A Molecular Phylogeny of Cavernicolous Oniscidea (Isopoda) Reveals Multiple Origins of Troglodytic Behavior (Oniscidea, Isopoda)

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Abstract. We infer the relationship of Oniscidea by utilizing two genetic markers, *COI* and *16S*, and build a topology using the Maximum Likelihood method. We found that troglobitic behavior to have arisen multiple times independently, and the genera *Spherillo* and *Burmoniscus* to be in need of revision.

Keywords: Genetic markers, convergent evolution, monophyly, caves, woodlice, Oniscidea.

1. Introduction

1.1. A global overview of Oniscidea

Oniscidea is a polyphyletic suborder within the Order Isopoda (Superorder Peracarida, Subclass Eumalacostraca, Class Malacostraca, Subphylum Crustacea, Phylum Arthropoda, Kingdom Animalia), and contributes to 50% of its species [1]. As of 2014, 3710 species belonging to 527 genera and 37 families within Oniscidea have been validated, with total number being estimated to be between 5000 and 7000[2]. Of these 37 families, the three with the highest species diversity in descending order are Armadillidae, Philosciidae, and Trichoniscidae [2].

Most Oniscidea species inhabit terrestrial habitats and serve as keystone detritivores, because of their high population density and phytosaprophagy [3], and have penetrated every biome of the world, apart from the poles and altitudes of above 4800 m [4]. Despite their high diversity, they are relatively poorly adapted to land, with rapid water loss to environment occurring in air that is not saturated [5]. The families that are best adapted to terrestrial environments, such as Oniscidae, Porcellionidae, and Armadillidiidae, all possess morphological specialization in the form of invaginated exopodite lungs to prevent desiccation [6]. Certain species, such as *Armadillidium vulgare*, are able to conglobate. They use this to reduce transpiration and as a defense mechanism against predation [7].

1.2. Molecular studies of Oniscidea Phylogeny

Despite the advancements in molecular phylogeny, generally considered to be the more accurate option as convergent evolution may cause distantly related clades to have similar characteristics, most modern-day studies of Oniscidea are still based on morphology. Molecular studies of Oniscidea are rare [1,8], and studies that take both into account are nonexistent.

Though Oniscidea was presumed to be monophyletic in the past based on morphological characteristics [9], molecular analyses have shed light on the likely polyphyly of both the suborder, and a family inside it [1,8]. Analyses of mito-nuclear genes suggests the families Tylidae and Ligiidae to be placed outside of the main Oniscidea group (Mesoniscidae, Crinocheta, and Synocheta), making it more likely that they colonized land independently [1], while molecular analyses of the family Porcellionidae based on the *COI, 16S, 18S, 28S* and *NAK* sequences reveals both it and its most diverse genus, *Porcellio*, to be polyphyletic [8]. Such large inconsistencies between molecular analysis and morphological analysis makes expanding current molecular studies a priority.

1.3. The distribution and research of Oniscidea within China

As of 2005, 72 species of terrestrial Oniscidea isopods have been described within China, of which 28% inhabit tropical regions, and 50% are unique to China [10]. This apparent lack of diversity is because of the initial lack of interest within the Oniscidea field in China, with new species being reported more frequently now. In contrast to this, the real diversity of Chinese Oniscidea seems to be quite large, with strong zonal characteristics [10].

Many Chinese studies of Oniscidea since the 1990s have focused on their ecological importance and morphological characteristics, with a lesser focus on their phylogeny [11,12,13]. Most studies of Oniscidea are restricted to south-eastern China, or other coastal areas, with especially many describing the species of the Yangtze river's downstream regions [14,15,16,17]. Though some studies of Oniscidea have been made in southern China, they are limited to the Yunnan province and the Hong Kong SAR [18], and do not study their phylogeny.

Despite the considerable diversity of cavernicolous Onscidea isopods within the Guangxi province [19,20], as well as the establishment of phylogenic studies of non-Chinese cavernicolous Oniscidea isopods [21], no studies so far have attempted to resolve the phylogeny between them, rock-face Oniscidea, and non-cavernicolous Oniscidea, as well as their position in the phylogenetic tree of Oniscidea as a whole. In addition to this, no studies have been made so far using phylogeny to describe Chinese cavernicolous Oniscidea. This paper aims to provide all three using molecular biology.

2. Materials and Methods

2.1. Sampling

Oniscidea specimens were collected in the Guangxi province in several regions. Three species were collected in the Detian International Waterfall Park (North 22 degrees 51'22'', East 106 degrees 43'28'', Altitude 450 meters, 2021/7/6) at the Sino-Vietnamese border near Chongzuo. Two species were procured in Nanning from a vendor which collected them in the Longzhou prefecture in Chongzuo (North 22 degrees 64'88'', East 106 degrees 89'57'', Altitude 158 meters, 2021/7/4). One species was collected in Beihai. Fresh specimens were kept alive initially and provided substrate of their region of collection, and placed in 99% alcohol when shipped. Genes of numerous other species from other regions of various different genera were also used in the phylogenetic analysis, as geography does not seem to affect phylogenetic relationships [8] (Dimitriou 2018). Some of these genes were found in the methods of other papers [1,8] (Dimitriou 2018, Lins 2017), while others were downloaded in the GenBank database (Table 1).

Table 1. Species which were collected, locality of origin and available sequence data.

Species	Locality	Genes	
		COI	*16s*
Burmoniscus sp. 1	Guangxi (Chongzuo)	√	
Ligiidae sp.	Guangxi (Detian)	√	√
Burmoniscus sp. 2	Guangxi (Detian)	√	
Spherillo sp.	Guangxi (Detian)		√
Porcellionides pruinosus	Guangxi (Beihai)	√	

Table.2. Species which were accessed through papers, locality of origin, available and used sequence data for them, and access number

Species (code)	Locality	Genes		Access number
		COI	*16s*	
Proporcellio vulcanius	Cyprus (Larnaca)	√	√	MG887933/MG887948
Agabiformius excavatus	Cyprus (Paphos)		√	-/MG887955
A. excavates	Cyprus (Paphos)		√	-/MG887956
Porcellio laevis	Cyprus (Lemesos)	√	√	MG887936/MG887957
Porcellio laevis	Cyprus (Lemesos)	√	√	MG887937/MG887958
Porcellio pruinosus	Cyprus (Larnaca)	√	√	MG887934/MG887949
Porcellio pruinosus	Cyprus (Larnaca)	√	√	MG887935/MG887950
Leptotrichus kosswigi	Cyprus (Paphos)		√	-/MG887963
Porcellio nasutus	Greece (Parnon)	√	√	MG887944/ MG887953
Porcellio nasutus	Greece (Parnon)		√	-/MG887954
Tura sp.	Kenya (Mombasa)	√	√	MG887946/MG887966
Caeroplastes porphyrivagus	France (Toulon)	√		MG887932/-
Uramba triangulifera	Kenya (Aberdare National Park)		√	-/MG887961
Thermocellio sp.	Tanzania (Dar es Salaam)		√	-/MG887962
Mica tardus	Italy (Sardinia)		√	-/MG887959
Acaeroplastes melanurus	Italy (Sardinia)	√	√	MG887945/G887960
Soteriscus laouensis	Morocco(Tirinesse)	√	√	MG887931/MG887964
Trachelipus aegaeus	Greece (Naxos)	√	√	EF659961/KF891440
Hemilepistus klugii	Iran (Isfahan)	√	√	MG887938/MG887951
Hemilepistus schirazi	Iran (Shahreza)	√	√	MG887939/MG887952
Armadillidium vulgare	Cyprus (Limassol)	√	√	KR424609/AJ419997
Chaetophiloscia elongata	Italy (Sardinia)	√	√	KJ668161/AJ388091
Actaecia euchroa	New Zealand	√	√	GQ302701/AJ388093
Cubaris murina	n.a.	√		AB861536/-
Armadillo officinalis	n.a.	√		FN824109/-
Spherillo dorsalis	n.a.	√		AB861899.1/-
Trichoniscus pusillus	n.a.	√		FN824124.1/-
Burmoniscus meeusei	n.a.	√		AB889796.1/-
Oniscus asellus	n.a.	√	√	KM611735/KX467635
Spherillo grossus	n.a.	√	√	KC706445/KC706402
Mongoloniscus sinensis	n.a.	√		KT424048/-
Haloniscus sp.	n.a.	√		KR424612/-
Spherillo obscurus	n.a.		√	-/AB861914
Merulana helmsiana	n.a.		√	-/KC706379
Burmoniscus sp. 3	n.a.		√	-/AB626480

2.2. DNA extraction and amplification

Specimens were used immediately upon arrival. Total genomic DNA was extracted from entire bodies using the AxyPrep Genomic DNA Miniprep Kit (AXYGEN), following the instructions of the manufacturer.

COI and *16S* rDNA were amplified using the following primer pairs respectively: LCO1490/HCO2198 [22], 16sar/16sbr [23]. All sequences were then put through PCR: pre-denaturation for 5 minutes at 94℃, 5 min at 94℃,followed by b) 5 cycles of 3 minutes equally separated at 94℃/60 ℃/72 ℃, c) 5 cycles of 3 minutes equally separated at 94 ℃/55 ℃/72℃, d) 10 cycles of 3 minutes equally separated at 94 ℃/50℃/72 ℃, e) 10 cycles of 3 minutes equally separated at 94℃/47℃/72 ℃, f) 10 cycles of 3 minutes equally separated at 94℃/42℃/72℃, and g) a final extension step of 72℃ for 10 min These products were then sent to a biotech company (BioSune, Shanghai, China) which sequenced them using sanger sequencing that corresponded to the primers.

2.3. Phylogenetic analyses

The wave function in ATGC was first used to manually correct and adjust nucleotides and to shorten strands. These amended strands for each sequence were then aligned with default parameters in MEGA7[24] through the muscle method, and manually cut into the same lengths by removing all parts where there were missing data. The sequences were then individually saved in the FASTA format, and put into PhyloSuite[25]. Within PhyloSuite, the flowchart function is used with the following parameters: sequence alignment with both MAFFT and MACSE, alignment cleaning with Gblocks, model selection with Concatenation and ModelFinder, Tree Reconstruction with IQ-TREE [26]. ModelFinder is preferred here over PartitionFinder, as even though it is slower, it is more accurate. Maximum likelihood analyses were made in IQ-TREE. This bilateral analysis with two models allows the final results to be compared to each other to check for inaccuracies.

3. Results

45% (18/40) of the species had both targeted genes successfully amplified and sequenced, 27.5% (11/40) only yielded usable *16S* results, and 27.5% (11/40) only yielded usable *COI* results.

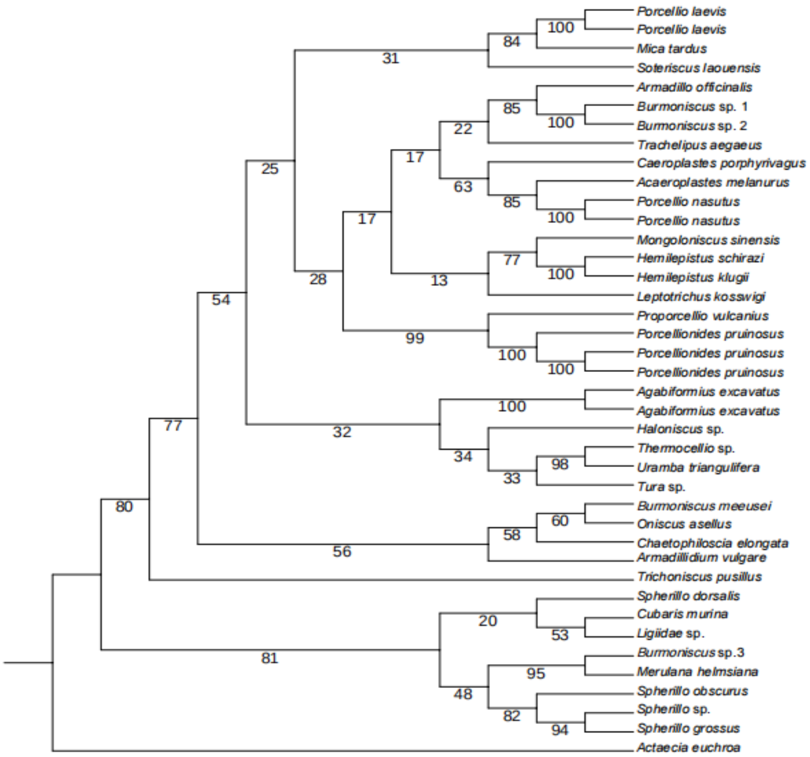

Figure. 1 Phylogram based on the genes *COI* and *16S*, generated using Maximum Likelihood through the IQ-Tree algorithm. Statistic support is given on the branch preceding divergence.

Two major clades are found in our phylogenetic tree. All *Spherillo*, *Cubaris*, *Merutana* species and *Burmoniscus* sp 3 are included in one clade. In another clade, *Thermocellio* sp. and *Uramba triangulifera*, *Proporcellio vulcanius* and *Porcellionides pruinosus*, *Acaeroplastes melanurus* and *Porcellio nasutus*, *Armadillo officinalis* and *Burmoniscus* sp. 1&2, *Porcellio laevis* and *Mica tardus* have close relationship.

Burmoniscus sp. 1 and *Burmoniscus* sp. 2 are found to be closely related to each other, but not to *Burmoniscus meeusei* nor *Burmoniscus* sp. 3. The Maximum Likelihood results, on the other hand, are extremely well supported (85), and suggest them to be most closely related to *Armadillo officinalis*.

4. Discussion

In this study, we analyzed the phylogentic relationships between different Guangxi Oniscidea along with related groups of non-Chinese Oniscidea. Our results show a multi-origin of cavernicolous Oniscidea, the need to revise genera within Oniscidea.

The close genetic relationships between the rock face species *Burmoniscus* sp. 1 and the non-cavernicolous *Burmoniscus* sp. 2 suggests that cave dwelling behavior evolved independently across different lineages. This is supported by evidence for independent colonization of caves by *Philosciidae oniscids* in Hawaii [27]. The specific niche partitioning and reason for cave colonization between different cavernicolous Oniscidea, however, is still left unresolved.

Figure. 2 Habitat display of Cavernicolous Oniscidea.

The monophyly of *Spherillo* being put into question is new, though not unexpected, as concerns have been raised regarding the monophyly of Oniscidea and genera inside of it as well [1,8]. A monophyletic *Spherillo* based on the Maximum Likelihood analysis would need to reclassify *Spherillo dorsalis*, and either revise *Merulana helsiana*, or remove *Spherillo obscurus* and *Spherillo sinoculus*. A monophyletic *Spherillo* based on Bayesian Analysis would either only include *Spherillo grossus* and *Spherillo* sp., or revise *Merulana helsiana* as well as *Cubaris murina*.

In addition to this, the monophyly of *Burmoniscus* is also put into question, with *Burmoniscus* sp.1 and *Burmoniscus* sp. 2 forming a group, *Burmoniscus* sp. 3 forming a group, and *Burmoniscus meeusei* forming a group. These three groups are phylogenetically distinct and occupy very different parts of the Oniscidea phylogram. The first group is most closely related to Armadillo officinalis, the second group most closely related to *Merulana helmsiana*, and the third group most closely related with *Oniscus asellus*. All three of these species belong to different families, being Armadillidae, Trachelipodidae, and Philosciidae.

With the still field of oniscid phylogeny still burgeoning, the extreme polyphyly of these two genera might suggest the necessity of taxonomic reform, especially when it comes to troglobites, which are often described through morphology alone [19,20].

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