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Including secondary structure, fossils and molecular dating in the centipede tree of life

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ABSTRACT

A well-corroborated morphological scheme of interrelationships for centipedes, once broadly accepted, has been in conflict with molecular data with respect to deep branching events. Expanded taxonomic coverage compared to previous analyses adds longer fragments for 28S rRNA and a structural alignment as part of a sample of four genes (two nuclear ribosomal and two mitochondrial) for 111 extant species; these sequence data are combined with morphology under parsimony and maximum likelihood, exploring both traditional multiple sequence alignment and direct optimization approaches. Novel automated procedures to incorporate secondary structure information are also explored. The molecular data in combination yield trees that are highly congruent with morphology as regards the monophyly of all centipede orders as well as the major groups within each of the large orders. Regardless of the optimality criterion or alignment strategy, the Tasmanian/New Zealand Craterostigmomorpha is resolved in a different position by the molecular data than by morphology. Addition of morphology overturns the placement of Craterostigmomorpha in favour of the traditional morphological resolution and eliminates the need to posit major character reversals with respect to developmental mode and maternal care. Calibration of the tree with Palaeozoic and Mesozoic fossils for a relaxed clock analysis corroborates the palaeontological signal that divergences between centipede orders date to the Silurian and earliest Devonian, and familial divergences are likewise almost wholly Palaeozoic.

1. Introduction

Centipedes (Chilopoda) serve as an intriguing test case for exploring the efficacy of standard genes and competing analytical techniques for phylogenetic inference. More so than is the case for other major arthropod groups, chilopods are a clade for which higher-level relationships are the subject of minimal discord from the perspective of morphology. The standard morphology-based hypothesis for how the five extant centipede orders are related emerged in the early 20th century (Pocock, 1902; Verhoeff, 1902-1925; Fahlander, 1938), was depicted in evolutionary trees several decades later (Prunescu, 1965; Shinohara, 1970), and was formalized when cladistic argumentation was applied to the problem (Dohle, 1985; Shear and Bonamo, 1988; Borucki, 1996). It has further withstood testing from numerical cladistic analyses that combined morphology with molecular sequence data (Edgecombe et al., 1999; Edgecombe and Giribet, 2004a; Giribet and Edgecombe, 2006a). Studies of individual anatomical character systems in recent years have endorsed and added new characters that fit the same tree (e.g., Hilken, 1997; Wirkner and Pass, 2002; Müller and Rosenberg, 2006).

The morphological hypothesis for higher-level centipede phylogeny splits Chilopoda into Notostigmophora (consisting of the order Scutigeromorpha, ca. 100 valid species) and Pleurostigmophora, a clade composed of the other four extant orders together with an extinct order known from Devonian fossils. Pleurostigmophora is itself divided into the order Lithobiomorpha (ca. 1100 species) and an unranked clade named Phylactometria (Edgecombe and Giribet, 2004a). Phylactometria, a group that shares maternal brood care, unites the order Craterostigmomorpha (two species, one in each of Tasmania and New Zealand) with a widely-endorsed clade named Epimorpha. The latter receives its name from a developmental character shared by its members; in Epimorpha, segment addition is confined to embryogenesis and hatchlings emerge from the egg with their complete adult segment number (see Edgecombe and Giribet, 2007). Epimorpha unites the orders Scolopendromorpha (ca. 700 valid species) and Geophilomorpha (ca. 1260 species).

The first surveys of nuclear ribosomal genes to include species of all five extant centipede orders found cladograms that were highly congruent with the morphological trees (Edgecombe et al., 1999; Giribet et al., 1999; Edgecombe et al., 2002). With the

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addition of mitochondrial markers and a taxonomic sampling of as many as 70 centipede species, incongruence with the morphological cladogram began to emerge (Edgecombe and Giribet, 2004a), in particular regarding the placement of Craterostigmomorpha. A complementary sampling of three nuclear protein-encoding genes by Regier et al. (2005) likewise yielded a centipede phylogeny that conflicted with the standard morphological tree. The position of Craterostigmomorpha again proved to be a source of incongruence, this order being resolved by the nuclear coding genes as sister group to all other chilopods. A combination of morphology, the three nuclear coding genes of Regier et al. (2005), and the two nuclear ribosomal genes and two mitochondrial genes of Edgecombe and Giribet (2004a), found two competing, highly incongruent topologies under different analytical conditions (Giribet and Edgecombe, 2006a). In several analyses, the nuclear protein-encoding genes dominated the signal and the total evidence cladogram for ordinal relationships matched that from those three genes alone. In other analyses the morphological cladogram emerged as optimal. Furthermore, although in a larger arthropod phylogeny context, Mallatt and Giribet (2006) used complete 28S rRNA in addition to 18S rRNA to find strong support for *Craterostigmus* being sister to all other pleurostigmophorans. The same relationship for Craterostigmomorpha was found in analyses of 62 nuclear protein coding genes (Regier et al., 2010), although without data from Geophilomorpha.

In order to explore the nature of incongruence between morphology and earlier molecular sequence data with respect to centipede phylogeny, here we added more complete 28S rRNA sequences (D1–D7 region), filled in some of the previous missing data for other markers, and added previously unsampled lineages in the context of centipede ordinal relationships. We also more fully explore the implications of incorporating secondary structure for the ribosomal genes. As recently noted, "the use of structural information and POY are not mutually exclusive" (Kjer et al., 2007) and its use has long been endorsed and applied (e.g. Giribet and Ribera, 2000; Giribet and Wheeler, 2001; Giribet, 2002). However, structural alignments, as well as their integration within the framework of direct optimization (Wheeler, 1996), still heavily rely on manual recognition of fragments and visual examination of characters to include/exclude (see Kjer et al., 2009, for a recent review). Recently, automated ways of implementing secondary structure information into alignments have been developed (Notredame et al., 1997; Gorodkin et al., 2001; Misof and Fleck, 2003; Hofacker et al., 2004; Holmes, 2004; Niehuis et al., 2006). In this study, we used novel automated procedures to incorporate secondary structure information both in classical two-step phylogenetic analysis (under parsimony and maximum likelihood) and in direct optimization. In addition, we used terminal and internal fossil calibrations in conjunction with molecular data to provide for the first time a dating scheme for the diversification of centipede groups.

2. Materials and methods

2.1. Sampling

A total of 112 species (111 extant and 1 extinct) were included in this study. The monophyly of Chilopoda is well established, and trees are rooted with members of Diplopoda, including exemplar species that span the broad variety of the group. Analyses of nuclear ribosomal genes repeatedly find anomalous placements of the other myriapod classes, Symphyla and Pauropoda (Mallatt et al., 2009; von Reumont et al., 2009), whereas a chilopod–diplopod clade is almost invariably stable and well supported in broadly sampled analyses of Arthropoda. Accordingly, diplopods are the obvious and appropriate outgroup for Chilopoda in datasets dominated by nuclear ribosomal genes. The core of the sampling is based on previous studies on centipedes (Edgecombe and Giribet, 2004a; Giribet and Edgecombe, 2006a). Additional species were included based on studies on Scutigeromorpha (Edgecombe and Giribet, 2006, 2009), Lithobiomorpha (Edgecombe and Giribet, 2004b; Giribet and Edgecombe, 2006b) and Craterostigmomorpha (Edgecombe and Giribet, 2008). The denser sampling allows questions such as the position of Scutigeromorpha in chilopod phylogeny to be evaluated based on much more data, e.g., 23 species herein versus five species in Edgecombe and Giribet (2004a).

Because the interrelationships of Geophilomorpha are one of the most pressing problems in centipede phylogenetics, we add several taxa in this study. Notably we provide the first sequence data for Oryidae, sampled by two species of *Orphnaeus* and one of *Orya*. In order to test the strength of support for previous resolutions of Mecistocephalidae as sister group to all other Geophilomorpha, existing data for *Mecistocephalus* spp. are supplemented with new data for two additional mecistocephalid genera, *Tygarrup* and *Dicellophilus*. An additional member of Himantariidae is likewise added, *Stigmatogaster souletina* (Brolemann, 1907). Newly added diversity in Scolopendromorpha includes members of two species-rich genera that were lacking in earlier studies, the scolopendrid *Otostigmus* and the scolopocryptopid *Newportia*. Previous studies of Lithobiomorpha sampled the mostly southern hemisphere Henicopidae much more densely than the mostly northern Lithobiidae, an imbalance that we redress with the addition of four more lithobiid species. All specimens were collected alive, either by sifting litter or by direct search, and preserved in 95% EtOH. Vouchers are deposited at the Museum of Comparative Zoology (MCZ), Department of Invertebrate Zoology DNA collection (Table 1).

2.2. Morphological data

The 222-character morphology matrix of Edgecombe and Giribet (2004a) was expanded by the addition of new characters that are informative for the relationships of newly added taxa, and also by an injection of data from recently studied character systems. These include characters for the fine structure of the eyes (Müller and Meyer-Rochow, 2006a,b; Müller and Rosenberg, 2006), the peristomatic structures (Koch and Edgecombe, 2006, 2008; Edgecombe and Koch, 2008), and the foregut (Koch et al., 2009). Recently published character matrices for the internal phylogeny of Scolopendromorpha (Koch et al., 2009; Edgecombe and Koch, 2009) and Scutigeromorpha (Edgecombe and Giribet, 2006, 2009) summarise available data for those groups, and characters employed in those analyses are coded for the relevant species herein (Supplementary material S1). The morphology matrix currently stands at 258 characters (Supplementary material S2).

Morphological coding for *Craterostigmus tasmanianus* and *C. crabilli* was based on specimens DNA102000 and DNA102004 respectively, supplemented extensively by anatomical data in the literature. Since intraspecific variation for the characters used has not been documented thus far, for the combined analyses, coding was duplicated for the remaining specimens of *Craterostigmus*.

2.3. DNA extraction, amplification and sequencing

The DNEasy tissue kit (Qiagen, Valencia, CA, USA) was used for tissue lysis and DNA purification following the manufacturer's protocol. Total DNA was extracted by incubating one appendage in the lysis buffer overnight.

Target genes were selected based on previous studies of centipedes and have proved to be informative at various levels in evolutionary studies. Nuclear 18S rRNA (18S hereafter) was

Table 1
Voucher numbers and GenBank accession numbers for the species included.

Family	Species	Voucher	18S	28S	16S	COI
Outgroup	*Polyxenus lagurus*	Plagurus	EU368619	EU376011	-	-
Outgroup	*Epicyliosoma* sp.	DNA100170	AF370785	-	AF370865	AF370841
Outgroup	*Polydesmus complanatus*	Polydesm	EU368620	EU376010	-	-
Outgroup	*Proteroiulus fuscus*	DNA100171	AF173236	AF370804	AF370866	AF370842
Outgroup	*Cylindroiulus caeruleocinctus*	Caeruleo	EU368621	EF199985	-	-
Outgroup	*Doratogonus* sp.	DNA100414	AY288687	AY288703	AY288715	AY288738
Outgroup	*Thyropygus* sp.	Thyropyg	X74822	-	NC_003344	NC_003344
Outgroup	*Narceus americanus*	DNA100173	AY288686	AF370805	AF370867	-
Scutigerinidae	*Scutigerina malagassa*	DNA101591	DQ222119	DQ222136	DQ222152	DQ222167
Scutigerinidae	*Scutigerina weberi*	DNA100455	AY288689	AY288705/DQ222135	AY288717	AY288741
Scutigerinidae	*Madagassophora hova*	DNA101592	DQ222120	DQ222137	DQ222153	-
Pselliodidae	*Sphendononema guildingii*	DNA101630	DQ222122	DQ222139	DQ222154	DQ222168
Scutigeridae, Scutigerinae	*Dendrothereua nubila*	DNA101791	FJ660704	FJ660744-5	FJ660785	FJ660817
Scutigeridae, Scutigerinae	*Dendrothereua homa*	DNA102576	FJ660705	FJ660746	FJ660786	FJ660818
Scutigeridae, Scutigerinae	*Scutigera coleoptrata*	DNA100259	DQ222124	EF199983	DQ222156	DQ222170
Scutigeridae, Scutigerinae	*Scutigera nossibei*	DNA102102	FJ660714-5	FJ660755	FJ660794	FJ660821
Scutigeridae, Scutigerinae	*Tachythereua* sp. SENEGAL	DNA102575	FJ660716	FJ660756	FJ660795	-
Scutigeridae, Thereuoneminae	*Allothereua bidenticulata*	DNA101589	FJ660717	FJ660757	FJ660796	FJ660822
Scutigeridae, Thereuoneminae	*Allothereua linderi*	DNA101463	DQ222128	DQ222147	DQ222160	DQ222174
Scutigeridae, Thereuoneminae	*Allothereua serrulata*	DNA100262	DQ222129	DQ222148	DQ222161	DQ222175
Scutigeridae, Thereuoneminae	*Allothereua maculata*	DNA101988	-	FJ660763	FJ660802	FJ660827
Scutigeridae, Thereuoneminae	*Parascutigera festiva*	DNA102584	FJ660725	FJ660766	FJ660803	FJ660828
Scutigeridae, Thereuoneminae	*Parascutigera guttata*	DNA102317	FJ660726	FJ660767	FJ660804	FJ660829
Scutigeridae, Thereuoneminae	*Parascutigera latericia*	DNA101046	DQ222131	DQ222150	DQ222164	DQ222178
Scutigeridae, Thereuoneminae	*Parascutigera nubila*	DNA103553	FJ660732	FJ660772	FJ660808	FJ660832
Scutigeridae, Thereuoneminae	*Parascutigera sphinx*	DNA101981	FJ660741	FJ660781	FJ660814	FJ660839
Scutigeridae, Thereuoneminae	*Pilbarascutigera incola*	DNA101997	FJ660742	FJ660782–3	FJ660815	-
Scutigeridae, Thereuoneminae	*Thereuonema tuberculata*	DNA101632	DQ222126	DQ222145	DQ222158	DQ222173
Scutigeridae, Thereuoneminae	*Thereuonema turkestana*	DNA101090	FJ660743	FJ660784	FJ660816	FJ660840
Scutigeridae, Thereuoneminae	*Thereuopoda clunifera*	DNA100260	AF173239	DQ222142	AY288716	DQ222171
Scutigeridae, Thereuoneminae	*Thereuopoda longicornis*	DNA101461	DQ222125	DQ222143	DQ222157	DQ222172
Lithobiidae	*Lithobius variegatus rubriceps*	DNA100283	AF000773	**HM453241**	AY084071	AF334311
Lithobiidae	*Lithobius forficatus*	Lforfica	EU368618	EF199984	AJ270997	AJ270997
Lithobiidae	*Lithobius obscurus*	Lobscuru	AF334271	**HM453242**	AF334333	-
Lithobiidae	*Lithobius castaneus*	DNA103939	**HM453233**	**HM453243**	**HM453214**	**HM453305**
Lithobiidae	*Lithobius giganteus*	DNA101089	-	**HM453244**	**HM453215**	**HM453306**
Lithobiidae	*Lithobius holstii*	DNA102106	**HM453234**	-	**HM453216**	**HM453307**
Lithobiidae	*Australobius scabrior*	DNA103925	AF173241	**HM453245**	DQ201424	-
Lithobiidae	*Eupolybothrus fasciatus*	DNA100281	AY213718	**HM453246**	AY214365	AY214420
Lithobiidae	*Bothropolys multidentatus*	Bmultide	AF334272	AF334293	AF334334	-
Lithobiidae	*Bothropolys xanti*	DNA100529	**HM453235**	**HM453247**	**HM453217**	**HM453308**
Henicopidae	*Anopsobius giribeti*	DNA100248	AY213721	**HM453248**	AY214368	AY214422
Henicopidae	*Dichelobius flavens*	DNA100380	AY213720	**HM453249**	AY214367	AY214421
Henicopidae	*Anopsobius neozelanicus*	DNA101035	AF173248	AF173274	AF334337	AF334313
Henicopidae	*Zygethobius pontis*	DNA100359	AY213722	-	AY214369	AY214423
Henicopidae	*Shikokuobius japonicus*	DNA100463	AY213719	**HM453250**	AY214366	-
Henicopidae	*Henicops maculatus* NEW ZEALAND	HmacuNZ	AF334277	AF334298	AF334342	AF334318
Henicopidae	*Henicops dentatus*	DNA100378	AY213724	**HM453251**	AY214370	AY214424
Henicopidae	*Lamyctes africanus*	DNA100287	AF334274	**HM453252**	**HM453218**	AF334314
Henicopidae	*Lamyctes emarginatus*	DNA101464	AF173244	**HM453253**	AF334338	-
Henicopidae	*Lamyctes caeculus*	DNA100288	AF334275	**HM453254**	AF334339	AF334315
Henicopidae	*Cermatobius japonicus*	DNA100265	AF334291	**HM453255**	AF334360	AF334332
Henicopidae	*Paralamyctes (Paralamyctes) asperulus*	DNA100401	AY213728	**HM453256**	AY214379	AY214432
Henicopidae	*Paralamyctes (Paralamyctes) rahuensis*	DNA101054	DQ201434	**HM453257**	**HM453219**	DQ201443
Henicopidae	*Paralamyctes (Thingathinga) grayi*	DNA100298	AF334288	**HM453258**	AF334356	AF334328
Henicopidae	*Paralamyctes (Thingathinga) validus*	DNA100297	AF334289	**HM453259**	AF334357	AF334329
Henicopidae	*Paralamyctes (Haasiella) subicolus*	DNA100292	AF334285	**HM453260**	AF334352	AF334327
Henicopidae	*Paralamyctes (Haasiella) cammooensis*	DNA101092	-	**HM453261**	DQ201446	DQ201444
Henicopidae	*Paralamyctes chilensis*	DNA100405	-	**HM453262**	AY214377	AY214430
Henicopidae	*Paralamyctes wellingtonensis*	DNA100408	-	**HM453263**	AY214378	AY214431
Henicopidae	*Paralamyctes (Edgecombegdus) mesibovi*	DNA100294	AF334284	**HM453264**	AF334350	AF334325
Craterostigmidae	*Craterostigmus tasmanianus*	DNA102000	EU024572	**HM453265**	EU024597	EU024611
Craterostigmidae	*Craterostigmus tasmanianus*	DNA102001	EU024573	**HM453266**	EU024598	EU024612
Craterostigmidae	*Craterostigmus tasmanianus*	DNA102003	EU024574	**HM453267**	EU024600	EU024614
Craterostigmidae	*Craterostigmus crabilli*	DNA102004	EU024575	**HM453268**	EU024602	EU024616
Craterostigmidae	*Craterostigmus crabilli*	DNA102005	EU024576	**HM453269**	EU024603	EU024617
Craterostigmidae	*Craterostigmus crabilli*	DNA102009	EU024577	**HM453270**	EU024607	EU024621
Craterostigmidae	*Craterostigmus crabilli*	DNA102012	EU024580	**HM453271**	EU024608	EU024624
Craterostigmidae	*Craterostigmus crabilli*	DNA102014	EU024582	**HM453272**	EU024610	EU024626
Scolopendridae	*Alipes* sp. SWAZILAND	DNA100454	AY288691	**HM453273**	AY288720	AY288742
Scolopendridae	*Cormocephalus monteithi*	DNA100274	AF173249	**HM453274**	AF370861	**HM453309**
Scolopendridae	*Scolopendra cingulata*	DNA100804	U29493	**HM453275**	**HM453220**	**HM453310**
Scolopendridae	*Scolopendra viridis*	DNA100675	DQ201419	DQ222134	DQ201425	DQ201431
Scolopendridae	*Ethmostigmus rubripes*	DNA100276	AF173250	**HM453276**	AY288721	AF370836

(*continued on next page*)

Table 1 (*continued*)

Family	Species	Voucher	18S	28S	16S	COI
Scolopendridae	*Rhysida nuda*	DNA100278	AF173252	**HM453277**	AY288722	**HM453311**
Scolopendridae	*Otostigmus astenus*	DNA102463	-	-	**HM453221**	**HM453312**
Cryptopidae	*Cryptops trisulcatus*	DNA100805	AF000775	AF000783	-	-
Cryptopidae	*Cryptops australis*	Caustral	AY288692	AY288708	AY288723	-
Cryptopidae	*Cryptops spinipes*	Cspinipe	AY288693	AY288709	AY288724	AY288743
Scolopocryptopidae	*Scolopocryptops nigridius*	DNA100807	AF173253	**HM453278**	AY288725	AY288744
Scolopocryptopidae	*Scolopocryptops sexspinosus*	DNA100808	AY288694	AY288710	AY288726	AY288745
Cryptopidae	*Theatops erythrocephalus*	DNA104805	AF000776	**HM453279**	**HM453222**	**HM453313**
Cryptopidae	*Theatops posticus*	DNA100806	AY288695	**HM453280**	AY288727	AY288746
Scolopocryptopidae	*Newportia longitarsis stechowi*	DNA102460	**HM453236**	**HM453281**	**HM453223**	**HM453314**
Oryidae	*Orya almohadensis*	DNA103730	-	**HM453282**	**HM453224**	**HM453315**
Mecistocephalidae	*Mecistocephalus guildingii*	DNA100809	AY288696	**HM453283**	AY288728	AY288747
Mecistocephalidae	*Mecistocephalus sp.*	DNA100524	AF173254	**HM453284**	AF370862	AF370837
Mecistocephalidae	*Dicellophilus carniolensis*	DNA102580	**HM453237**	**HM453285**	**HM453225**	-
Mecistocephalidae	*Tygarrup javanicus*	DNA103936	**HM453238**	**HM453286**	**HM453226**	-
Oryidae	*Orphnaeus brevilabiatus*	DNA101998	**HM453239**	**HM453287**	**HM453227**	**HM453316**
Oryidae	*Orphnaeus brasilianus*	DNA103937	**HM453240**	-	**HM453228**	-
Himantariidae	*Himantarium gabrielis*	DNA100646	AY288697	**HM453288**	AY288729	AY288748
Himantariidae	*Himantarium mediterraneum*	DNA100803	AF000778	**HM453289**	-	-
Himantariidae	*Bothriogaster signata*	DNA100645	AY288698	**HM453290**	AY288730	AY288749
Himantariidae	*Stigmatogaster souletina*	DNA103938	-	**HM453291**	**HM453229**	**HM453317**
Ballophilidae	*Ballophilus australiae*	DNA100247	AF173258	**HM453292**	-	-
Schendylidae	*Schendylops pampeanus*	DNA100268	AF173257	**HM453293**	AY288737	-
Schendylidae	*Pectiniunguis argentinensis*	DNA100269	AF173256	**HM453294**	**HM453230**	-
Schendylidae	*Plesioschendyla confossa*	DNA100633	AY288699	**HM453295**	AY288731	-
Geophilidae	*Geophilus electricus*	DNA100166	AY288700	**HM453296**	AY288732	AY288750
Geophilidae	*Tuoba sydneyensis*	DNA100264	AF173260	**HM453297**	**HM453231**	AY288751
Geophilidae	*Tasmanophilus opinatus*	Tasmanop	AF173259	**HM453298**	**HM453232**	AY288752
Geophilidae	*Tuoba poseidonis*	Tposeido	AF000777	AF000785	-	-
Geophilidae	*Zelanophilus provocator*	Zprovoca	AY288701	AY288714	-	-
Geophilidae	*Steneurytion antipodum*	DNA100249	AF173261	-	AY288734	-
Geophilidae	*Steneurytion* sp.	DNA100253	AF173262	**HM453299**	AY288735	-
Geophilidae	*Ribautia* n.sp.	DNA100250	AF173263	**HM453300**	AY288736	AY288755
Geophilidae	*Pachymerium ferrugineum*	DNA100266	AY288702	**HM453301**	AF370863	AF370838
Aphilodontidae	*Aphilodon weberi*	DNA100811	AF173264	**HM453302**	-	-
Linotaeniidae	*Strigamia maritima*	DNA100812	AF173265	**HM453303**	AY288733	AY288753
Dignathodontidae	*Henia (Chaetechelyne) vesuviana*	DNA100810	AF173255	**HM453304**	-	AY288754

amplified by the 1F/5R, 3F/18Sbi and 18Sa2.0/9R primer pairs (Giribet et al., 1996; Whiting et al., 1997). The nuclear 28S rRNA (28S hereafter) was amplified using a set of overlapping primer pairs as in Dell'Ampio et al. (2009). A map of the 28S primers can be found in Supplementary Fig. S3. The mitochondrial 16S rRNA (16S hereafter) was amplified using the primer pair 16Sa/16Sbi (Xiong and Kocher, 1991). The mitochondrial protein-encoding gene cytochrome *c* oxidase subunit I (COI hereafter) was amplified using the primer pair LCO1490/HCO2198 (Folmer et al., 1994) or alternatively with the reverse primer HCOoutout (Prendini et al., 2005; Schwendinger and Giribet, 2005).

Polymerase chain reactions (PCRs; 50 μL) included 2 μL of template DNA, 1 μM of each primer, 200 μM of dinucleotide-triphosphates (Invitrogen, Carlsbad, CA, USA), 1× PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Branchburg, NJ, USA) and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). PCRs were carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems), and involved an initial denaturation step (5 min at 95 °C) followed by 35 cycles including denaturation at 95 °C for 30 s, annealing (ranging from 44 to 49 °C) for 30 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min.

The double-stranded PCR products were verified by agarose gel electrophoresis (1% agarose) and purified with a Perfectprep PCR Cleanup 96 system (Eppendorf, Westbury, NY, USA). The purified PCR products were sequenced directly with the same primer pairs as used for amplification. Each sequence reaction contained a total volume of 10 μL including 2 μL of PCR product, 1 μM of one of the PCR primer pairs, 2 μL ABI BigDye 5× sequencing buffer, and 2 μL ABI BigDye Terminator v3.0 (Applied Biosystems). The sequencing reactions involved an initial denaturation step for 3 min at 95 °C, and 25 cycles (95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min). The BigDye-labelled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD). The sequence reaction products were then analyzed using an ABI Prism 3730×l Genetic Analyzer (Applied Biosystems).

2.4. Sequence editing

Chromatograms were edited and overlapping sequence fragments were assembled using Sequencher 4.7 (Gene Codes Corporation 1991–2007, Ann Arbor, MI). Blast searches (Altschul et al., 1997), as implemented in the NCBI website (http://www.ncbi.nlm.nih.gov/), were conducted to check for putative contamination. In addition, a distance tree using the BioNJ algorithm (Gascuel, 1997) was built with PhyML 2.4 (Guindon and Gascuel, 2003). The visual observation of branch lengths in this tree provides an additional quick way to assess the potential presence of contaminants or reverse complements. All new sequences have been deposited in GenBank under the accession numbers specified in Table 1.

2.5. Multiple sequence alignment

For the 16S, 18S and COI genes, all sequences were aligned in one batch using Muscle 3.7 (Edgar, 2004) with the most accurate algorithm (muscle -in gene1.fas -out gene1.ali). For the 28S, the use of the above strategy was avoided as it tends to produce an alignment in which short sequences (amplified with primer pairs 28Sa/28Sb, for example) are stretched over the entire length of

the alignment. In a first step, complete sequences and short sequences were aligned in two different datasets using Muscle 3.7 (Edgar, 2004). This step was performed using the fast speed options (muscle -in in.fa -out out.fas -maxiters 1 -diags1). The alignment produced was then visually inspected for short sequences' "misbehavior" using Jalview 2.4 (Waterhouse et al., 2009). In the case of an obvious alignment issue, sequences were manually readjusted and realigned using ClustalW (Thompson et al., 1994). The two datasets were then combined using the profile-to-profile approach implemented in Muscle (muscle -profile -in1 short.fas -in2 long.fas -out chilo.fas). This preliminary alignment was divided into five fragments based on the position of the following primers: D2a, 28Sa, D3b.rev.MOD, 28See.MOD, D7aCA (see Supplementary Fig. S3). Each of those fragments was aligned using the most accurate algorithm of Muscle (muscle -in frag1.fas -out frag1.ali) and were then reassembled using Phyutility (Smith and Dunn, 2008) (phyutility -concat -in frag1.fas frag2.fas frag3.fas -out 28S.nex).

2.6. Structural alignment

In the present study, information on ribosomal RNA secondary structure was integrated in a "structural alignment" using RNAsalsa 0.7.4 (Stocsits et al., 2009a,b) based on the Muscle alignment. As opposed to manually aligning the sequences (e.g., Mallat et al., 2009), the method implemented in RNAsalsa provides an open and repeatable framework to automatically take into account a known accurate secondary structure, primary sequence information and well-established folding algorithms. This approach has been successfully applied at various taxonomic levels in recent studies (von Reumont et al., 2009; Letsch et al., 2009).

Randomly similar sections (RSS hereafter) within the structural alignments were identified using Aliscore (Misof and Misof, 2009). The method provides some advantages over Gblocks (Castresana, 2000) used in previous studies (Murienne, 2009; Murienne et al., 2010). A window size of 6 positions was used and gaps were treated as ambiguous characters. A neighbour-joining tree was used as a guide tree.

Alignments were checked for compositional base heterogeneity using the 5% level chi-square-test implemented in Tree-Puzzle 5.2 (Schmidt et al., 2002, 2003). The chi-square-test compares the nucleotide composition of each sequence to the frequency distribution assumed in the distance model used in the maximum likelihood analysis. Phylogenetic information in the datasets was analysed using likelihood mapping (Strimmer and von Haeseler, 1997) as implemented in Tree-Puzzle 5.2. Accumulation of dots in corners of the likelihood mapping diagram and absence of dots in the central region have been interpreted as indicators of phylogenetic structure in the dataset (see Supplementary Fig. S4).

In order to recognize fragments within sequences for subsequent direct optimization (Wheeler, 1996), delimiters (# symbols) were introduced in the RNAsalsa alignment at specific positions using Biopython (Cock et al., 2009). Both RSS (identified by Aliscore) and structural features (identified by RNAsalsa) were taken into account. Fragments corresponding to RSS were deactivated. In POY, the following command will include fragments 0 (fragments are counted from 0 to x) and 2 of 16S but not fragment 1 [select(characters, names:("16S.poy:0", "16S.poy:2")]. The procedure described here provides a fully repeatable and objective way to establish and choose fragments based on secondary structure information, thus bridging the gap between "structural alignment" and direct optimization.

2.7. Phylogenetic analyses

At first, a classical two-step analysis was performed. Concatenation of the separate datasets was performed with Phyutility (Smith and Dunn, 2008). A Nexus matrix was manually edited to code missing characters as N's instead of the 'dash' (-) symbol. This has no importance under most current implementations of maximum likelihood, which ignore indel information, but can influence parsimony if indels are treated as a fifth state. For use with TNT, the Nexus matrix was edited to replace "N" (read as [ATGC]) with "? " (read as [ATGC-]).

The resulting matrix was submitted to a maximum likelihood analysis following Murienne et al. (2010) using RAxML 7.0.4 (Stamatakis, 2006) with a GTR + Γ model (Yang, 1993) applied to each partition and a rapid bootstrap procedure (Stamatakis et al., 2008). The analyses were performed on the cluster of the CIPRES project (Miller et al., 2009) at the San Diego Supercomputer Center http://www.phylo.org/sub_sections/portal/ (last accessed October 15, 2009).

Parsimony analyses were also performed on the same matrix. One hundred replicates of random addition sequence followed by tree refinement with SPR and TBR were performed in TNT (Goloboff et al., 2008) followed by a combination of Ratchetting (Nixon, 1999) and Tree Fusing (Goloboff, 1999). Group support was assessed with 500 replicates of bootstrapping (Felsenstein, 1985).

For the morphological data, a first analysis was performed as above and a second analysis was performed removing the fossil *Devonobius* in order to observe the effect on support values. A third analysis was performed with implied weighting (Goloboff, 1993b) with the concavity constant $k = 3$. There is no theoretical justification for choosing a particular value of k (Turner and Zandee, 1995) but extreme values are contraindicated (Goloboff, 1993b, 1995). Mild functions ($k = 5+$) are close to equal weighting and very strong functions ($k = 1$) are difficult to justify (Prendini, 2000). The TNT default value ($k = 3$) is an acceptable compromise and has been used as a sole value in previous studies (e.g. Goloboff, 1993a; De Jong et al., 1996; Griswold et al., 1998).

Alternatively, phylogenetic analyses were performed in a one-step fashion under direct optimization (Wheeler, 1996) with the program POY 4.1.2 (Varón et al., 2010). Instead of manually defining a specific tree search strategy (see Murienne et al., 2008, for commands), we used the max_time command (see Murienne et al., 2010, for details). The search was implemented under equal weighting on 40 nodes of the Harvard odyssey cluster for a total time of 1 day (plus additional time for fusing and final refinement).

Whenever possible, trees are depicted so that they show both the monophyly of Chilopoda and Diplopoda.

2.8. Molecular dating–dating constraints

A few Palaeozoic fossil occurrences constrain the timing of divergences between chilopod clades (for a review of the myriapod fossil record see Shear and Edgecombe, 2010). The Siluro–Devonian genus *Crussolum* (Shear et al., 1998) is identified as a stem-group scutigeromorph. It posses apomorphic characters of total-group Scutigeromorpha but lacks some apomorphies that are shared by all members of the scutigeromorph crown-group (e.g., tarsal papillae and resilient sole hairs; four pairs of maxillipede spine bristles that oppose a single spine bristle on the trochantero-prefemur). *Crussolum* includes fossils of latest Silurian (Pridolí), Early Devonian (Pragian) and Middle Devonian (Givetian) age (Shear et al., 1998; Anderson and Trewin, 2003). The oldest fossil of *Crussolum* in the Ludlow Bone Bed in western England (at least 416 million years, dating the end of the Přidolí) constrains the split of Scutigeromorpha from Pleurostigmophora. We used an upper limit of 450 million years for the same node because the terrestrial arthropod record and the only evidence for Myriapoda at that time is confined to trace fossils (Johnson et al., 1994).

The divergence of Devonobiomorpha and Epimorpha is constrained by the occurrence of *Devonobius delta* in the Middle

Devonian of New York, at least 385 million years (date for the end of the Givetian). The basal divergence in Epimorpha, i.e., the split between Scolopendromorpha and Geophilomorpha is constrained by the oldest scolopendromorph, *Mazoscolopendra richardsoni* in Upper Carboniferous (Westphalian D) deposits of Mazon Creek, Illinois (at least 305 million years ago). Available character data for *Mazoscolopendra* are insufficient to establish whether it is a stem- or crown-group scolopendromorph, and it only provides a minimum age for total-group Scolopendromorpha. A minimum age for Scolopendridae is provided by the Early Cretaceous *Cratoracricus oberlii*, dating to the latest Aptian-Early Albian (at least 110 million years).

The millipede fossil record predates that of Chilopoda, and mid-Silurian body fossils of Diplopoda provide some constraints on the divergence of Chilopoda and Diplopoda, as well as deep divergences within Diplopoda. Total-group Helminthomorpha (in this study the clade that unites all millipedes except for *Polyxenus* and *Epicyliosoma*) is dated by the occurrence of Archipolypoda (stem-group helminthomorphs) in the late Wenlock or early Ludlow (Wilson and Anderson, 2004), at least 422 million years.

2.9. Molecular dating–dating methodology

Molecular dating was performed on the combined molecular and morphological tree obtained under parsimony with TNT. Branch lengths were optimized under a likelihood framework using Garli0.96r396.partMkTest.OSXintel (Zwickl, 2006) with a standard variable model – corresponding to the mkv model of Lewis (2001) – for the morphological partition and a GTR + Γ applied to the molecular partition.

The ages of clades were estimated using standard likelihood methods as implemented in the program r8s 1.71 (Sanderson, 2003, 2006). We used a cross-validation procedure (Sanderson, 2002) to select the best method among those offered by the program. We tested one clock-like method, the Langley and Fitch (1974) method, and two relaxed-clock methods, nonparametric rate smoothing (Sanderson, 1997) and penalized likelihood (Sanderson, 2002). For the penalized likelihood method, the degree of autocorrelation within lineages was estimated using cross-validation, and the smoothing parameter λ defined accordingly. We also tested the performance of two penalty functions, the additive penalty function, which penalizes squared differences in rates across neighbouring branches in the tree, and the log penalty function, which penalizes the squared difference in the log of the rates on neighbouring branches. All fossil calibrations described above were included as hard bounds (Ho, 2007; Ho and Phillips, 2009) except for *Devonobius delta*, which was included as a point calibration at a terminal node, as the fossil was represented as a terminal in the morphological partition.

A search was then performed using the commands num_time_guesses = 3 (3 initial starting conditions) and checkGradient in order to validate the results. Garli was used to generate 100 bootstrap datasets based on the optimal topology. Those 100 topologies thus only vary in branch lengths. Divergence estimates were then calculated for each of the 100 bootstrap replicates using r8s 1.71 to obtain standard deviations on each node using the profile command.

In order to check the influence of the inclusion of morphological characters (see also Lee et al., 2009), we performed an analysis on the combined molecular tree obtained in maximum likelihood with RaxML. We used the date obtained for Chilopoda in the previous analysis (optimal data for the Pleurostigmophora–Notostigmophora split) as a calibration point and dates of diversification of the chilopod orders were checked.

3. Results

3.1. Morphology

The morphological dataset yielded 11 equally parsimonious trees of 500 steps, the consensus of which is depicted in Supplementary Fig. S5. The consensus tree shows the monophyly of Chilopoda with 100% bootstrap frequency (BF hereafter) as well as the monophyly of all centipede orders with high BF. As already reported in previous morphological analyses, Scutigeromorpha appears as sister to the remaining groups, Pleurostigmophora, followed by Lithobiomorpha and Craterostigmomorpha. *Devonobius* is sister to the Scolopendromorpha–Geophilomorpha group (=Epimorpha), as resolved by Shear and Bonamo (1988), but with only 27% BF. Missing data for the fossil taxon *Devonobius* weaken support for deep nodes in Phylactometria: the grouping of *Craterostigmus*, *Devonobius* and Epimorpha is retrieved in 81% of bootstrap replicates (against 89% support for *Craterostigmus* as sister to Epimorpha when the data are analyzed without the fossil *Devonobius*), and Epimorpha in 72% (against 96% without *Devonobius*). When the same dataset was analyzed under implied weighting with ($k = 3$), 18 trees were found with a best score of 33.27619, the consensus of which is shown in Supplementary Fig. S6. Ordinal relationships are stable to different weighting regimes. The main differences between equally weighted and implied weighted analyses are the more explicit resolution of blind Scolopendromorpha as a grade in the latter, and the internal relationships of Geophilomorpha. Under equal weights, Ballophilidae + Schendylidae are the sister group of Oryidae + Himantariidae, and Geophilidae constitute a grade at the base of Adesmata (Supplementary Fig. S5), whereas under implied weights Geophilidae is monophyletic and ballophilids and schendylids are more closely related to Geophilidae than to Oryidae and Himantariidae (Supplementary Fig. S6).

3.2. Molecular data under ML

The COI dataset comprises 84 sequences. This dataset yielded a Muscle alignment of 815 positions with no gaps, showing 499 distinct alignment patterns. This alignment was kept in one block and considered as prealigned in direct optimization. The average composition of ambiguous characters (N's) was of 10.89%, mainly due to the shorter amplification (LCO1490/HCO2198) of some sequences (sequences with deviating base composition are indicated in boldface in Supplementary Fig. S7). The likelihood mapping of phylogenetic signal shows 7.8% unresolved and 11.1% partly resolved quartets. The resulting likelihood tree ($\ln L = -24697.13$) is shown in Supplementary Fig. S7. Bootstrap search stopped after 400 replicates. Species with deviating composition are found in all the groups (mainly in Geophilomorpha, affecting 70% of the species), but this does not seem to affect the results. The tree does not show monophyly of Chilopoda because the diplopod *Epicyliosoma* sp. groups with Scolopendridae. Scutigeromorpha is monophyletic (100% BF), as are Craterostigmomorpha (100% BF), Lithobiomorpha (69% BF) and Geophilomorpha (59% BF). As in the morphological analysis, Scutigeromorpha is sister to all the remaining chilopods (Pleurostigmophora), apart from the anomalous placement of *Epicyliosoma* in Pleurostigmophora. The only order for which monophyly is unsupported is Scolopendromorpha, wherein blind species (Cryptopidae and Scolopocryptopidae) and ocellate species (Scolopendridae) occupy distant positions.

The 16S dataset comprises 101 sequences of an average length of 487 base pairs (min = 463, max = 526). This dataset yielded a Muscle alignment of 619 positions. Structural alignment was performed based on the secondary structure of the 16S sequence of

Apis mellifera (available from the RNAsalsa website at http://www.zfmk.de/ last accessed Oct 15, 2009). After alignment filtering with Aliscore, 520 characters were retained (86.8% of the original structural alignment) presenting 431 site patterns. The average composition of ambiguous characters (indels and N's) was of 12.10% (sequences with deviating base composition are depicted in boldface in Supplementary Fig. S8). The likelihood mapping of phylogenetic signal shows 19.9% unresolved and 5.6% partly resolved quartets. For direct optimization, the 16S gene was segmented into 89 fragments and fragments corresponding to RSS were deactivated. The resulting likelihood tree ($\ln L = -20178.70$) is shown in Supplementary Fig. S8. The bootstrap search stopped after 350 replicates. As for the COI gene, most of the species with deviating base compositions are members of Geophilomorpha but this does not seem to affect the results. Chilopoda is monophyletic (42% BF) as well as Scutigeromorpha (99% BF), Craterostigmomorpha (100% BF) and Geophilomorpha (98% BF). As for morphology and COI, Scutigeromorpha is sister to the remaining chilopods. Non-monophyly of Scolopendromorpha, as for COI data, again involves blind and ocellate taxa being split into separate groups, but the node contributing to paraphyly of Scolopendromorpha at the base of Pleurostigmophora is weakly supported (40% BF). Lithobiomorpha is also paraphyletic in the 16S tree, Lithobiidae being sister to *Craterostigmus* (rather than to Henicopidae) and that node receiving moderate support (79% BF).

The 18S dataset comprises 107 sequences of an average length of 1771 base pairs (min = 516 for partial sequences, max = 2291). This dataset yielded a Muscle alignment of 3727 positions. Structural alignment was performed based on the secondary structure of the 18S sequence of *Anopheles albimanus* (available from the RNAsalsa website at http://www.zfmk.de/ last accessed Oct 15, 2009). After visual evaluation of the structural alignment in Jalview (Waterhouse et al., 2009), the 18S sequences of *Orphnaeus brevilabiatus* (DNA101998) and *O. brasilianus* (DNA103937) clearly appear misaligned in the first region while they were well aligned in the Muscle alignment. It is apparent that the sequences were "stretched" over the first region that was missing for those two sequences. Since we tried to avoid additional manual readjustment, the misaligned regions were removed from these two species in subsequent analyses. After alignment filtering with Aliscore, 2172 characters were retained (78.6% of the original structural alignment) presenting 1175 site patterns. The average composition of ambiguous characters (indels and N's) was of 23.35%. Sequences with deviating base composition are *Zygethobius pontis* and *Narceus americanus*. The likelihood mapping of phylogenetic signal shows 5.9% unresolved and 7.1% partly resolved quartets. The 18S sequence of *Z. pontis* (GenBank accession number AY213722) is missing the middle fragment and is of relatively low quality, resulting in a long branch in the separate analysis (Supplementary Fig. S9). The sequence was nevertheless retained for further analysis as it groups with the other members of Henicopidae, indeed in a morphologically expected position in Henicopinae. For direct optimization, the 18S gene was segmented into 652 fragments and those corresponding to RSS were deactivated. The resulting likelihood tree ($\ln L = -21088.75$) is shown in Supplementary Fig. S9. Bootstrap search stopped after 350 replicates. The tree does not show the monophyly of Chilopoda as *Epicyliosoma* sp. groups with Lithobiomorpha. Scutigeromorpha is monophyletic (96% BF) as well as Craterostigmomorpha (95% BF), Lithobiomorpha (39% BF) and Scolopendromorpha (78% BF). A morphologically anomalous result is the resolution of Geophilomorpha as a grade at the base of Chilopoda, but it must be noted that the four nodes contributing to geophilomorph paraphyly are all very weakly supported (6–12% BF). The behaviour of Geophilomorpha in this tree appears to contribute to a re-rooting of chilopod ordinal relationships that are not found with morphology or the mitochondrial markers, i.e., yielding groupings of Scutigeromorpha + Craterostigmomorpha and Lithobiomorpha + Scolopendromorpha.

The 28S dataset comprises 103 sequences of 1731 base pairs average length (min = 310, max = 3134). The shorter length (ca. 300 bp) corresponds to species for which the 28S was amplified with the 28Sa/28Sb primer pair in previous studies and for which extra amplification was unsuccessful. Due to the presence of missing fragments, it was impossible to estimate the degree of variation for the total length. This dataset yielded a Muscle alignment of 5400 positions. Structural alignment was performed based on the secondary structure of the 28S sequence of *Anopheles albimanus* (available from the RNAsalsa website at http://www.zfmk.de/ last accessed Oct 15, 2009). After visual evaluation of the structural alignment in Jalview, the 28S sequence of *O. brevilabiatus* (DNA101998) clearly appears misaligned despite appearing well aligned when using Muscle. This is probably due to the large amount of missing data in this sequence, not enabling the RNA structure folding algorithm to perform well. The sequence was submitted to GenBank but removed from further analyses. After alignment filtering with Aliscore, 1050 characters were retained (23.9% of the original structural alignment) presenting 687 site patterns. The average composition of ambiguous characters (gaps and N's) was of 32.88%. No sequence with deviating base composition was detected. The likelihood mapping of phylogenetic signal shows 12.6% unresolved and 10.5% partly resolved quartets. For direct optimization, the 28S gene was segmented into 245 fragments and fragments corresponding to RSS were deactivated. The resulting likelihood tree ($\ln L = -11495.55$) is shown in Supplementary Fig. S10. Bootstrapping stopped after 450 replicates. The tree does not show the monophyly of Chilopoda due to the geophilomorph *Orya almohadensis* grouping with the outgroups. Craterostigmomorpha is monophyletic (92% BF) as is Scutigeromorpha (92% BF). Lithobiomorpha is not monophyletic as it does not include *Bothropolys multidentatus*. *Craterostigmus* is resolved as sister to all other Chilopoda (apart from the spuriously placed *Orya* noted above), but the node isolating *Craterostigmus* is weakly supported (11% BF).

The maximum likelihood analysis for the combination of all four markers gave a tree of $\ln L = -81061.16$ (Fig. 1). The resulting tree shows the monophyly of Chilopoda (95% BF) as well as the strong support for the monophyly of all extant centipede orders, Scutigeromorpha (100% BF), Craterostigmomorpha (100% BF), Lithobiomorpha (98% BF), Scolopendromorpha (95% BF) and Geophilomorpha (100% BF). The topology conforms to morphology in finding Pleurostigmophora monophyletic (55% BF) but deviates from the morphological cladogram with respect to ordinal interrelationships within that group. The molecular tree places *Craterostigmus* as the sister to other pleurostigmophorans with moderate support (73% BF), and resolves Lithobiomorpha rather than Geophilomorpha as sister group of Scolopendromorpha (65% BF). The combined likelihood tree thus conflicts with the monophyly of Phylactometria and Epimorpha.

3.3. Parsimony analyses

The parsimony analysis of the static alignment for the combination of all four markers gave a tree shown in Supplementary Fig. S11 (22,976 steps). The topology is very similar to the one obtained in maximum likelihood (Fig. 1). The only major difference is the fact that Scolopendromorpha is not retrieved as a monophyletic group, its ocellate members (Scolopendridae) being resolved as sister to Geophilomorpha.

During the 24 h of tree searching under direct optimization, POY conducted 183 builds + TBR, 193 fusing rounds and 88 ratchet rounds. Shortest trees were found four times for a tree length of 20,434, resulting in a single most parsimonious tree (Supplementary

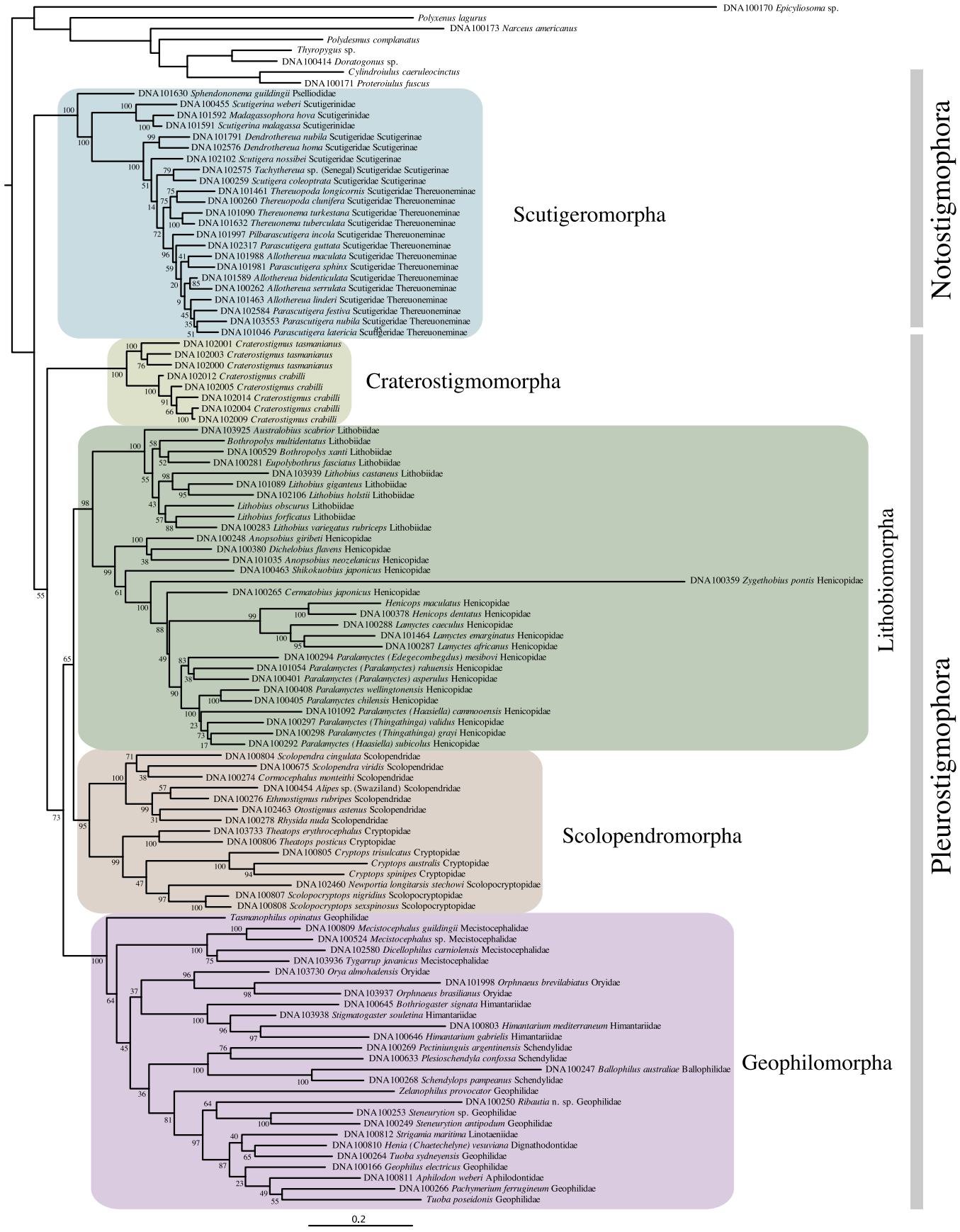

Fig. 1. Combined molecular dataset analyzed under maximum likelihood with RaxML (ln L = −81061.16).

Fig. S12). All chilopod orders are monophyletic, and Scolopendromorpha and Geophilomorpha unite as a clade, Epimorpha. In contrast to the combined ML and TNT analyses, monophyly of Pleurostigmophora is violated by the union of *Craterostigmus* with Scutigeromorpha (a grouping otherwise observed only in the 18S tree under ML).

The parsimony analysis using TNT for the combination of all four markers and morphology gave a tree shown in Supplementary Fig. S13 (23,586 steps). The contribution of the morphological data is apparent in that all inter-ordinal groupings are precisely congruent with the cladograms based on morphology alone (Supplementary Figs. S5 and S6). Based on the fact that the corresponding molecular analysis (Supplementary Fig. S11) resolved *Craterostigmus* as sister to other Pleurostigmophora rather than in Phylactometria, the support for Phylactometria in the total evidence analysis is low (20% BF), as is that for Epimorpha (34% BF). Here we caution that support at these nodes is likely deflated by missing data for the fossil taxon *Devonobius delta* (see discussion of the morphology cladograms above). Comparing molecules-only (Supplementary Fig. S11) and molecules + morphology (Supplementary Fig. S13), the morphological data provide additional resolution within Scutigeromorpha and contribute to the monophyly of Scolopendromorpha.

During the 24 h of tree searching under direct optimization for the combined molecular and morphological dataset, POY conducted 192 builds + TBR, 212 fusing rounds and 78 ratchet rounds. The same shortest tree was found twice for a tree length of 21,029 (Supplementary Fig. S14). Its topology closely corresponds to the parsimony tree for the combined dataset with TNT; again, Pleurostigmophora, Phylactometria and Epimorpha are clades. Differences between the one-step and two-step approaches concern details of relationships within orders. In a clade of blind Scolopendromorpha, the two-step analysis resolves Cryptopinae as sister to Plutoniuminae + Scolopocryptopidae, whereas Direct Optimization instead resolves Plutoniuminae as sister to the rest of the blind clade and favours paraphyly of Scolopocryptopidae (these differences dictate whether a 23-segmented trunk in Scolopocryptopidae from a 21-segmented ancestor was reversed or unreversed). The two-step analysis (Supplementary Fig. S13) places Mecistocephalidae as sister to other Geophilomorpha (apart from the oddly placed geophilid *Tasmanophilus*), but the POY analysis unites Mecistocephalidae with Oryidae. Broadly, however, the congruence between the two analyses is substantial.

Dates (in millions of years) for the basal split in the crown groups of the five extant centipede orders are as follow (based on the bootstrap procedure): Scutigeromorpha (mean = 359.0, SD = 21.1, min = 259.1, max = 394.5), Lithobiomorpha (mean = 398.2, SD = 23.0, min = 245.5, max = 421.6), Craterostigmomorpha (mean = 270.2, SD = 26.3, Min = 179.8, max = 317.8), Scolopendromorpha (mean = 374.5, SD = 20.2, min = 280.4, max = 408.7) and Geophilomorpha (mean = 350.4, SD = 24.1, min = 225.7, max = 381.2). The chronogram obtained is shown if Fig. 2, depicting the optimal dates (optimal dates and mean dates from the bootstrap procedure may vary). When the molecular ML tree was analyzed instead of the combined morphological and molecular TNT tree, those dates were younger; nodes outside the 95% confidence intervals were Craterostigmomorpha (231.9 mya), Lithobiomorpha (362.5 mya) and Scutigeromorpha (325.2 mya), all of which are close to the lower 95% limit and still within the total range observed.

4. Discussion

The combination of the four molecular markers and analysis with maximum likelihood yielded a phylogenetic hypothesis (Fig. 1) that conforms to morphology (Supplementary Figs. S5 and S6) in finding the five extant orders of Chilopoda monophyletic, and strongly supported (95–100% BF). The internal relationships within the four large orders in the combined ML and parsimony trees are for the most part readily interpreted with respect to morphology-based classifications. For example, both data sources divide Scutigeromorpha into Pselliodidae and a clade that unites Scutigerinidae and Scutigeridae (though these relationships are more strongly supported by the molecular data). Likewise, for Lithobiomorpha, the ML and molecular parsimony trees divide the order into two clades that correspond to the traditional families Lithobiidae (100% and 98% BF for ML and parsimony, respectively) and Henicopidae (99% and 96% BF), both also monophyletic in the morphological cladogram. Scolopendromorpha splits basally into blind and ocellate groups (with 99% and 100% BF, respectively, in the combined ML tree), as classified by Attems (1930) and found in some contemporary morphological analyses under particular character weighting regimes (Koch et al., 2009). Within Scolopendromorpha, the ML and parsimony trees are congruent with morphology in identifying such fundamental groups as Scolopendridae, Scolopendrinae, Otostigminae, Cryptopinae and Scolopocryptopidae. In Geophilomorpha, groupings in the ML tree such as Mecistocephalidae, Oryidae + Himantariidae, and Schendylidae + Ballophilidae are all congruent with morphology. The basal split of the geophilomorphs into Placodesmata (Mecistocephalidae) and Adesmata with morphology (Supplementary Fig. S5) is mirrored in the combined ML tree (Fig. 1) and parsimony trees (Supplementary Fig. S11) apart from the placement of the geophilid *Tasmanophilus* in an unexpected basal position in the order. Morphology appears to differ considerably from the molecular data with respect to relationships of Geophilidae, but this part of the tree is sensitive to character weighting. The equally-weighted morphological cladogram (Supplementary Fig. S5) depicts Geophilidae as a paraphyletic group, a result similarly found in a previous morphological analysis (Foddai and Minelli, 2000). The molecular data instead unite geophilids (apart from *Tasmanophilus*) in a clade with other families (Aphilodontidae, Dignathodontidae and Linotaeniidae) (Figs. 2, Supplementary S11) that were classified as geophilid subfamilies by Attems (1929). When the morphological data were analyzed with implied character weighting (Goloboff, 1993b), Geophilidae unites as a clade, with the members of Aphilodontidae, Dignathodontidae and Linotaeniidae, their closest relatives (Supplementary Fig. S6), as in the molecular tree (Fig. 1). Thus, with respect to relationships within centipede orders, we reiterate the conclusion drawn in earlier studies (Edgecombe and Giribet, 2004a; Giribet and Edgecombe, 2006a) that available nuclear ribosomal and mitochondrial data, when combined, show a high degree of congruence with morphology. An even denser sampling may be required within Geophilomorpha to stabilize their interrelationships.

Apart from both data sources recognising the basal split of Notostigmophora and Pleurostigmophora, the combined molecular trees under either likelihood (Fig. 1) or parsimony (Supplementary Fig. S11) and morphology differ substantially with respect to how centipede orders relate to each other. The molecular trees conflict with two of the cornerstones of morphology-based cladograms and classifications of Chilopoda: Phylactometria is non-monophyletic for the combined data under all three optimality criteria as well as for each individual gene, and Epimorpha is non-monophyletic under likelihood (Fig. 1) and ambiguous under parsimony (Supplementary Fig. S11), though it is monophyletic under direct optimization (Supplementary Fig. S12). The molecular resolutions of *Craterostigmus* and Lithobiomorpha force considerable homoplasy on behavioural and developmental characters that have long served as the basis for centipede systematics. If *Craterostigmus* is sister group to all other Pleurostigmophora, then a loss of maternal

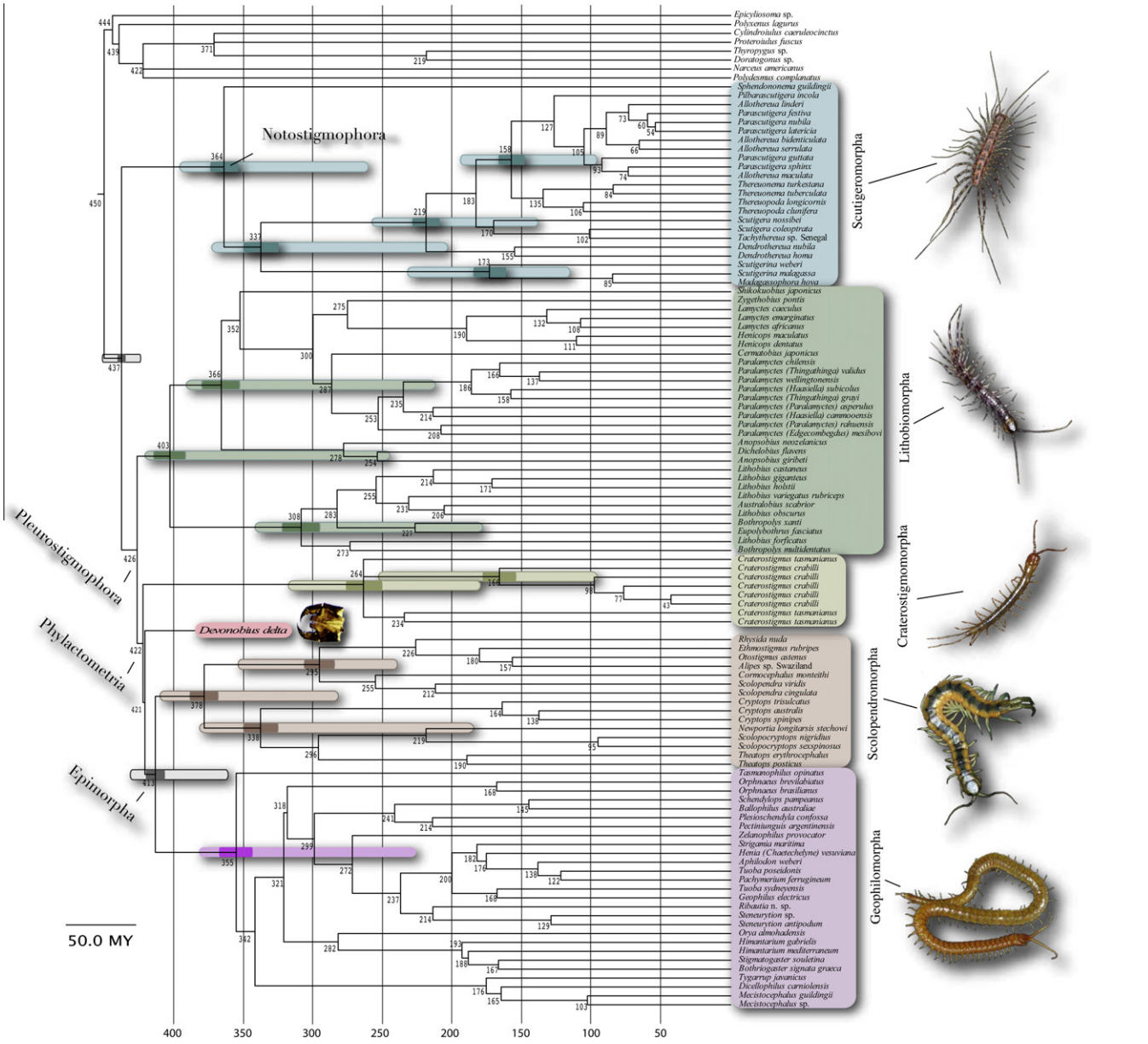

Fig. 2. Chronogram inferred under penalized likelihood showing the topology obtained for the combined molecular and morphological data under parsimony. Standard deviation in depicted as a dark error bar while minimum and maximum bootstrap values are depicted as a light error bar. Photographs are (from top to bottom): *Parascutigera guttata* (by G. Hormiga), *Henicops dentatus* (by G. Giribet), *Craterostigmus crabilli* (by G. Giribet), *Devonobius delta* (by W. A. Shear), *Scolopendra cingulata* (by G. Giribet) and *Zelanophilus provocator* (by G. Giribet).

brood care is forced in Lithobiomorpha. Similarly if Scolopendromorpha is sister to Lithobiomorpha rather than Geophilomorpha, then epimorphic development would have to either have been lost in Lithobiomorpha or else it was independently acquired in scolopendromorphs and geophilomorphs. These conspicuous homoplasies that are forced by trees that conflict with Phylactometria would be joined by numerous other anatomical features shared by *Craterostigmus* and Epimorpha (Wirkner and Pass, 2002; Edgecombe and Giribet, 2004a,b; Müller and Meyer-Rochow, 2006a,b). Although we note the tendency for the molecular analyses to resolve *Craterostigmus* more basally in Chilopoda than is indicated by morphology, the precise position of *Craterostigmus* in the molecular analyses is sensitive to marker selection and analytical methods (e.g., *Craterostigmus* as sister to other Pleurostigmophora for ML and parsimony versus sister to Scutigeromorpha in the POY molecular analysis). This sensitivity, coupled with the fact that support for the sister group relationship between *Craterostigmus* and all other Pleurostigmophora in the combined ML (Fig. 1) and molecular parsimony (Supplementary Fig. S11) is at best moderate (but see Mallatt and Giribet, 2006, for a reduced taxonomic sample), is reflected in the fact that the inclusion of morphological data is sufficient to overturn the "molecular" position of *Craterostigmus* in favour of the morphological position, irrespective of whether a two-step (Supplementary Fig. S13) or one-step (Supplementary Fig. S14) approach is used.

Examining the molecular dates of nodes, we see very few shallow (Cenozoic) divergences but this is in part affected by sampling design in that we selected species to sample taxonomically for deep events. In the case of congeners with Mesozoic divergences, the *Scolopendra* species are from the New World and Old World

groups, which could well predate the breakup of Pangaea. Likewise, *Paralamyctes* species include representatives from most Gondwanan fragments (Giribet and Edgecombe, 2006b). Nonetheless, several nodes appear excessively older than expected based on their distribution or taxonomic status. Notably, the two *Craterostigmus* species split at least by the Jurassic, which seems old in comparison with the very similar morphology of the species and their distribution in Tasmania and New Zealand. The ages for *Craterostigmus* divergences contradict the possibility of *C. crabilli* being a geologically young introduction to New Zealand. Dating on an alternate topology and using the molecular dataset alone did not provide a substantially younger age for *Craterostigmus*. The same is true for some other examples where shallow divergences might have been suspected based on taxonomy and distributions, such as the two *Paralamyctes* species in southern Chile, the three *Parascutigera* species in New Caledonia and the two Neotropical *Dendrothereua* species. This pattern of deep splits should be further investigated using a denser sampling of the terminal taxa.

Despite the geological antiquity of its stem-group and its basal phylogenetic position in Chilopoda, Scutigeromorpha is inferred to have diversified more recently that did some of the orders in its sister group, Pleurostigmophora: Scolopendromorpha and especially Lithobiomorpha have earlier crown group diversification. A similar pattern is observed in Opiliones (Giribet et al., 2010), in which the suborder Cyphophthalmi (sister to the remaining clades) likewise diversified more recently than the other three suborders. The pattern of deep divergences but later diversification is repeated within Scutigeromorpha. The split of Pselliodidae from Scutigerinidae and Scutigeridae dates to the Late Devonian (ca. 364 mya, SD = 21.1), and the scutigerinid-scutigerid split is Early Carboniferous (ca. 337 mya, SD = 25.4). The basal splits within each of the latter two families are, however, considerably younger, Late Triassic in the case of Scutigeridae (ca. 219 mya, SD = 19.9) and Middle Jurassic for Scutigerinidae (173 mya, SD = 19.4).

In this study we have re-evaluated the systematics of the myriapod class Chilopoda to explore the conflict between morphology and molecules that had been identified in earlier studies. In addition we have dated the origins of each of the extant and extinct centipede orders and show that the group is indeed composed of six ancient clades that diversified before the breakup of Pangaea. We hope that our better understanding of the origins and diversification of this arthropod group opens up new perspectives in the study of these terrestrial predators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.06.022.

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