG)*	0	trnR	25.13	16.96
Pseudoniphargus cupicola	*trnH2(GUG)*	39.88	trnY	40.76	0.88
Stygobromus foliatus	*trnY(GUA)*	43.17	trnL2	49.91	6.74

Canbäck, 2007; Schattner et al., 2005).

We have applied the covariance model (cm) scoring implemented in the Infernal software package (Nawrocki and Eddy, 2013) to test the identity of all predicted tRNA genes and to detect potential cases of remolding. Amphipod tRNA genes were assessed for similarity to the metazoan models of 22 mt tRNA families of MiTFi (Jühling et al., 2012), which incorporate both sequence and secondary structure information. Recently remolded tRNA genes are expected to display the highest similarity to the profile of their original tRNA gene family. In the analysis with the metazoan mt tRNA models, over 14% of amphipod tRNA genes (279 out of 1933) yielded the highest bit score values outside of their isoacceptor family (Supplementary Table S3). A large proportion of these cases correspond to the proposed ancestral remolding events of *trnW* to *trnG* and *trnC* to *trnY* at the root of Amphipoda (Sahyoun et al., 2015). *TrnW* to *trnG* remolding is supported by the similarity measure in all analysed amphipod species, reinforcing the ancestral status of the event. *TrnC* to *trnY* remolding receives less consolidated support: 11 out of 86 amphipod trnY genes retained the highest score to the trnY isoacceptor family. Another large group of remolding candidates includes the amphipod trnL1 genes. The analysis does not recover a high similarity of amphipod *trnL1* to the metazoan trnL1 model of MiTFi, instead conferring the highest scores to the metazoan trnL2 model. To the exclusion of these major reassignments, the analysis identifies 28 remolding candidates and several tRNA genes with no significant or very low similarity to any of the models (Supplementary Table S3). For the 28 candidates, the average difference between the highest and second highest bit score values is 2.8 bits, and for 7 of these candidates, the difference is less than 1 bit, which indicates a weak distinction between competing models.

3.2. Amphipod-specific models identify additional cases of tRNA gene duplication

To improve the fidelity of remolding discoveries and account for amphipod-specific features of tRNA evolution, we have constructed covariance models for tRNA families using amphipod sequences (Methods) (Supplementary file 3). These models were used instead of metazoan models in MiTFi to detect tRNA genes in amphipod mt genomes. In the majority of mt genomes, this search resulted in the same tRNA gene sets as the ones predicted with the metazoan models; however, the amphipod-specific models detected 27 additional tRNA genes in 23 mt genomes (Supplementary Table S2).

The similarity analyses involving amphipod-specific models were performed for the whole set of tRNA genes, including the 27 newly predicted ones. This analysis narrowed the list of remolding candidates to 19 tRNA genes (Supplementary Table S4). Another 17 tRNA genes, most of which were annotated as *trnV*, exhibit low similarity to any of the amphipod models (maximal bit score value $<$ 20) and might represent divergent tRNA genes or erroneous predictions (Supplementary Table S5). The duplicated tRNA genes and tRNA genes that differ in the MiTFi outputs are summarized in Table 1.

The mt genomes of seven out of ten Baikalian amphipod species possess duplicated tRNA genes, whereas seven out of 78 non-baikalian species have tRNA duplications. *P. cupicola* has the largest number of duplicated tRNA genes (four) in its mt genome among all studied species. The coordinates of some tRNA genes predicted by amphipod-specific models (*trnV* of *G. fasciatus*, *trnV* of *O. nanseni, trnV* of *P. sorbasiensis*, and *trnS1* of *S. indentatus*) do not coincide with the coordinates predicted by the metazoan models. The predictions with new coordinates show much greater bit score values to their tRNA types (from 29.67 to 51.32) than the corresponding tRNA genes predicted by the metazoan models (from 7.72 to 10.70) (Supplementary Table S3, S4). This suggests that the tRNA genes predicted by the metazoan models are erroneous. The *trnI* in the genome of *O. nanseni* was predicted only by the amphipod-specific models with a bit score of 27.43. In the mt genome of *Haploginglymus* sp. JP-2016, the tRNA gene located at the same coordinates was identified as *trnN* by the metazoan models and as *trnI* by the amphipod-specific models. This gene showed the greatest score with the *trnI* model (47.09) and a very low score (9.36) with the *trnN* model. This indicates that the prediction based on amphipod-specific models is likely correct. The amphipod-specific models also identified an unusual tRNA in *C. tuberculatus* with an impaired anticodon with two bases (GU).

Of the 17 remolding candidates identified, nine involve Baikalian amphipod species (Table 2). These include *A. victorii trnH* to *trnD2*, *trnI* to *trnA2*, and *trnD* to *trnY2*, *E. vittatus trnL1* to *trnP2*, *G. fasciatus trnH* to *trnQ2* and *trnI* to *trnN2*, *P. kessleri trnG* to *trnF/I*, *C. tuberculatus trnM* to *trnX(GU)*, and *B. grewingkii trnY* to *trnF2*. The latter case shows the weakest differentiation between the best scoring models among the remolding candidates in Baikalian species (Table 2). *P. kessleri trnF/I* is predicted to originate from the trnG/trnW family, which is itself a product of an ancestral remolding event, resulting in just a 5-bit score difference between the trnG and trnW models for this tRNA (24.2 vs 18.8). The cm scoring with amphipod models suggests the origin of *trnX* from *trnM* in *C. tuberculatus*. A somewhat weaker case is seen in *P. cupicola* with a possible remolding of *trnY* to *trnH2*. Both copies of *trnX (UUA)* in *G. antarctica* were identified as the remolded *trnW*. It is notable that a single *trnI* in both *M. remyi* and *Metacrangonyx* sp. 4 MDMBR-2012 displayed the greatest similarity with trnF; however, the difference between the next suitable model trnI in both cases was very

low (0.69 and 2.21, respectively) (Table 2, Supplementary Table S4). The remolding of a single *trnY* from *trnL2* in *S. foliates* is predicted with better certainty considering the larger value between the next suitable model trnY.

3.3. Phylogeny supports several recent cases of duplication and remolding

To confirm the novel tRNA gene predictions and the results of cm scoring, we performed phylogenetic inference with tRNA gene sequences of amphipod mt genomes. Considering the number of tRNA gene sequences (1960 predicted tRNA genes in total) and the uneven species sampling, we separated all species into several subsets according to their phylogenetic relationships (Methods): Baikalian species, gen. *Metacrangonyx* species, gen. *Pseudoniphargus* species, amphipod species group 1 and amphipod species group 2 (Supplementary Table S1). We removed the tRNAs of *Gammarus* species to reduce the dataset. The phylogenies were inferred separately for each group of species. The sequences identified as tRNA genes with low cm scores (< 20) were discarded. This caused the disappearance of long branches from the phylogenies.

The topology of the phylogenetic tree based on tRNA gene sequences may depend significantly on the alignment accepted; thus, we have chosen the best alignment for every group of species using the BIC criterion (see Methods). As a result, the G-INS-i method of MAFFT turned out to be the best for the tRNA set of Baikalian species and the set of amphipod species of group 1, and ClustalW alignment was selected as the best for the tRNA set of the *Metacrangonyx* species group, the *Pseudoniphargus* species group and the set of species of group 2 (Supplementary Table S6).

It is interesting to note that for both of the tested groups, the alignment involving the deduced tRNAs secondary structure did not yield the optimal tree topology. Therefore, we may conclude that consideration of the secondary structure of tRNA for alignment is redundant, even in datasets from relatively distant species. The most likely phylogenetic tree obtained for each group of species is in Supplementary file 4. We also note that short sequences of tRNA genes and low identity between tRNA gene families, which result in ambiguous alignment, do not allow us to draw strong conclusions about the relationship between tRNA isoacceptor families.

The most likely phylogenetic tree for tRNA genes of Baikalian amphipods shows that isoacceptor tRNA genes form well-defined clades with the exception of some cases that may correspond to remolding events (Fig. 1, Supplementary file 4). There are at least seven likely tRNA gene remolding cases, where the tRNA gene falls outside of its isoacceptor class and groups with a different family of tRNA genes. The *trnP2* from *E. vittatus* falls into a clade of *trnL1* sequences. The *trnD2* from *A. victorii* and *trnQ2* from *G. fasciatus* fall inside the *trnH* clade. The *trnA2* from *A. victorii* and *trnN2* from *G. fasciatus* fall inside the *trnI* clade; however, the latter has a longer branch indicating a higher degree of sequence degeneration. The *trnY2* from *A. victorii* is in the *trnD* clade. The *trnX* from *C. tuberculatus* groups with the *trnM* clade. The *trnF/I* of *P. kessleri* branches as a sister to the *trnW/G* clade. The long branch of *trnF/I* demonstrates a high degree of divergence from this tRNA class. The phylogenetic placement of all of these remolded copies matches the ancestry defined by the cm analysis. The *trnF2* from *B. grewingkii* belongs to the *trnL1* clade, but it has a very long branch that shows either a high degree of degeneration or indicates an erroneous prediction of this tRNA gene. Taking into account that this tRNA gene was found inside the Nad2 gene, the latter assumption seems more probable. Multiple copies of the *trnL* gene from *G. cabanisii*, *trnL1-1, trnL1-2*, and *trnL1-3*, cluster with other *trnL1* sequences in the tree. The *trnV1* and *trnV2* from *P. kessleri* also belong to the clade of their isoacceptor type *trnV*. These cases illustrate tRNA duplication events without remolding.

The phylogenetic tree with tRNA gene sequences of mt genomes of gen. *Metacrangonyx* species (Fig. 2, Supplementary file 4) confirmed the remolding case of *trnA2* in *M. spinicaudatus*, which falls inside the *trnR* clade. However, this gene has a very long branch. The *trnM2* that clusters with other *trnM* also has a very long branch. Both of these additional tRNAs might be false positive predictions or highly degenerated copies. The *trnI* genes from *M. remyi* and *Metacrangonyx* sp. 4 MDMBR-2012 cluster in the clade of their isoacceptor type *trnI*. This finding refutes the results of cm analysis, which suggested higher similarity of these genes to the *trnF* family. Additional evidence is needed to resolve this contradiction.

The topology of the most likely ML tree for tRNA genes of gen *Pseudoniphargus* (Fig. 3, Supplementary file 4) shows one case of remolding of *trnH2* from *P. cupicola*, which falls inside the *trnY* clade. The other duplicated tRNA genes of this species (*trnI1* and *trnI2*, *trnC1* and *trnC2*, *trnQ1* and *trnQ2*) are placed inside the well-defined clades of their isoacceptor classes, indicating relatively recent duplication events. The *trnV* of *P. sorbasiensis* predicted exclusively by amphipod-specific models clusters inside the *trnV* clade, which validates its prediction.

The phylogenetic tree of tRNA genes from mt genomes of amphipod species group 1 (Fig. 4, Supplementary file 4) supports one case of remolding of *trnQ2* from *P. japonica,* which branches as a sister to the *trnR* clade. The origin of the two copies of *G. antarctica trnX1* and *trnX2* from a *trnW* duplication is supported by clusterisation of these genes inside the *trnW* clade. Two copies, *trnC1* and *trnC2*, from *H. lucifugax* fall into the clade of their isoacceptor type. The *trnV* and *trnI* from *O. nanseni* and *trnI* from *Haploginglymus* sp. JP-2016 predicted by the amphipod-specific models also group with their isoacceptor families, confirming the predictions.

The phylogenetic inference of tRNA genes from amphipod species group 2 (Fig. 5, Supplementary file 4) shows one case of tRNA gene remolding: *trnW2* of *S. foliatus*, which falls inside a clade of *trnC* sequences. These data contradict the results of cm analysis, which identified this tRNA as *trnW*. The *trnY* of *S. foliatus* clusters with the isoacceptor family *trnY* in the tree; however, cm analysis identified this tRNA as remolded *trnL2*. Both copies of *trnL2* (*trnL2-1* and *trnL2-2*) from *P. daejeonensis* are placed at the bases of the *trnL1* and *trnL2* clades. Additional data are needed to resolve these conflicting results. The *trnS1* from *S. indentatus* predicted by amphipod-specific models is located inside the clade of its isoacceptor type, validating this prediction.

3.4. Pairwise identity analysis results

To obtain additional evidence on the evolutionary origin of all duplicated tRNA genes, the single tRNA genes predicted only by metazoan or amphipod-specific models and to resolve cases of conflicting results between cm predictions and phylogenetic inference, we analysed the distributions of pairwise genetic distances in the candidate progenitor tRNA families. The results of this analysis are shown in Supplementary Table S7 and summarized in Table 3.

The majority of tRNA genes under examination showed concordance with the tRNA gene types identified by the tRNA anticodons; however some duplicated tRNA genes have a strong affiliation with other tRNA gene types. In Baikalian species, the analysis confirms several cases of tRNA gene remolding: *trnD2* from *trnH*, *trnA2* from *trnI* and *trnY2* from *trnD* of *A. victorii*, *trnP2* from *trnL1* of *E. vittatus*, *trnQ2* from *trnH* and *trnN2* from *trnL1* of *G. fasciatus* and *trnF/I* from *trnL1* of *P. kessleri.* The origination of *trnF2* of *B. grewingkii*, *trnX* of *C. tuberculatus,* and *trnY* of *G. fasciatus* could not be established precisely due to contradictory results from the analysis, which is likely a consequence of the higher degree of degeneration among these tRNA gene copies.

The non-baikalian species display three tRNA gene remolding cases. The *trnH2* of *P. cupicola* originated from *trnY*, and *trnL2-2* from *P. daejeonensis* originated from *trnR*, although the latter case is much less strongly supported considering the weak discrimination with the other nearest tRNA gene type (Supplementary Table S7). A single copy of *trnI* from *O. nanseni* displays the greatest resemblance with *trnL2*.

Two additional tRNA gene copies of *M. spinicaudatus*, *trnA2* and

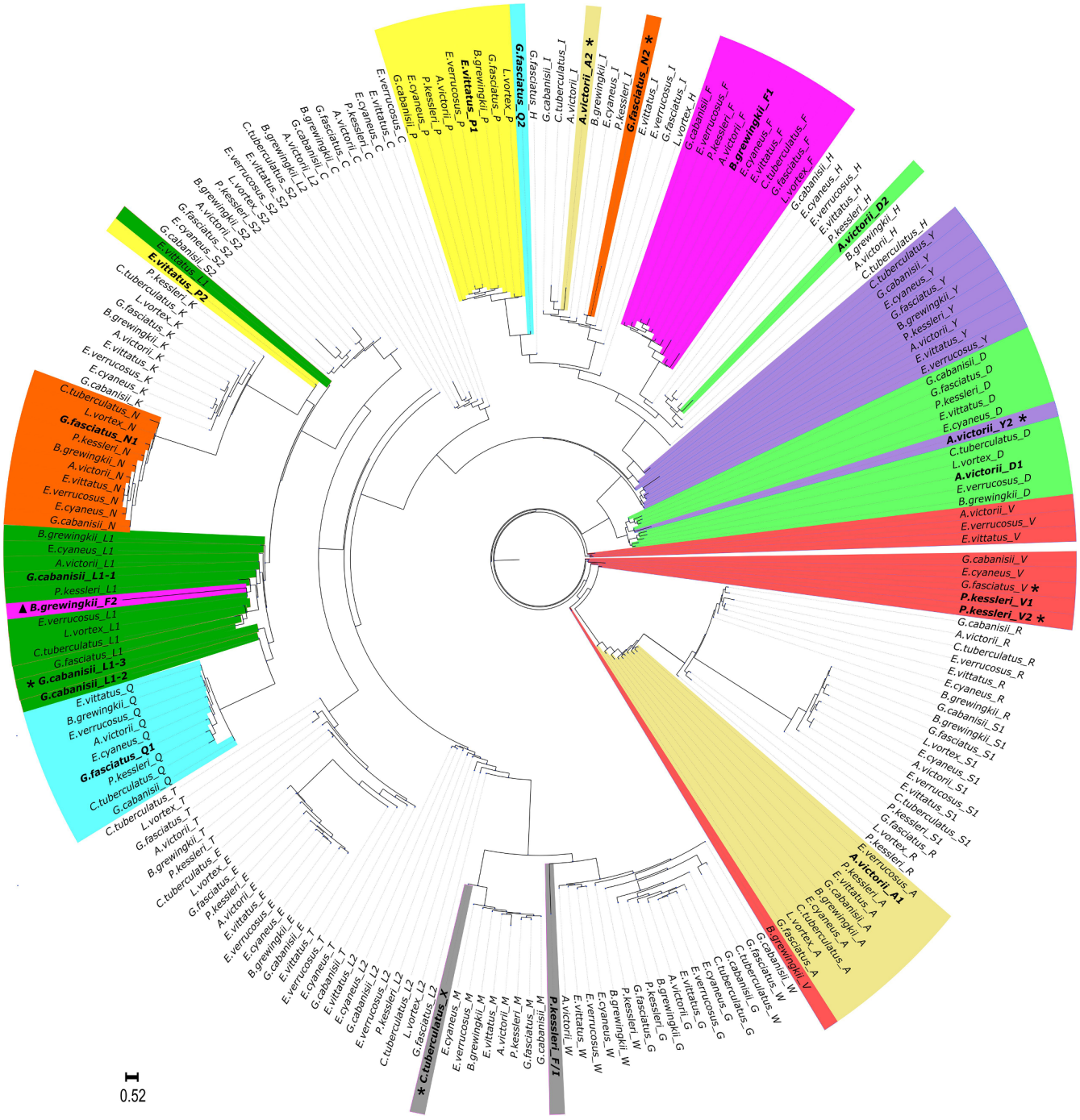

Fig. 1. The ML tree of tRNA genes from mt genomes of Baikalian amphipods. Isoacceptor tRNA gene groups of interest are marked in different colours. The tRNAs with undefined anticodons are marked in grey. The tRNA genes predicted only by amphipod-specific models are indicated with asterisks, and the tRNA gene predicted by only metazoan models is indicated with a triangle.

trnM2, display ambiguous results from the analysis. As these tRNA genes possess very long branches on the phylogenetic tree, the ambiguity may presumably be explained either by a profound degeneration of these sequences or by a false prediction of these tRNA genes by amphipod-specific models.

Most cases of ambiguous identification are in amphipod species of group 1 and group 2 (Table 3, Supplementary Table S7). As these two groups comprise tRNA genes from very distant amphipod species, the genetic diversity in every isoacceptor group is much higher than that in Baikalian species, species of gen. *Metacrangonyx* and species of gen. *Pseudoniphargus* and is close to intergroup genetic diversity. Such diversity may lead to ambiguous identification of some tRNA genes, especially if it is necessary to discriminate between tRNA gene types that have relatively recent common origins in amphopods such as *trnG* and *trnW* or *trnC* and *trnY* (Sahyoun et al., 2015). However, it is notable that the analysis unambiguously related most original tRNA copies from these diverse amphipod groups to their tRNA anticodon type, and contradictory results were mainly obtained for the second copies with apparently accumulated substitutions. To perform the correct identification of tRNA genes, one should also consider the results of other tests.

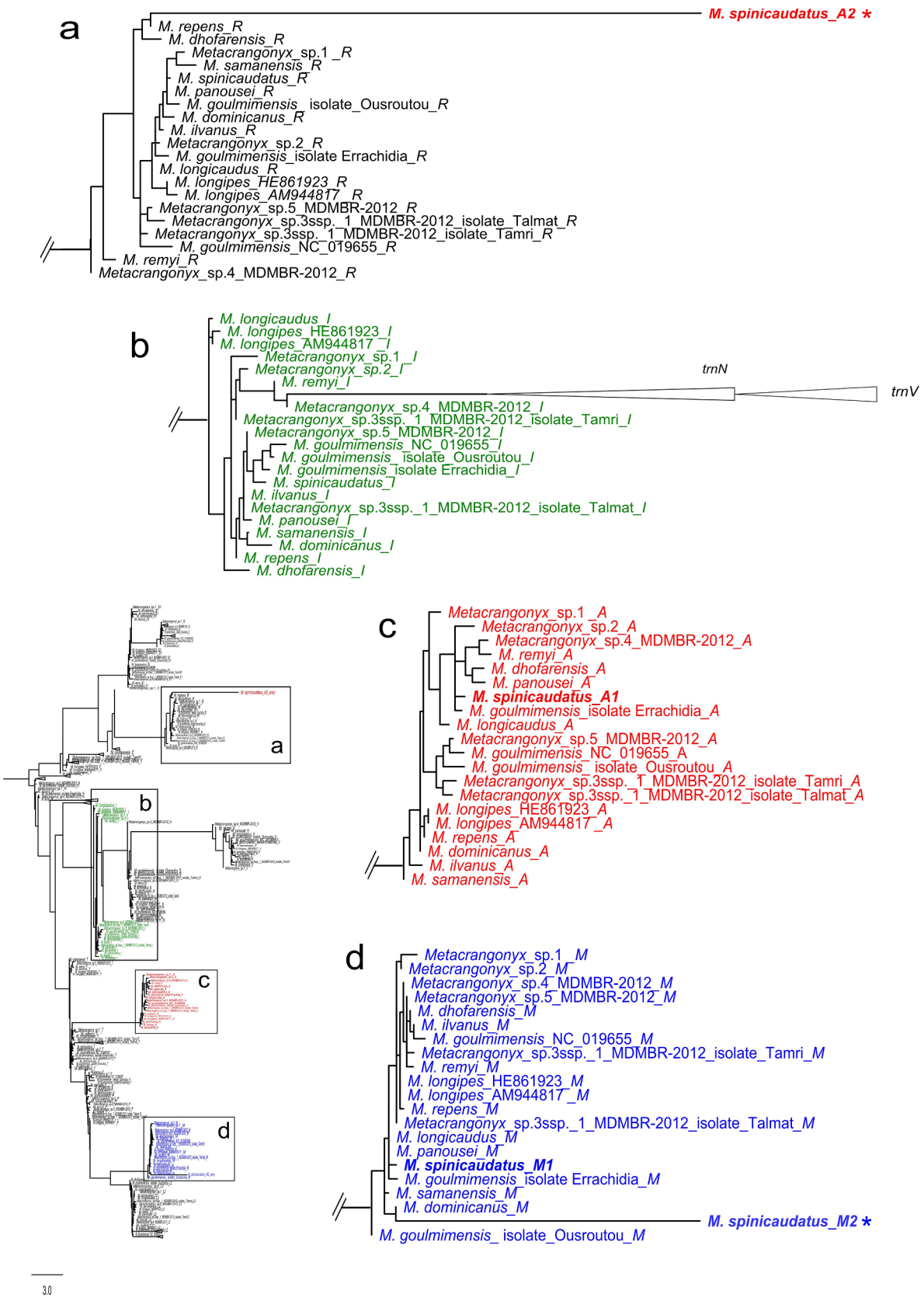

Fig. 2. Selected clades from the ML tree of mt tRNA genes of gen *Metacrangonyx* amphipods, with the full tree shown as a thumbnail sketch. Isoacceptor tRNA gene groups are marked in colours. The duplicated tRNA genes are shown in bold. The tRNA genes predicted only by amphipod-specific models are indicated with asterisks.

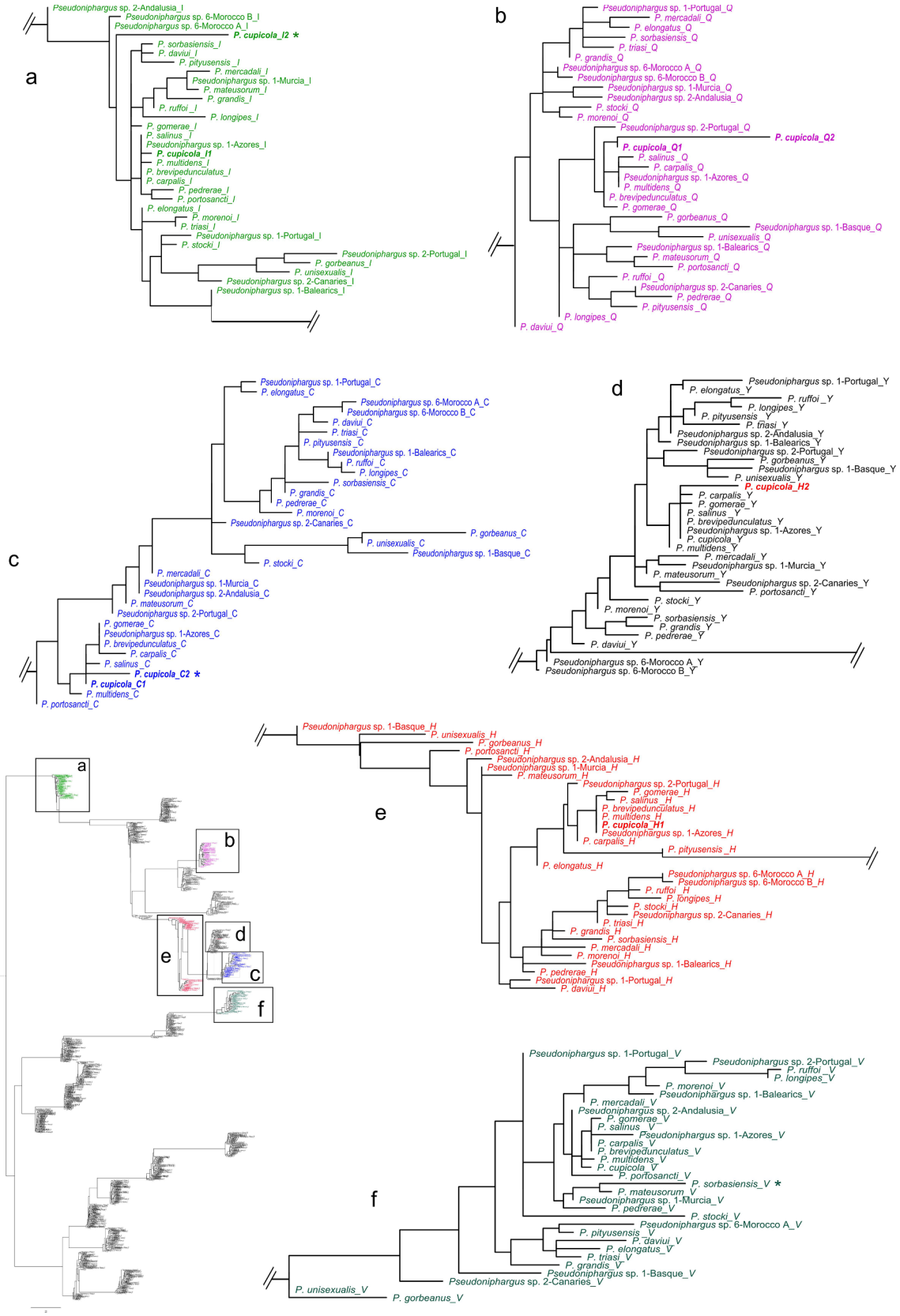

Fig. 3. Selected clades from the ML tree of mt tRNA genes of gen *Pseudoniphargus* amphipods, with the full tree shown as a thumbnail sketch. Isoacceptor tRNA gene groups are marked in colours. The duplicated tRNA genes are shown in bold. The tRNA genes predicted only by amphipod-specific models are indicated with asterisks.

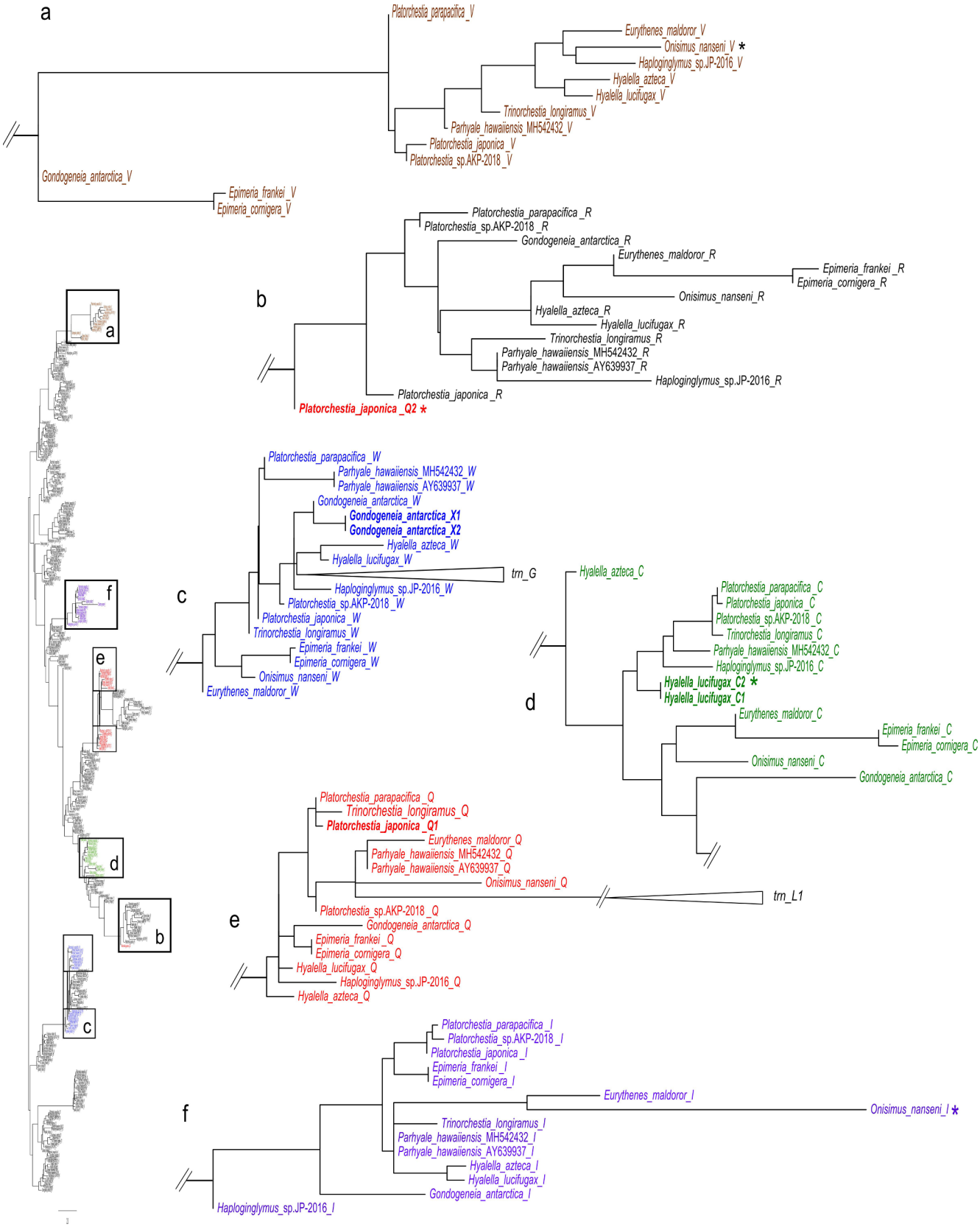

Fig. 4. Selected clades from the ML tree of mt tRNA genes of amphipod species group 1, with the full tree shown as a thumbnail sketch. Isoacceptor tRNA gene groups are marked in colours. The duplicated tRNA genes are shown in bold. The tRNA genes predicted only by amphipod-specific models are indicated with asterisks.

Fig. 5. ML tree of tRNA genes from mt genomes of amphipod species group 2. Isoacceptor tRNA gene groups of interest are marked in colour. The duplicated tRNA genes are shown in bold. The tRNA genes predicted only by amphipod-specific models are indicated with asterisks.

Table 3
Summary of pairwise identity analysis. For every tRNA gene under examination *p*-value and mean distance ratio were evaluated by comparing to the tRNA genes of their isoacceptor family. In case if the isoacceptor family does not yield the best similarity scores, we show these values for the alternative best scoring families.

Species	tRNA genes	tRNA genes set values of anticodon isoacceptor group		tRNA gene type sets with best values	
		p-value	Mean genetic distance ratio	*p*-value	Mean genetic distance ratio
Acanthogammarus victorii	*trnD1(GUC)*	D	D	–	–
		0.9882	0.1964	–	–
	trnD2(GUC)	D	D	H	H
		0.1508	0.5874	0.7070	0.3055
	trnA1(UGC)	A	A	–	–
		0.9921	−0.1080	–	–
	trnA2(UGC)	A	A	I	I
		0.1438	0.6352	0.4662	0.2480
	trnY1(GUA)	Y	Y	–	–
		0.8803	−0.2136	–	–
	trnY2(GUA)	Y	Y	D	D
		0.1142	0.6632	0.5781	0.3590
Brachyuropus grewingkii	*trnF1(GAA)*	F	F	–	–
		0.9728	−0.1441	–	–
	trnF2(GAA)	F	F	C	L1[a]
		0.1574	0.4810	0.3436	0.4578
Crypturopus tuberculatus	*trnX(G-U)*	–	–	C[a]	S2[a]
		–	–	0.1585	0.5213
Eulimnogammarus vittatus	*trnP1(UGG)*	P	P	–	–
		0.9905	−0.1288	–	–
	trnP2(UGG)	P	P	L1	L1
		0.0245	0.8232	0.4192	0.3198
Garjajewia cabanisii	*trnL1-1(UAG)*	L1	L1	–	–
		0.9963	−0.3267	–	–
	trnL1-2(UAG)	L1	L1	–	–
		0.9866	0.1852	–	–
	trnL1-3(UAG)	L1	L1	–	–
		0.9906	0.0897	–	–
Gmelinoides fasciatus	*trnQ1(UUG)*	Q	Q	–	–
		0.9083	0.1643	–	–
	trnQ2(UUG)	Q	Q	H	H
		0.1301	0.7863	0.9905	0.2929
	trnV(UAC)	V	V	–	–
		0.4185	0.4316	–	–
	trnN1(GUU)	N	N	–	–
		0.9183	−0.1536	–	–
	trnN2(GUU)	N	N	L1	L1[a]
		0.0423	0.7137	0.1822	0.5305
	trnY(GUA)	Y	Y	–	H
		0.5030	0.5324	–	0.4566
Pallaseopsis kessleri	*trnF/I (GAAU)*	–	–	L1	L1
		–	–	0.2310	0.5145
	trnV1(UAC)	V	V	–	–
		0.7987	0.2118	–	–
	trnV2(UAC)	V	V	–	–
		0.7486	0.2105	–	–
Metacrangonyx spinicaudatus	*trnM1(CAU)*	M	M	–	–
		0.9999	−0.0150	–	–
	trnM2(CAU)	M	M	Y[a]	S2[a]
		0.0004	0.8590	0.0025	0.6617
	trnA1(UGC)	A	A	–	–
		0.9995	0.0246	–	–
	trnA2(UGC)	A	A	S2[a]	R[a]
		0.0002	0.7773	0.0056	0.6092
Metacrangonyx sp. 4 MDMBR-2012	*trnI(GAU)*	I	I	–	–
		0.9999	0.2863	–	–
Metacrangonyx remyi	*trnI(GAU)*	I	I	–	–
		0.9503	0.3856	–	–
Pseudoniphargus cupicola	*trnH1(GUG)*	H	H	–	–
		0.9996	−0.1280	–	–
	trnH2(GUG)	H	H	Y	Y
		8.25E-05	0.6089	0.1991	0.3237
	trnQ1(UUG)	Q	Q	–	–
		0.9999	−0.0896	–	–
	trnQ2(UUG)	Q	Q	–	–
		0.1044	0.5353	–	–
	trnI1(GAU)	I	I	–	–
		0.9999	−0.2888	–	–
	trnI2(GAU)	I	I	–	–
		0.4460	0.4708	–	–

(*continued on next page*)

Table 3 (*continued*)

Species	tRNA genes	tRNA genes set values of anticodon isoacceptor group		tRNA gene type sets with best values	
		p-value	Mean genetic distance ratio	*p*-value	Mean genetic distance ratio
	trnC1(GCA)	C	C	–	–
		0.9999	−0.0920	–	–
	trnC2(GCA)	C	C	–	–
		0.9998	0.1387	–	–
Pseudoniphargus sorbasiensis	*trnV(UAC)*	V	V	–	–
		0.2499	0.2473	–	–
Gondogeneia antarctica	*trnX1(UUA)*	–	–	C	S1[a]
		–	–	0.6771	0.2372
	trnX2(UUA)	–	–	A	G[a]
		–	–	0.7544	0.2373
Haploginglymus sp. JP-2016	*trnI(GAU)/ trnN2(AUU)*	I	I	–	–
		0.9648	0.3312	–	–
Hyalella lucifugax	*trnC1 (GCA)*	C	C	Y[a]	–
		0.9590	−0.1119	0.9886	–
	trnC2 (GCA)	C	C	Y[a]	–
		0.9304	−0.2534	0.9633	–
Onisimus nanseni	*trnV(UAC)*	V	V	–	–
		0.6101	0.1402	–	–
	trnI(GAU)	I	I	L2	L2[a]
		0.2450	0.5701	0.4693	0.3642
Platorchestia japonica	*trnQ1(UUG)*	Q	Q	–	–
		0.9970	−0.3160	–	–
	trnQ2(UUG)	Q	Q	Y	R
		0.0344	0.6727	0.8576	0.1641
Pseudocrangonyx daejeonensis	*trnL2-1(UAA)*	L2	L2	–	–
		0.9807	−0.0269	–	–
	trnL2-2(UAA)	L2	L2	R[a]	R[a]
		0.5054	0.3571	0.9684	0.2409
Stygobromus foliatus	*trnW1(UCA)*	W	W	–	–
		0.9990	0.0423	–	–
	trnW2(UCA)	W	W	Y[a]	A[a]
		0.1898	0.4933	0.7619	0.2873
	trnY(GUA)	Y	Y	–	–
		0.9612	0.1308	–	–
Stygobromus indentatus	*trnS1(UCU)*	S1	S1	–	–
		0.7949	0.0382	–	–

[a] Weak discrimination between different tRNA genes sets.

3.5. Summary of tests for tRNA gene identity. Recommendations for amphipod-specific model usage

We performed a series of tests to define the evolutionary origin of tRNA genes predicted in the mt genomes of amphipods using metazoan- and amphipod-specific models. Decisions about the validity of prediction and about possible scenarios of their evolution in genomes (duplication, remolding, or degeneration) were made on the basis of the results of all tests (Table 4). We considered that the congruence of conclusions about the origin of a tRNA gene in all three tests indicated that it is a robust prediction.

The copy with the lowest E-value was more likely to be functional if there were no differences in the results of other tests. This is likely the case for *trnL1* copies of *G. cabanisii*, *trnV* copies of *P. kessleri*, *trnI* and *trnC* copies of *P. cupicola,* and *trnC* copies of *H. lucifugax*. Similarly, there were seven cases of strongly supported tRNA gene remolding: *trnH* to *trnD2*, *trnI* to *trnA2* and *trnD* to *trnI2* in *A. victorii*, *trnL1* to *trnP2* of *E. vittatus, trnH* to *trnQ2* of *G. fasciatus*, *trnY* to *trnH2* in *P. cupicola* and *trnR* to *trnQ2* in *P. japonica*. Apparently, the duplication of these additional tRNA genes occurred recently, which helped to identify them unambiguously. The additional tRNA genes found in this study and robustly supported by the analyses were added to the annotation of appropriate mt genomes of Baikalian amphipods. All novel tRNA genes were annotated according to the anticodon type of their tRNAs.

The tRNAs for which only two out of three tests gave an identical result were assumed to be partially degraded and probably non-functional copies. In *G. fasciatus*, alteration in the anticodon changed the identity of *trnI* to *trnN2*; in *C. tuberculatus*, a nucleotide deletion in the anticodon sequence of *trnM* and a substitution in the first position of the anticodon turned this sequence into a *trnX* pseudogene; in *P. daejeonensis*, *trnL2-2* is partially degenerated; and two additional *trnX* copies of *G. antarctica* were identified as *trnW*. The tRNA gene sequences without duplicated copies that demonstrated such a combination of test results (*trnI* of *O. nanseni*, *trnY* of *S. foliatus*, *trnI* of *Metacrangonyx* sp. 4, and *trnI* of *M. remyi*) were acknowledged as correctly identified. In three of these cases, the results of cm analysis were incorrect.

The other five tRNA genes studied here, for which all three tests pointed to different isoacceptor types, were considered to be cases of false predictions or cases of extreme degeneration. For these cases, the identification of progenitor tRNA families was not possible (Table 4) Additional evidence suggesting erroneous prediction of *trnM2* from *M. spinicaudatus*, *trnW2* from *S. foliatus* and *trnF2* from *B. grewingkii* is the location of these genes inside the PCG sequences. It is also notable that the majority of tRNA genes (16 out of 22) defined as erroneously predicted according to the cm scoring test are located inside different PCGs and ribosomal genes (Supplementary Table S5).

The applied tests corroborated the valididy of most tRNA genes predicted only by the amphipod-specific models (Table 4). Thus, we find the application of amphipod-specific models to be useful for a more accurate annotation of tRNA genes in amphipod mt genomes in general.

In case of routine annotation of mt genomes when the evolution of tRNA genes is not discussed, we recommend that one does not take into consideration the extra tRNA genes predicted with amphipod-specific models with an E-value threshold of 1exp-04 or higher to avoid false positive results. However, the presence of genuine single tRNA genes in complete mt genomes with higher E-values is possible. To verify such

Table 4
Summary of the results of different analyses for identification of tRNA genes types.

tRNA gene type predicted by MitFi	tRNA gene types inferred in different tests			Decision
	Cm scores evaluation	Phylogenetic inference	Genetic distance analysis	
A. victorii trnD1(GUC)	*D*	*D*	*D*	Original *trnD(GUC)*
A. victorii trnD2(GUC)	*H*	*H*	*H*	Strongly supported remolding of *trnH(GUG)* to *trnD (GUC)*
A. victorii trnA1(UGC)	*A*	*A*	*A*	Original *trnA(UGC)*
A. victorii trnA2(UGC)[e]	*I*	*I*	*I*	Strongly supported remolding of *trnI(GAU)* to *trnA(UGC)*
A. victorii trnY1(GUA)	*Y*	*Y*	*Y*	Original *trnY(GUA)*
A. victorii trnY2(GUA)[e]	*D*	*D*	*D*	Strongly supported remolding of *trnD(GUC)* to *trnY (GUA)*
B. grewingkii trnF1(GAA)	*F*	*F*	*F*	Original *trnF(GAA)*
B. grewingkii trnF2(GAA)[f]	*Y*[a]	*L1*[b]	*C/L1*	Identification is impossible. False prediction.
C. tuberculatus trnX(G-U)[e]	*M*	*M*	*Y/S2*	Degenerating copy of *trnM(CAU)*
E. vittatus trnP1(UGG)	*P*	*P*	*P*	Original *trnP(UGG)*
E. vittatus trnP2(UGG)	*L1*	*L1*	*L1*	Strongly supported remolding of *trnL(UAG)* to *trnP (UGG)*
G. cabanisii trnL1-1(UAG)	*L1*	*L1*	*L1*	Recent duplication of *trnL(UAG)*
G. cabanisii trnL1-2(UAG)	*L1*	*L1*	*L1*	Recent duplication of *trnL(UAG)*
G. cabanisii trnL1-3(UAG)[e]	*L1*	*L1*	*L1*	Recent duplication of *trnL(UAG)*
G. fasciatus trnQ1(UUG)	*Q*	*Q*	*Q*	Original *trnQ(UUG)*
G. fasciatus trnQ2(UUG)	*H*	*H*	*H*	Strongly supported remolding of *trnH(GUG)* to *trnQ (UUG)*
G. fasciatus trnV(UAC)[e]	*V*	*V*	*V*	Correct prediction of *trnV(UAC)*
G. fasciatus trnN1(GUU)	*N*	*N*	*N*	Original *trnN(GUU)*
G. fasciatus trnN2(GUU)[e]	*I*	*I*	*L1*[b]	Degenerating copy of *trnI*. Remolding of *trnI(GAU)* to *trnN(GUU)*.
G. fasciatus trnY(GUA)	*Y*[d]	*Y*	*Y/H*	Perhaps, native *trnY(GUA)*
P. kessleri trnF/I (GAAU)	*G*	*W*[b]	*L1*[c]	Identification is impossible. Either false prediction or a very degenerated copy.
P. kessleri trnV1(UAC)	*V*	*V*	*V*	Recent duplication of *trnV(UAC)*
P. kessleri trnV2(UAC)[e]	*V*	*V*	*V*	Recent duplication of *trnV(UAC)*
M. spinicaudatus trnM1(CAU)	*M*	*M*	*M*	Original *trnM(CAU)*
M. spinicaudatus trnM2(CAU)[e]	*M*	*M*[b]	*Y/S2*[c]	Identification is impossible. False prediction.
M. spinicaudatus trnA1(UGC)	*A*	*A*	*A*	Original *trnA(UGC)*
M. spinicaudatus trnA2(UGC)[e]	*R*[a]	*R*[b]	*S2/R*	Identification is impossible. Either false prediction or a very degenerated copy.
Metacrangonyx sp. *4 MDMBR-2012 trnI(GAU)*	*F*[a]	*I*	*I*	Original *trnI(GAU)*.
M.remyi trnI(GAU)	*F*[a]	*I*	*I*	Original *trnI(GAU)*.
P. cupicola trnH1(GUG)	*H*	*H*	*H*	Original *trnH(GUG)*
P. cupicola trnH2(GUG)	*Y*[a]	*Y*	*Y*	Strongly supported remolding of *trnY(GUA)* to *trnH (GUG)*
P. cupicola trnI1(GAU)	*I*	*I*	*I*	Recent duplication of *trnI(GAU)*
P. cupicola trnI2(GAU)[e]	*I*	*I*	*I*	Recent duplication of *trnI(GAU)*
P. cupicola trnC1(GCA)	*C*	*C*	*C*	Recent duplication of *trnC(GCA)*
P. cupicola trnC2(GCA)[e]	*C*	*C*	*C*	Recent duplication of *trnC(GCA)*
P. sorbasiensis trnV(UAC)[e]	*V*	*V*	*V*	Original *trnV(UAC)*
G. antarctica trnX1(UUA)	*W*	*W*	*C/S1*	Degenerating copy of *trnW(UCA)* with altered anticodon to *X(UUA)*
G. antarctica trnX2(UUA)	*W*	*W*	*A/G*	Degenerating copy of *trnW(UCA)* with altered anticodon to *X(UUA)*
Haploginglymus sp. *JP-2016 trnI(GAU)*[e]/ *trnN2(AUU)*[f]	*I*	*I*	*I*	Original *trnI(GAU)*
H. lucifugax trnC1 (GCA)	*C*	*C*	*C/Y*	Recent duplication of *trnC(GCA)*
H. lucifugax trnC2 (GCA)[e]	*C*	*C*	*C/Y*	Recent duplication of *trnC(GCA)*
O. nanseni trnV(UAC)[e]	*V*	*V*	*V*	Original *trnV(UAC)*
O. nanseni trnI (GAU)[e]	*I*	*I*	*L2*[c]	Original *trnI(GAU)*
P. japonica trnQ1(UUG)	*Q*	*Q*	*Q*	Original *trnQ(UUG)*
P. japonica trnQ2(UUG)[e]	*R*	*R*	*Y/R*	Strongly supported remolding *R(UCG)* to *Q(UUG)*
P. daejeonensis trnL2-1(UAA)	*L2*	*L1/L2*	*L2*	Duplication of *trnL2(UAA)*
P. daejeonensis trnL2-2(UAA)[e]	*L2*	*L1/L2*	*R*[c]	Duplication of *trnL2(UAA)*
S. foliatus trnW1(UCA)	*W*	*W*	*W*	Original *trnW(UCA)*
S. foliatus trnW2(UCA)[e]	*W*	*C*	*Y/A*	Identification is impossible. False prediction.
S. foliatus trnY(GUA)	*L2*	*Y*	*Y*	Original *trnY(GUA)*
S. indentatus trnS1(UCU)[e]	*S1*	*S1*	*S1*	Original *trnS1(UCU)*

[a] Weak discrimination between competitive models.
[b] Very long branch/very distat sister taxon on phylogenetic trees.
[c] Weak discrimination between different sioacceptor tRNA genes sets.
[d] Very low cm bitscores
[e] tRNA genes predicted only by amphipod-specific models
[f] tRNA genes predicted only by metazoan models

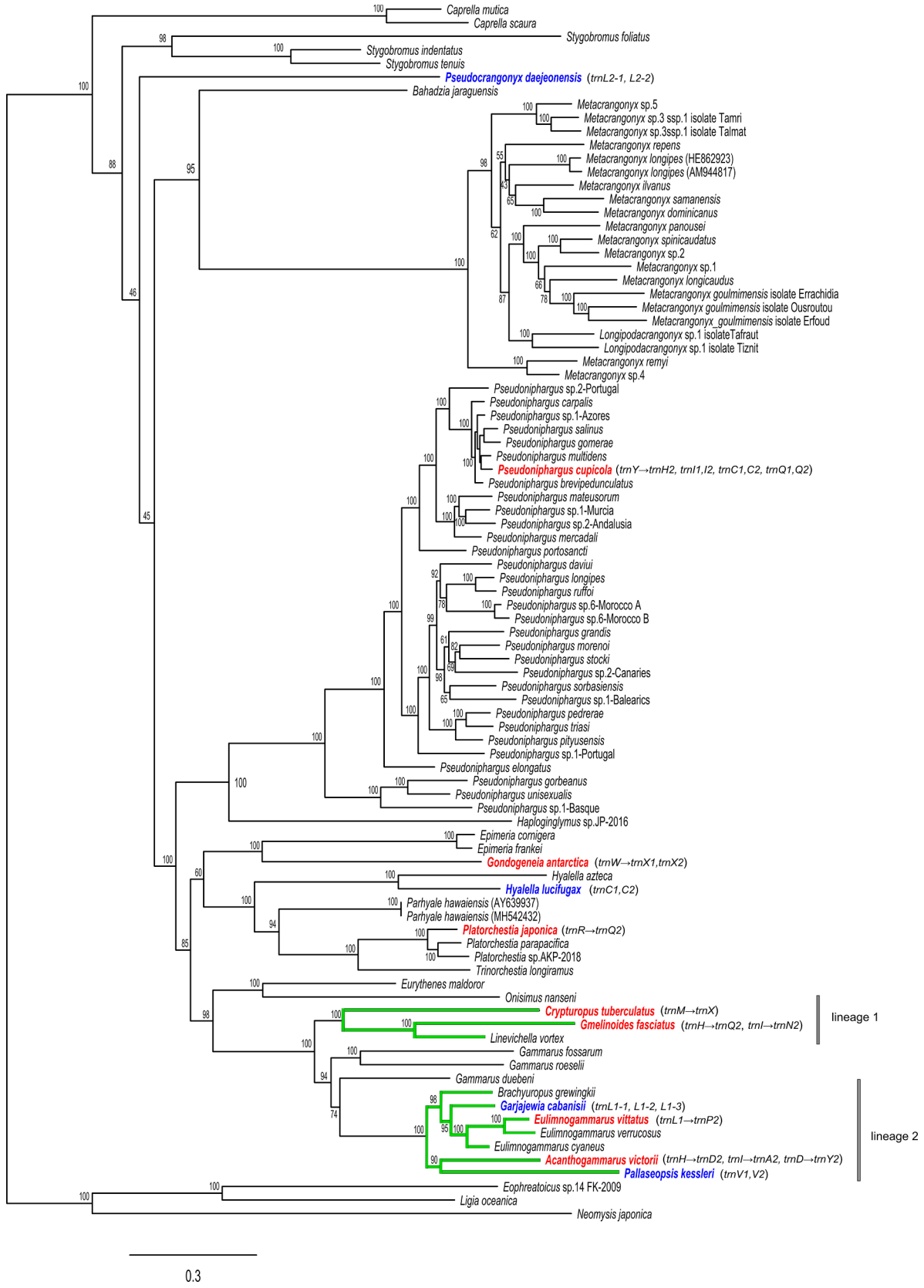

Fig. 6. ML phylogenetic tree of amphipods based on amino acid sequences of 13 mt PCG amino acid sequences. Numbers at the branches correspond to ultrafast bootstrap support (%). The species that possess duplicated tRNA genes in their mt genomes are highlighted in blue colour, and the species that possess tRNA genes with altered anticodon sequences are highlighted in red colour. All cases of duplicated tRNA genes are listed beside the appropriate species. The clades of Baikalian species are highlighted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

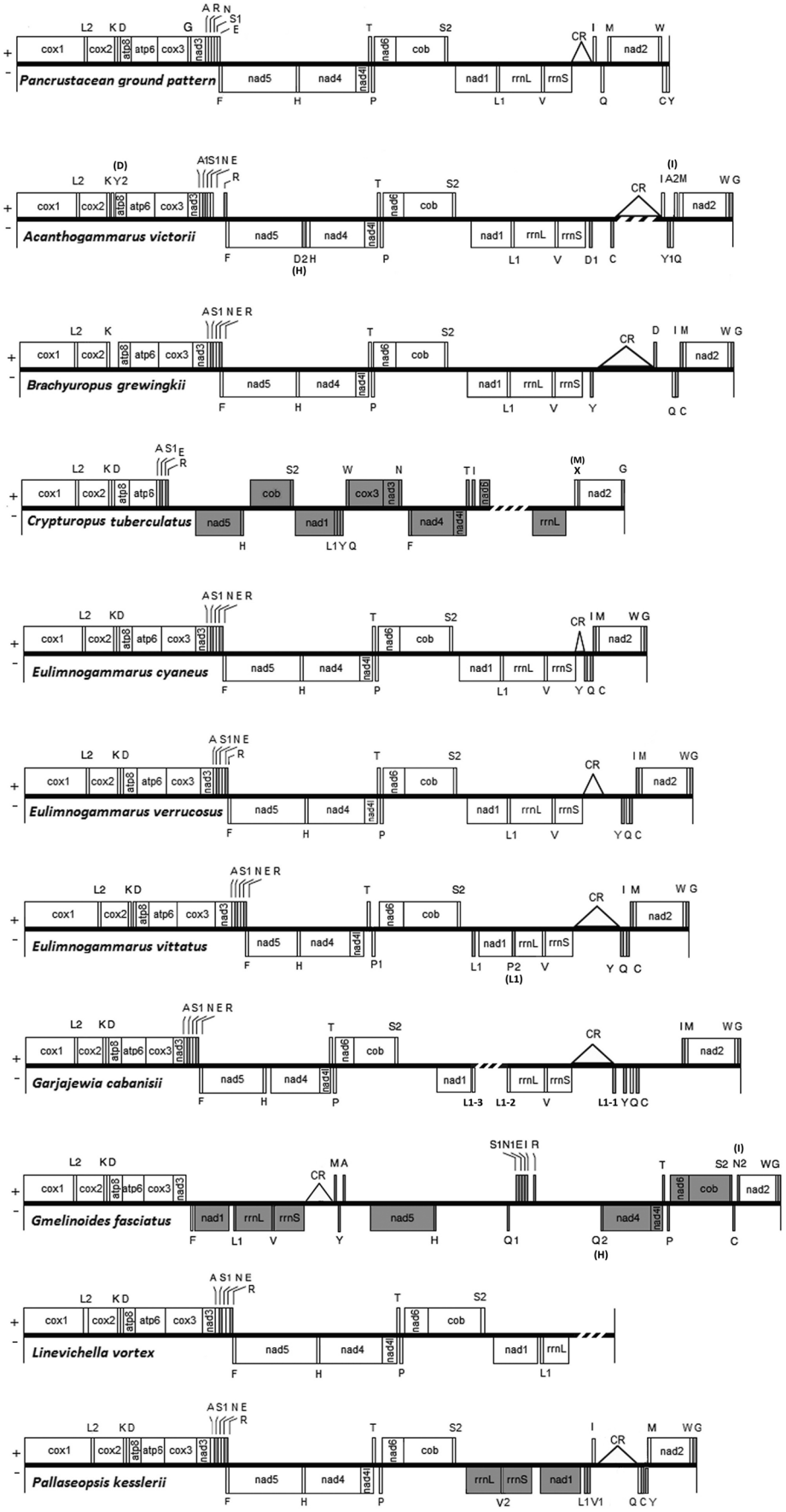

Fig. 7. Corrected map of mt genomes of Baikalian amphipods. tRNA genes are labelled by their single-letter amino acid code. tRNA genes that underwent to remolding are shown with their progenitor tRNA gene types in brackets.

predictions, additional analytical methods described in this paper may be applied. For correct tRNA gene annotation, one should also consider the positions of the nearest protein-coding and ribosomal genes in mt genomes.

As an additional consequence of the application of amphipod-specific models, several new tRNA gene remolding cases were detected. The tRNA similarity score estimation based on amphipod-specific models considered both nucleotide sequence similarity and secondary structure information. The tRNA remolding candidates were identified by higher similarity scores to the tRNA family models other than the isoacceptor family model. This approach is similar but more streamlined compared to that described in Sahyoun et al., 2015, in which candidate cases were first detected as exceptionally similar pairs of alloacceptor tRNAs contained in the same mt genome. Both methods rely on covariance model scoring and allow the detection of tRNA remolding cases in large datasets. Additionally, similar to Sahyoun et al., 2015, the cases detected by covariance model scoring are further scrutinized here by phylogenetic analysis, which in this study involves reconstruction of the tRNA gene phylogeny. Verification of tRNA remolding cases by phylogenetic inference or genetic distance analyses is a commonly used approach with smaller tRNA datasets (Belinky et al., 2008; Lavrov and Lang, 2005; Rawlings et al., 2003; Wang and Lavrov, 2011; Wu et al., 2012, 2014). The lineage-specific models for MiTFi are a valuable instrument for obtaining more accurate predictions of tRNA gene duplications and remolding cases than the annotations relying on the default Metazoa-wide models.

4. Discussion

In our study, we found some cases of duplicated tRNA genes in the mt genomes of amphipods. The application of phylogenetic and statistical methods of analysis to predicted tRNA genes enabled us to define the stages of their evolution. Additional tRNA genes were found in eleven amphipod species, six of which are Baikalian endemics occurring in different biotopes (see Romanova et al., 2016a). All of the remaining are species from different parts of the world, including two subterranean species, *P. cupicola* (Stokkan et al., 2018) and *P. daejeonensis* (Lee et al., 2018), the sublittoral Antarctic species *G. antarctica* (Shin et al., 2012), the freshwater American species *H. lucifugax* (Juan et al., 2016), and a terrestrial and supra-littoral inhabitant of the Pacific region, *P. japonica* (Juan et al., 2016). The species with tRNA gene duplications are emphasized in colour on the phylogenetic tree of amphipods with available mt genomes (Fig. 6). The separation of Baikalian species into two lineages on the tree supports the results of previous phylogenetic studies (Macdonald et al., 2005; Naumenko et al., 2017).

Some of the species with additional tRNA genes are sisters to those without tRNA duplications but otherwise similar gene orders. These are *E. vittatus* and *E. verrucosus*, *P. cupicola* and *P. multidens* and species of gen. *Platorchestia*. These examples point to the possibility of tRNA gene duplications and remoldings occurring in a relatively short span of time. According to the study of Stokkan, the split of *P. cupicola* and *P. multidens* occurred approximately 3 Mya at the boundary between Pliocene and Pleistocene (Stokkan et al., 2018).

The presence of additional tRNA gene copies indicates gene order rearrangements of varying complexity. Mt genomes of *A. victorii, E. vitttatus, G. cabanisii, P. cupicola*, *H. lucifugax,* and *G. antarctica* have the same order of PCGs and ribosomal genes as the Pancrustacean ground pattern (PGP) but differ by the positions of their tRNA genes. The gene orders in the mt genomes of *G. fasciatus, P. kessleri, C. tuberculatus, P. daejeonensis,* and *P. japonica* are considered to be profoundly altered by rearrangements of large blocks of PCGs and/or ribosomal genes relative to the PGP (Lee et al., 2018; Romanova et al., 2016a; Yang et al., 2017). Mt genomes of *A. victorii, G. cabanisii, G. fasciatus* and G. *antarctica* have large noncoding regions (Romanova et al., 2016a; Shin et al., 2012), which might be vestiges of former duplication events. The extra tRNA gene copies are also a possible consequence of active gene order rearrangements. It is not clear why the additional tRNA genes are retained in the mt genomes more frequently than the large protein coding or ribosomal genes.

The ability to distinguish between cases of simply duplicated or remolded tRNA genes is useful for analyses of gene order rearrangements and reconstruction of the scenarios of such rearrangements. For example, *trnP2* of *E. vittatus*, which was identified as a *trnL1* copy, is located between *nad1* and *rrnL*, which is a typical position for this tRNA gene in the mt genomes of most amphipod species and PGP (Boore et al., 1998; Krebes and Bastrop, 2012). Considering the mt gene order of its sister species, *E. verrucosus,* we may assume that the gene order of *E. vittatus* is more likely to occur through the duplication of the *nad1-rrnL* region and the subsequent deletion of *nad1* (Fig. 7). The *trnY2* of *A. victorii*, identified as *trnD*, is in close proximity to *atp8*, where the *trnD* is usually located in PGP. The *trnD2* identified as *trnH* is situated next to another *trnH,* and *trnA2*, which originated from *trnI*, is also located at the usual position of *trnI* (next to *trnM* and *nad2*) in the mt genomes of most Baikalian amphipods (Romanova et al., 2016a). The *trnQ2* of *G. fasciatus*, which was identified as *trnH*, is also located at its typical place for *trnH* (between *nad4* and *nad5*), while *trnN2*, which originated from *trnI*, is situated at its regular location near *nad2* in the mt genomes of most amphipod species (Romanova et al., 2016a). It is also interesting to note that *trnX* has an impaired anticodon sequence that originated from *trnM* in the mt genome of *C. tuberculatus* and is located at the position typical for *trnM* of PGP (before *nad2*). The position of the unimpaired *trnM* copy, however, is unknown due to the incomplete sequence of this mt genome. The *trnL1-3* and *trnL1-2* of *G. cabanisii* are in a usual site occupied by this gene in the PGP - between *nad1* and *rrnL*, but these copies are separated by a noncoding region of an unknown size. The *trnV2* of *P. kessleri* is located between *rrnL* and *rrnS*, which is common for the PGP (Fig. 7).

Thus, we observed that all remolded tRNA genes and duplicated tRNA genes with more divergent sequences are located in positions typical for their progenitor genes in the mt genomes of most amphipod species and in PGP, whereas their more conserved copies are more likely to end up with unusual positions in the genome. According to the phylogenetic inference, the ancestral mt gene pattern of Baikalian amphipods from the second lineage should be similar to the species of gen. *Gammarus,* which is in turn very similar to the PGP, differing only by six tRNA gene positions (Cormier et al., 2018; Krebes and Bastrop, 2012; Macher et al., 2017). Thus, we deduce that mt gene order rearrangements presumably involved progressive alterations of the 'common' gammarus-like ancestral pattern to the more varied patterns of Baikalian species from the second lineage. The ancestral mt gene order of Baikalian species of the first lineage is more difficult to deduce; however, it should probably share common features with the mt gene orders of *O. nansenii, E. maldoror* and species of gen. *Gammarus.* To elucidate the scenarios of these changes, more precise analysis taking into account all mt gene features is needed.

The tRNA gene duplications in the mt genomes of non-Baikalian amphipods also accompany various types of rearrangements. The mt genomes of *H. lucifugax, G. antarctica* and *P. cupicola* have the same order of protein-coding and ribosomal genes as the PGP; however, the order of tRNA genes in all three genomes varies significantly (Juan et al., 2016; Shin et al., 2012; Stokkan et al., 2018). In all three cases, the additional tRNA genes are located relatively close to their progenitor tRNA genes. Both copies of *trnC* from the mt genome of *H. lucifugax* are located between *trnY* and the control region. In the mt genome of *G. antarctica*, *trnX1* and *trnX1* are situated in the large noncoding area between *trnW* and *trnG*. The four additional tRNA gene copies of *P. cupicola* are situated between *trnM* and *nad2* (Supplementary Table S2). The origin of *trnH2* from *trnY* allows us to predict the appearance of the mt gene order of this species. The ancestor of *P. cupicola* underwent a tandem duplication of the *trnI-trnY-trnQ-trnC* region, and then the *trnY* of the first copy remolded to *trnH*. In the modern *P. cupicola*, this region is annotated as *trnI1-trnH2-trnQ1-trnC1-trnI2-*

trnY-trnQ2-trnC2 (Supplementary Table S2). This scenario looks very likely considering that the mt genome of the sister species *P. multidens* possesses one copy of the region *trnI-trnY-trnQ-trnC*.

The mt genomes of *P. japonica* and *P. daejeonensis* possess major rearrangements relative to the PGP. In the former species, the additional *trnQ2,* identified as the remolded copy of *trnR,* is located in the immediate vicinity of its progenitor *trnR*. Two copies of *trnL2* from *P. daejeonensis* are situated at a large distance from each other and are separated by other genes (Lee et al., 2018, Supplementary Table S2). In this case, we cannot propose any scenario for this mt gene order appearance because its closest sister species were not reliably identified (Fig. 6). Finding additional or remolded tRNA genes at their common locations or in proximity to their progenitor tRNA genes provides additional support for the correctness of their annotation.

The mt genomes of most Baikalian species studied here (six out of ten) contain extra tRNA gene copies, whereas in the 78 non-Baikalian amphipod species, we found only five species with duplicated tRNA genes. Although the current sample of species is biased towards species of gen. *Metacrangonyx* and *Pseudoniphargus*, we may nevertheless conclude that the presence of extra tRNA genes is more typical for mt genomes of Baikalian species.

It is generally assumed that the majority of redundant gene copies rapidly accumulate mutations and lose their functions (Macey et al., 1997), as selection is directed towards mt genome compactization (Lynch and Conery, 2000). If we assume the deleterious or slightly deleterious nature of the additional mt tRNA genes for organism fitness, we should expect that the presence of extra tRNA genes may be more common for mt genomes of species with populations subjected to profound decline of genetic diversity (Kimura, 1983). A recent study by Bukin et al., (2018) reconstructed the evolutionary history of Baikalian populations of *G. fasciatus*. The research revealed that the southwest population of this species, which the sequenced individual of *G. fasciatus* belongs to, underwent directional selection, leading to its low genetic diversity and a very low estimated effective population size (Bukin et al., 2018). The data on the genetic diversity of the *A. victorii* population in Baikal, on the contrary, discovered the process of neutral evolution (Daneliya et al., 2011). These controversial results may be explained either by the neutral effect of additional tRNA genes in mt genomes on the viability of amphipods in Lake Baikal or rather by the more significant impact of protein-coding and ribosomal gene rearrangements. We may also hypothesize that the emergence and maintenance of additional tRNA gene copies as well as the presence of other unusual features in the mt genomes of amphipods (Romanova et al., 2016a) are favoured by environmental factors peculiar to Lake Baikal, such as the high concentration of oxygen in Baikalian water. To discover why Baikalian species are tolerant to the additional and remolded tRNA genes in their mt genomes, more extensive knowledge of the population history is needed.

Another possible explanation for the prevalent tRNA gene duplications in mt genomes of some Baikalian species is their possible functional significance. For instance, the secondary structure of mt tRNA gene sequences is thought to assist with the correct processing of primary mt transcripts by functioning as punctuation marks (Ojala et al., 1981). Mabuchi et al. (2004) propose that tRNA pseudogenes (i.e., redundant tRNA genes that underwent partial degeneration) may also act as punctuation marks the in the mt genomes of Scaridae fishes (Mabuchi et al., 2004). In mt genomes of Baikalian species and a non-baikalian *P. daejeonensis*, the duplicated and remolded tRNA genes separate different protein coding or ribosomal genes (Fig. 6, Supplementary Table S2). Thus, the additional tRNA genes in amphipods may function as punctuation marks in place of their predecessors. It is important that most of these tRNAs retain their typical cloverleaf secondary structure (Supplementary Fig. S3), which is in concordance with one of the criteria for tRNA gene functionality (Ojala et al., 1981; Kumazawa and Nishida, 1993).

Although the existence of remolded tRNAs in an organism can lead to translation errors and is strongly selected against (Sengupta and Higgs, 2015), mistranslation under specific conditions such as oxidative stress or adaptation to a new environment might be beneficial for organism viability as it increases the diversity of the protein pool (Mohler and Ibba, 2017; Netzer et al., 2009; Schwartz and Pan, 2017; Yona et al., 2013). In cases when translation of the codon by the alternative reassigned tRNA becomes more advantageous, this can be gradually fixed by natural selection. Thus, tRNA gene remolding is the one of the mechanisms of genetic code alteration in the course of evolution (reviewed in Kollmar and Mühlhausen, 2017; Moura et al., 2010; Watanabe and Yokobori, 2011). The probability of such a scenario for amphipods is supported by the detection of several cases of tRNA gene remolding in mt genomes of the arthropod lineage (Abascal et al., 2012; Rawlings et al., 2003; Sahyoun et al., 2015). For instance, the origination of *trnL(CUN)* from duplicated and remolded *trnL(UUR)* was shown in some decapod taxa and the isopod *Eophreatoicus* sp.-14 on the basis of sequence similarity and phylogenetic inference (Kilpert and Podsiadlowski, 2010; Rawlings et al., 2003). Sahyoun et al., 2015 identified a remolding event of trnW(UCA) to trnG(UCC) in the ancestor of Amphipoda, and trnC(GCA) to trnY(GUA) remolding in the Peracarida lineage (except Isopoda) (Sahyoun et al., 2015). Mutation in the anticodon is responsible for the reassignment of the AGG codon in arthropods from lysine to serine (Abascal et al., 2012).

A detailed analysis of the tRNA gene pool in the human genome and the genomes of other eukaryotes revealed a large percentage of isodecoders (tRNAs that share the same anticodon but have differences in their body sequence) (Geslain and Pan, 2010; Goodenbour and Pan, 2006; Velandia-Huerto et al., 2016), which may be indirect evidence of their potential functionality. Indeed, different additional functions of tRNAs have been discovered recently. Misacytelated and poorly acetylated tRNAs, pseudo tRNAs and tRNAs fragments are involved in various cellular processes such as regulation of transcription, antibiotic resistance, RNA biosynthesis, RNA silencing, etc. (reviewed in Hamashima and Kanai, 2013).

The recently discovered abundance of additional tRNAs in mt and nuclear genomes and the diversity of their functions make their correct identification very important. In our study, we found many cases of additional tRNA genes in mt genomes of amphipods that illustrate the complex pattern of their evolution. The bioinformatic tools applied in this study for the identification and verification of mt tRNA genes (the amphipod-specific models for mt tRNA genes were searched in MiTFi, and R scripts were used to determine the most appropriate mode of alignment and to estimate genetic distances in groups of tRNA genes) may be used in future studies of mt genomes of amphipods.

5. Conclusion

In our study, we performed the identification of tRNA genes in available mt genomes of amphipods. The amphipod-specific models for MiTFi revealed novel cases of tRNA gene duplication and allowed us to refine annotations of amphipod mt genomes. Application of covariance model scores, phylogenetic inference and genetic distance estimation provides a robust framework for the validation of predictions and establishing the evolutionary origins of duplicated tRNA genes. Thus, we identified eight cases of recent tRNA gene remolding events and the ancestry of tRNA genes with impaired anticodon sequences. It is notable that an excess of tRNA gene remolding cases was detected in the mt genomes of Baikalian amphipods relative to other amphipod species. The observed abundance of tRNA gene duplication and remolding events in amphipod mt genomes suggests complex patterns of tRNA gene evolution. The data on additional duplicated and remolded tRNA genes are essential for the correct annotation of mt genome sequences and for studying the gene order dynamics and evolution of tRNA genes in mt genomes of amphipods and other groups of species.

CRediT authorship contribution statement

Elena V. Romanova: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Yurij S. Bukin:** Software, Formal analysis. **Kirill V. Mikhailov:** Software, Formal analysis. **Maria D. Logacheva:** Data curation, Investigation. **Vladimir V. Aleoshin:** Project administration, Funding acquisition. **Dmitry Yu. Sherbakov:** Conceptualization, Methodology, Resources, Writing - review & editing.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106710.

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