New insights into Oniscidea (Crustacea: Isopoda) mitogenome structural features and phylogenetic placement of targeted taxa using mitogenomic and nuclear data

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Abstract

Among Isopoda suborders the Oniscidea has the highest species richness, and is also the largest terrestrial group in the Crustacea. Terrestrial isopods are an excellent case to study adaptations related to sea-land transition. However, the monophyly of Oniscidea and the relationships of the main lineages has been debated over the last three decades. Aiming to further explore structural features of mitochondrial genome and investigate the phylogenetic relationships within Oniscidea, the mitogenomes and a series of nuclear markers of the oniscids *Ligia exotica* and *Mongoloniscus sinensis* were sequenced. The nuclear genome was represented by four nuclear genes analyzed in a separate dataset. The mitogenomes of *L. exotica* and *M. sinensis* were 16,018 and 14,978 bp in length, respectively. Both included 13 protein-coding genes, 2 rRNA genes, and 21 and 19 tRNA genes respectively, missing one and three tRNA genes respectively compared to the isopod ground pattern. The *M. sinensis* mitogenome had higher average A+T content (~75.3%) than any other isopod studied to date. Phylogenetic analyses confirmed the assignment of *M. sinesis* to Agnaridae, as well as the sister-group relationship of the family with Porcellionidae, one of the more derived Crinochaeta clades. On the other hand, the basal position of *Ligia* within Oniscidea and the close evolutionary relationship with the aquatic groups Valvifera, Shaperomatida and some Cymothoidea that were included in our analysis, is indicated.

Introduction

The Isopoda, with more than 10,300 described species, is the largest order within the Peracarida, and includes an amazing diversity in morphology and ecology [1,2]. Isopods are diverse in the ocean, land, and freshwater, include free-living to parasitic taxa, and range from the deep oceans to at least 4,700m above sea level. Currently, the Isopoda is divided into 11 suborders, with Oniscidea being the most speciose suborder consisted of 39 families, 523 genera and more than 3700 species [3-5]. From an ecological point of view, although Oniscidea can be found across most terrestrial biomes, from nearshore marine habitats to moist forests and arid deserts, they exhibit a strong preference for humid micro-habitats [5]. These phytosaprophagous animals are among the most important decomposers [6]. The suborder is currently separated into five lineages: Diplocheta, Tylida, Microcheta, Synocheta and Crinocheta [7-9]. The most basal members of the group, in the families Ligiidae, Ligidiidae, and Tylidae are amphibious and restricted to littoral marine habitats [10]. *Ligia* Fabricius 1798 is of particular interest as it is considered to be an intermediate form with morphological, physiological and behavioral characteristics that exhibit similarities with marine ancestors and fully terrestrial Oniscidea [8,11]. Schmidt (2008) in a detailed morpho-phylogenetic study of oniscids argued that the Ligiidae was the most basal clade in the Oniscidea, suggesting a marine origin for the suborder. Hence the phylogenetic relationship of Ligiidae with the rest of Oniscidea and aquatic relatives is essential in our effort to understand the evolutionary steps towards Isopods terrestrialization.

Although a series of synapomorphies have been proposed to support the monophyly of the group [7,8,10,12,13], molecular phylogenetic analyses have questioned the common origin of all Oniscidea and also recovered varied topologies for the relationship of oniscids to other isopods [9,14-23]. Recently, in a study based on four highly conserved nuclear genes Dimitriou et al. (2019) failed to recover a monophyletic Oniscidea, with *Ligia* recovered with valviferan and sphaeromatid isopods.

Extremely high genetic distances in both nuclear and mitochondrial genome reaching up to 50.3% between confamiliar genera and 20.3% between individuals of the same species were identified [24,25]. These results highlight the vivid divergence between even closely related taxa indicating the need of phylogenetic assessment using genetic data where possible.

Dataset of mitochondrial sequences is widely used to infer phylogenetic relationships and resolve taxonomic debates [26-38].

Isopods mitogenomes exhibit substantial diversity and unusual structures with unique linear/circular organization in some oniscids and inverted GC skew in cymothoids [39,40]. The majority of isopods studied exhibit an inversed strand asymmetry (positive GC skew), while a negative GC skew in some is attributed to double-inversion [22,23]. So far mitogenomic data are currently available for only 10 Oniscidea, including one species of *Ligia* (Table 1). The phylogenetic placement of this taxon is crucial to determine whether terrestrial isopods invaded in land once in the past, consisting a monophyletic group, or more times.

Table 1. All known mitogenomes of suborder Oniscidea.

Species	Genebank acession number	Mt genome Length (bp)	Entire genome number	Protein-coding gene Length(bp)	Protein-coding gene number	tRNAs number	Miss tRNA	Complete or Partial mitogenomes	Refere or N Data author
Mongoloniscus sinensis	**MG709492**	**16018**	**34**	**11088**	**13**	**19**	**TrnA,trnE,trnL1**	**Complete**	**This st**
Ligia exotica	**MK028672**	**14978**	**36**	**11113**	**13**	**21**	**TrnG**	**Complete**	**This st**
Ligia oceanica	DQ442914	15289	36	11064	13	21	TrnR	Complete	Kilpert al. (20
Armadillidium vulgare	EF643519	13939	28	10291	12(Miss nad2)	12	TrnR,trnE,trnI,trnL1,trnS1,trnL2,trnK,trnN,trnA	Partial	Marca et (2007)
Cylisticus convexus	KR013002	14154	33	11041	13	18	TrnG,trnV,trnF,trnE(trnC repetition)	Partial	Chand et (2015)
Trachelipus rathkii	KR013001	14129	29	10776	13	14	TrnA,trnL2,trnY,trnK,trnE,trnS1,trnT,trnQ	Partial	Chand et (2015)
Armadillidium album	KX289585	13812	29	11187	13	15	Trna,trnI,trnW,trnR,trnH,trnE,trnS1,trnL1,16S (trnK repetition)	Partial	Marca et al
Armadillidium nasatum	MF187611	13943	34	10846	12(Miss atp8)	20	TrnA,trnI,trnR,trnK,trnE,trnQ(trnD,trnW,trnF repetition,three TrnL)	Partial	Peccou et (2017)
Oniscus asellus	KX289581	13515	29	7310	13	15	trnV,trnI,trnW,trnR,trnE,trnL2,trnS2,16S,trnL1	Partial	Marca et al
Porcellionides pruinosus	KX289584	14078	31	11696	13	16	trnG,trnV,trnL2,trnE,trnS2,16s	Partial	Marca et al
Procellio dilatatus petiti	KX289583	13343	30	10304	13	15	trnG,trnA,trnL2,trnW,trnE,trnS2	Partial	Marca et al
Procellio dilatatus dilatatus	KX289582	14103	31	11235	13	17	trnG,trnA,trnL2,trnE,trnS2,12S	Partial	Marca et al

Aiming to further explore oniscid mitogenome characters and investigate the phylogenetic relationships within the group and between terrestrial isopods and aquatic relatives, the complete mitogenomes of *L. exotica* and *M. sinensis* were sequenced. Furthermore, we also sequenced 18s, 28s ribosomal RNA genes, protein-coding Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genes of *M. sinensis* and *Ligia cinerascens* to supplement the assignment based on mitogenome dataset. Mitogenome and nuclear data were kept separately taking into account the limited overlap in the taxa and the fact that they originate from organelles under different evolutionary pressure.

Materials And Methods

2.1 Sampling

Specimens of the endemic to China *Mongoloniscus sinensis* (Dollfus, 1901) were collected from Tao village in Yantai, Shandong province (37°28.8' N, 121°27.6' E), while congeneric *Ligia exotica* Roux, 1828 and *Ligia cinerascens* Budd-Lunde, 1885 were collected from Qingdao, Shandong province (36°18' N, 120°06' E) and Huludao, Liaoning province (40˚44' N, 120°58' E) respectively. All collected specimens were kept in ethanol (>99.7%) until DNA extraction and vouchers deposited in the collections of the Shanxi Normal University.

2.2 DNA extraction, gene annotation and sequence analysis

Total genomic DNA was extracted from the pereopods/pleopods of specimens using the gDNA rapid extraction kit (Aidlab Biotechnologies Co., Ltd) according to the manufacturer's instructions. Libraries were constructed using Whole Genome Shotgun (WGS) strategies with libraries sequenced using paired-end (PE) approaches on the Illumina MiSeq sequencing platform.

Raw data quality was tested using the Read Quality Inspection Tool FastQC (http://www.bioinformatics.ac.uk/projects/fastqc). High-quality NGS data were assembled with A5-miseq v20150522 and SPAdesv3.9.0 to construct contigs and scaffold sequences. The complete mitochondrial sequences were identified and annotated by BLAST v2.2.31, Mummer v3.1 and Pilon v1.18. The complete mitochondrial sequences (*M. sinensis* and *L. exotica*) were annotated using the MITOS webserver [41]. The secondary structure of tRNAs were identified using tRNAscan-SE v1.21 [42] and ARWEN v1.2.3.c [43]. The complete genome sequences have been submitted to NCBI (GenBank acc. no.: MG709492 and MK028672).

2.3 Nuclear DNA primer design, LA-PCR amplification and sequencing

Primers targeting the 18s rRNA, 28s rRNA, Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genes were designed according available sequences of closely related species (S1 Table). PCR conditions were as follows: initial denaturation 94°C for 2 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min/kb, followed by the final extension at 72°C for 10 min. The total volume for PCR and LA-PCR was 50 μl, of which Takara LATaq (5 U/μl) was 0.5 μl, 10×LATaq Buffer II (Mg2+) was 5 μl, dNTP mixture (2.5 mM) was 8 μl, template was 60 ng,

and the total volume was reached with distilled water. The final concentration of the forward and reverse primers was 0.2~1.0 μM, and that of MgCl2 was 2.0 mM. The PCR products were sequenced directly, or if needed first cloned into a pMD18-T vector (Takara, JAP) and then sequenced, by the dideoxynucleotide procedure, using an ABI 3730 automatic sequencer (Sanger sequencing) using the same set of primers.

2.4 Phylogenetic analysis

Phylogenetic analyses were conducted using concatenated amino acid sequences of all 13 protein-coding genes (PCGs) from 2 new sequenced and 28 currently available isopod mitogenomes from GenBank. Amino acid sequences were aligned using MAFFT in batch mode under L-INS-i strategy [44,45]. Aligned loci were concatenated with PhyloSuite. Bayesian analysis using the probabilistic CAT-GTR model was implemented in PhyloBayes-MPI 1.7a through CIPRES server [46]. The analysis run using default parameters until maxdiff < 0.1 and minimum effective size > 300 were reached.

The nuclear genes dataset was further enriched with species *Platorchestia japonica* (Tattersall, 1922), *Gammarus troglophilus* Hubricht & Mackins, 1940 and *Sinogammarus chuanhui* Hou & Li, 2002 included to serve as outgroup.

Multiple sequence alignments were performed using MAFFT wih Q-INS-i algorithm for not-coding 18s rRNA and 28s rRNA and '–auto' option for NAK and PEPCK. Ambiguously or poorly aligned regions were removed using Gblocks allowing smaller final blocks, less strict flanking positions and presence of gaps at the final alignment [47,48]. The best-fit partitioning scheme and evolutionary model for each partition were selected with PartitionFinder2 under BIC criterion [49]. The final concatenated dataset set was partitioned by gene into four distinct data blocks. Bayesian inference analysis (BI) was implemented using MrBayes v.3.2.6 [50], with the selected model of nucleotide evolution for each gene, allowing rate heterogeneity between partitions.

Results

3.1 Complete mitochondrial genomes

The complete mitochondrial genome of *M. sinensis* and *L. exotica* are circular, double-stranded DNA molecules, 16,018 and 14,978 bp in length, respectively. The *M. sinensis* mitogenome is comprised of 13 protein-coding genes, 2 rRNA genes and 19 tRNAs, with trnA, trnE and trnL1 are missing. In addition, 18 overlapping regions and 13 intergenic regions were identified (Table 2). The average A+T content of the *M. sinensis* mt genome is approximately 75.3% (S2 Table), higher than any other known isopod mitogenomes (range: 54.4□-71.2□). The mitogenome of *L. exotica* is composed of 13 PCGs, 2 rRNA genes and 21 tRNA genes, only lacking the trnG gene. In total 17 overlapping regions and 14 intergenic regions were detected (Table 3). The average A+T content is approximately 59.1% (S2 Table). The PCG size and start/stop codons of both species is typical of other isopod mitogenomes (Tables 2 and 3). Consistent with the inversed strand asymmetry shown in most isopods, GC skews of the mitochondrial genomes of 12 examined Oniscidea was positive (S2 Table).

Table 2. Gene content of the *Mongoloniscus sinensis* mitochondrial genome.

Feature	Strand[a]	Position	Length (bp)	Initiation Codon	Stop Codon	Anticodon	Intergenic nucleotide
cob	-	1-1,107	1,107	ATG	TAA	–	64
nad5	+	1,172-2,884	1,713	ATT	TAA	–	*
trnF	+	2,885-2,952	68	–	–	GAA	-33[b]
trnH	-	2,920-2,984	65	–	–	GTG	5
nad4	-	2,990-4,333	1,344	ATG	TAA	–	*
nad4l	-	4,334-4,612	279	ATT	TAA	–	-5
trnP	-	4,608-4,665	58	–	–	TGG	-4
nad6	+	4,662-5,153	492	ATT	TAA	–	-2
trnS2	+	5,152-5,202	51	–	–	TGA	-9
rrnL	-	5,194-6,336	1,143	–	–	–	21
trnQ	-	6,358-6,424	67	–	–	TTG	47
trnM	+	6,472-6,529	58	–	–	CAT	-16
nad2	+	6,514-7,518	1,005	ATG	TAG	–	-10
trnC	-	7,509-7,562	54	–	–	GCA	-18
trnY	-	7,545-7,600	56	–	–	GTA	4
cox1	+	7,605-9,140	1,536	ATG	TAA	–	1
trnL2	+	9,142-9,208	67	–	–	TAA	-10
cox2	+	9,199-9,870	672	ATC	TAA	–	-2
trnK	+	9,869-9,926	58	–	–	TTT	-13
trnD	+	9,914-9,975	62	–	–	GTC	-8
atp8	+	9,968-10,123	156	ATT	TAA	–	-13
atp6	+	10,111-10,785	675	ATG	TAA	–	*
cox3	+	10,786-11,613	828	ATG	TAA	—	-41
trnR	+	11,573-11,633	61	–	–	TCG	21
nad3	+	11,655-12,011	357	ATT	TAA	–	-41
trnV	+	11,971-12,025	55	–	–	TAC	-9
nad1	-	12,017-12,940	924	ATA	TAA	–	1
trnN	+	12,942-13,008	67	–	–	GTT	-2
rrnS	+	13,007-13,723	717	–	–	–	-17
trnI	+	13,707-13,768	62	–	–	TCA	4
trnW	+	13,773-13,826	54	–	–	TCA	292
trnG	+	14,119-14,176	58	–	–	TCC	485
trnT	+	14,662-14,725	64	–	–	TGT	879
trnS1	+	15,605-15,677	73	–	–	TCT	341

*Gene borders are defned based on borders with adjacent genes.

[a]Plus strand (+)/minus strand (−).
[b]Negatve values represent overlapping nucleotdes.

Table 3. Gene content of the *Ligia exotica* mitochondrial genome.

Feature	Strand[a]	Position	Length (bp)	Initiation Codon	Stop Codon	Anticodon	Intergenic nucleotide
trnE	+	663-724	62	–	–	TTC	–
trnS1	+	725-787	63	–	–	TCT	17
cob	-	805-1,938	1,134	ATA	TAA	–	*
trnT	-	1,939-1,997	59	–	–	TGT	-8[b]
nad5	+	1,990-3,717	1,728	ATT	TAG	–	-8
trnF	+	3,710-3,768	59	–	–	GAA	-2
trnH	-	3,767-3,828	62	–	–	GTG	*
nad4	-	3,829-5,158	1,330	ATG	T	–	-7
nad4l	-	5,152-5,448	297	ATA	TAA	–	6
trnP	-	5,455-5,516	62	–	–	TGG	1
nad6	+	5,518-6,024	507	ATT	TAG	–	-2
trnS2	+	6,023-6,084	62	–	–	TGA	*
rrnL	-	6,085-7,268	1,184	–	–	–	-7
trnV	-	7,262-7,320	59	–	–	TAC	2
trnQ	-	7,323-7,377	55	–	–	TTG	4
trnM	+	7,382-7,445	64	–	–	CAT	-21
nad2	+	7,425-8,441	1,017	ATG	TAG	–	-15
trnC	-	8,427-8,479	53	–	–	GCA	-1
trnY	-	8,479-8,540	62	–	–	GTA	6
cox1	+	8,547-10,079	1,533	CGA	TAA	–	-5
trnL2	+	10,075-10,136	62	–	–	TAA	*
cox2	+	10,137-10,820	684	ATA	TAG	–	-2
trnK	+	10,819-10,880	62	–	–	TTT	-2
trnD	+	10,879-10,938	60	–	–	GTC	9
atp8	+	10,948-11,097	150	ATA	TAA	–	-7
atp6	+	11,091-11,762	672	ATG	TAA	–	-1
cox3	+	11,762-12,565	804	ATG	TAA	–	-17
trnR	+	12,549-12,608	60	–	–	TCG	9
nad3	+	12,618-12,962	345	ATT	TAG	–	-2
trnA	+	12,961-13,021	61	–	–	GCA	24
nad1	-	13,046-13,957	912	ATC	TTA	–	18
trnL1	-	13,976-14,035	60	–	–	TAG	-4
trnN	+	14,032-14094	63	–	–	GTT	1
rrns	+	14,096-14,794	699	–	–	–	2
trnI	+	14,797-14,860	64	–	–	GAT	14
trnW	+	14,875-14,938	64	–	–	TCA	39

*Gene borders are defned based on borders with adjacent genes.
[a]Plus strand (+)/minus strand (−).
[b]Negatve values represent overlapping nucleotdes.

3.2 Transfer RNA genes

Nineteen tRNAs were found in *M. sinensis*, ranging from 51 bp (trnS2) to 73 bp in size (trnS1), totaling 1158 bp. In *L. exotica*, 21 tRNAs were identified, ranging from 53 bp (trnC) to 64 bp in size (trnI, trnM, trnW), summing up to 1278 bp. Transfer RNA genes are distributed throughout the mitogenome and found on both strands. The proposed secondary structures of all identified tRNAs are shown in Figs 1 and 2. The majority of tRNAs have a common t-shaped or clover-leaf secondary structure. In *M. sinensis* exceptions include the absence of DHU-arm in trnC, and TΨC-arm in trnG, trnK, trnP, trnS2, trnW and trnV. In *L. exotica* exceptions are the absence of DHU-arm in trnC and trnV, and TΨC-arm in trnF, trnP, trnM, trnL2, trnK, trnD, trnR.

3.3 Gene translocations of mitogenomes

The comparison of mitogenomes of *M. sinensis* and *L. exotica* with the known ground pattern of isopods revealed four and three gene rearrangements respectively (Fig 3). In *M. sinensis* i) trnR is located between cox3 and nad3 instead of trnA and nad1 where it is usually found; ii) trnV is between nad3 and nad1, instead of between 16s rRNA and trnQ, iii) trnG is between trnW and trnT, instead of between cox3 and nad3 and iv) trnT is located between trnG and trnS1, instead of between cob and nad5. Similarly, in *L. exotica*, trnR was translocated between cox3 and nad3, while trnW and trnE are interchanged. Compared with the known found pattern of isopods, both *M. sinensis* and *L. exotica* have two and one gene missing respectively. In *M. sinensis* i) trnA is missing instead of between nad3 and trnR; ii) trnL1 is missing instead of between nad1 and trnN. In *L. exotica*, trnG is missing instead of between cox3 and nad3.Gene translocations as well as loss of tRNA genes are well known in isopod mitogenomes. Some tRNA genes are missing in every oniscids studied to date. Some of the genetic rearrangements are attributed to the missing tRNA genes.

3.4 Phylogenetic analyses

3.4.1 Amino Acids Dataset (13PCGs)

Phylogenetic reconstruction based on the concatenated amino acid sequences of all 13 PCGs recovered a statistically well supported monophyletic Oniscidea clade, with Ligiidae in basal position. *M. sinensis* was recovered as sister to a clade to Porcellionidae represented by *Porcellionides* and *Porcellio* (Fig 4).

3.4.2 Nuclear Dataset (18s-28s-NAK-PEPCK)

The aligned data for the combined nuclear genes was 1719 base pairs (bp). The best substitution models were SYM+I+G for 18s, GTR+G for 28s, SYM+I+G for NAK, and GTR+I+G for PEPCK. The Oniscidea was recovered in two separate statistically well supported clades, with *Ligia* species in one, and the rest of oniscids in another including *Sphaeroma*. Although the basal phylogenetic relationships of the group are not fully resolved, the close evolutionary relationship of Oniscidea excluding *Ligia*, with *Sphaeroma* is statistically supported. *Typhloligidium* Verhoeff, 1918, *Ligidium* Brandt, 1833 and *Taurologidium* Borutzky, 1950 all members of the newly established family Ligidiidae are grouped together [9], Agnaridae, Platyarthridae, Trichoniscidae, Trachelipodidae and Porcellionidae were all recovered as polyphyletic. *Mongoloniscus sinensis* was sister to *Agnara madagascariensis* and in turn with to three species of *Hemilepistus*, but in a separate branch than *Protracheoniscus* aff. *fossuliger* the only other agnarid included.

Discussion

4.1 Characteristics of mitogenomes

The aim of this work was to determine the mitogenome structure of two Oniscidea species and exploit these data for the phylogenetic assignment of targeted species and possibly revisit phylogenetic relationships within Oniscidea and/or Isopoda. The mitogenomes of *L. exotica* and *M. sinensis* are circular double stranded, with incomplete or totally missing tRNA genes. A recently published work speculated that these tRNA gene fragments may actually be transcribed and edited into functional tRNAs, since their presence is common in isopods [23]. Structural rearrangements of these two species' mitogenomes may be explained by linearized and fragmented events [51].

Unfortunately, the pattern of specific genes loss and rearrangement, and the exact mechanism behind it, had not been found in the 12 known species of the suborder Oniscidea, possibly due to insufficient data. It is hoped that the pattern, exact mechanism and the meaningful information behind the pattern can be found in future further studies.

4.2 Data Processing

Considering the confounding phylogenies resulting from different datasets based on nuclear or/and mitochondrial data [9,14,15,17,23], we compiled two different inclusive datasets in order to assess the patterns yielding from genetic loci with different evolutionary history. Herein we investigated the phylogenetic assignment of the targeted species within Oniscidea combining the results from separate analyses of genomic data from different organelles. Taking into account the long evolutionary history of the group, in order to avoid saturation events due to high mutation rates of mitogenomes, only PCGs were included in mitochondrial datasets analyses [52]. Furthermore, these loci were translated in AA sequences and run under a heterogeneous CAT-GTR model. In this way we avoided phylogenetic artifacts attributed to biases due to similar GC skews among distant taxa [22]. On the other hand, nuclear non-coding genes were treated with Gblocks in order to eliminate poorly aligned regions. With this treatment we excluded from our analyses DNA fragments accumulating mutations at higher rates, compared to their flanking regions, which could result in misleading phylogenies.

4.3 Phylogenetic relationships of Oniscidea

Phylogenetic relationships within Oniscidea and other isopod groups, even at high taxonomic scales, based on morphological characters or molecular data were repeatedly debated [9,15,53-55]. The monophyly of Oniscidea is well supported by morphological characters such as the complex water-conducting system and reduced first antenna with only three articles [8,12,13,15,56,57]. Although it was alternatively suggested as a possible homoplasy, the first antenna with rudimentary three articles has been regarded as a prominent synapomorphy for Oniscidea [8,12]. Furthermore, taking into account the existence of two types of water conducting systems (i.e "Ligia type" and "Porcellio type") [10], we could speculate that these two characters might not be attributed to the monophyletic origin of Oniscidea but to convergent evolution as a response to environment challenges [15].

4.4 Phylogeny based on mitogenome dataset

Monophyly of Oniscidea have been questioned by several phylogenetic analyses including molecular data [9,14,22,23,58]. Solely based on mitochondrial data recently published works failed to support the monophyly of Oniscidea [20,21,51]. The monophyly of Oniscidea mainly relies on the phylogenetic origin of the key genera *Ligia*, *Ligidium*, *Tylos* and *Helleria* whose close evolutionary relationship with marine ancestors was repeatedly highlighted in the past [9,15,22]. Therefore, two species of the genus *Ligia* were added in this paper to explore the monophyly of the suborder Oniscidea. Particular focus was given in *Ligia*, as that genus have been proposed to represent an intermediate form in the evolution of terrestriality in isopods [11]. However, the phylogenetic placement of *Ligia* within Oniscidea was debated in the past [8,9].

According to our mitogenome analysis results, *Ligia* appears to form a statistically well supported monophyletic group with Crinocheta, the more evolutionary recent Oniscidea lineage whose representatives exhibiting some of the most pioneer adaptations to terrestrial life. These results are also supported by a series of well described Oniscidea synapomorphies [7,8]. Based on the same mitochondrial loci, running analyses under different evolutionary substitution models recently published works came up with different results [15,23]. In contrast to Lins et al. (2017) our results are in agreement with Zou et al. (2020) findings whose analysis was conducted under a CAT-GTR model (Fig 4).

Furthermore, our analysis revealed the close evolutionary relationship of Oniscidea with Sphaeromatidea, Valvifera and some Cymothoidea. Sphaeromatidae and Valvifera were also shown to be the more closely related marine taxa to terrestrial isopods by recently published works based on mitochondrial and nuclear data [9,23]. All Asellota members are grouped together with strong statistical support while Cymothoa although represented by a small number of species appear to be paraphyletic. Phreatoicidea should be considered as the more ancestral isopod lineage rooting the mitogenome tree.

4.5 Nuclear markers Phylogeny

Although the mitochondrial genome data were mainly used in this paper to elucidate the phylogenetic relationship of Oniscidea, nuclear gene data were also used for supplement, as detailed in the appendix materials. The main differences between the nuclear dataset presented by Dimitriou et al. (2019) and the one analyzed herein, mainly enriched with outgroups, are located at the basis of the constructed phylogenetic tree and added a key genus species, *L. cinerascens*.

The basal position of Phreatoicidea as well as Asellota at Isopods phylogeny is also supported by data of nuclear origin. On the other hand, we failed to save the monophyly of Oniscidea as *Sphaeroma* appear to be more closely related to terrestrial isopods than *Ligia* (S3 Fig.) (Hence the monophyly of Diplocheta could not be saved as well. Numerus taxonomic revisions at lower taxonomic levels especially withing the more apical sister groups Crinocheta and Synochetawere proposed [5,24].

The familiar assignment of *Mongoloniscus* was debated in the past as it was proposed to be a member of both Agnaridae and Porcellionidae families [3,59]. Our results corroborate the assignment of *Mongoloniscus* to Agnaridae family as well as the close evolutionary relationship with Porcellionidae family. This relationship is also confirmed by a recently published study undermining the monophyly of Porcellionidae [24].

At family lever our results provide evidence against the monophyly of Agnaridae, Platyarthridae, Trachelipodidae, Porcellionidae and Trichoniscidae families (S3 Fig.). Based on morphological or/and genetic data previous studies came to the same results questioning also the monophyly of Scyphacidae, Philosciidae, Dubioniscidae, Trachelipodidae and Porcellionidae [8,9,17,24]. Aforementioned studies commended on the validity of traditionally used morphological characters for taxonomic purposes and shown the impact of the selected genetic loci on phylogenetic reconstructions.

Conclusions

Present Oniscidea classification was determined by morphological characters which probably cannot adequately portray the evolutionary history of the group and its relation with aquatic relatives. This is highlighted by the numerus taxonomic revisions, even at high taxonomic levels, considering new morphological or/and molecular data.

Incongruent patterns were produced using different characters or combination of genetic loci. Herein, keeping separately the genetic data from different organelles, that are not under the same evolutionary pressure, we reached at different phylogenetic patterns even regarding the monophyly of Oniscidea. Previously published studies indicated the important influence of taxonomic sampling, outgroup selection, targeted loci and selected models of nucleotide evolution on constructed phylogenies [9,15,23]. Analyses with deviations on any of these parameters could lead in different tree topologies, even between the basal Oniscidea clades, indicating alternative scenarios about the origin and terrestrialization history of the group. Even exploiting similar datasets depending on aforementioned factors we might conclude having different phylogenies [15,23].

Although recently there are growing evidence against the monophyly of Oniscidea [9,15] crucial aspects on taxon's terrestriallity history still remain to be explored. From the phylogenetic point of view, a more inclusive mitogenome dataset could give new insights related to the evolution of both the taxon and the Isopods special mitochondrial structure and organization. A combination of better representation of taxa and nuclear loci should also be applied for more robust and reliable results. In this direction next generation sequencing techniques (NGS) targeting the whole genome, in combination with bioinformatic progress on the development of new advanced tools are anticipated to take advantage of high throughput data and shed light in various facets of terrestrial isopods evolutionary history.

Abbreviations

bp: base pair

DNA: Deoxyribonucleic acid

g DNA: genome Deoxyribonucleic acid

L.exotica: *Ligia exotica*

M.sinensis: *Mongoloniscus sinensis*

NCBI: National Center of Biotechnology Information

NCR: non-coding regions

NGS: Next Generation Sequencing

PCGs: protein-coding genes

PE: paired-end

rRNA: ribosomal Ribonucleic Acid

rrnL: 16s ribosomal Ribonucleic Acid

rrns: 12s ribosomal Ribonucleic Acid

RSCU: relative synonymous codon usage

tRNA: Transfer Ribonucleic Acid

WGS: Whole Genome Shotgun

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Genetic data used in the present study are deposited at GenBank and publicly accessible through the provided accession numbers, and other relevant data are presented within paper and Suppl. Materials.

Competing interests

The authors declare that they have no competing interests.

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