The molecularization of centipede systematics

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Abstract

The injection of molecular data over the past 20 years has impacted on all facets of centipede systematics. Multi-locus and transcriptomic datasets are the source of a novel hypothesis for how the five living orders of centipedes interrelate but force homoplasy in some widely-accepted phenotypic and behavioural characters. Molecular dating is increasingly used to test biogeographic hypotheses, including examples of ancient vicariance. The longstanding challenge of morphological delimitation of centipede species is complemented by integrative taxonomy using molecular tools, including DNA barcoding and coalescent approaches to quantitative species delimitation. Molecular phylogenetics has revealed numerous instances of cryptic species. "Reduced genomic approaches" have the potential to incorporate historic collections, including type specimens, into centipede molecular systematics.

Introduction

Centipedes – the myriapod Class Chilopoda – are an ancient group of soil predators, with a >420 million year fossil history and about 3150 described extant species (Minelli, 2011). They are of interest to students of arthropods more broadly for conserved elements of their relatively compact genome (Chipman *et al.*, 2014), for their insights into the position of myriapods in Arthropoda (Rehm *et al.*, 2014), and for the data available on their mechanisms of segment proliferation (e.g., Brena, 2014), in light of the systematic variability in their numbers of trunk segments and modes of postembryonic development (Minelli *et al.*, 2000). These questions have all been profoundly impacted by conceptual and technological advances in molecular biology, and the same is true of other fields of biology, including systematics. This essay explores the impact of molecular methods on reconstructing the evolutionary relationships of centipedes, dating centipede diversification, and recognizing species.

Just over a decade ago, we reviewed the evolutionary biology of centipedes (Edgecombe and Giribet, 2007) and developments in centipede systematics over the previous 25 years (Edgecombe, 2007). At the time, the higher – level phylogeny of centipedes was considered a given and a textbook example of the congruence between morphology and molecules. The past 10 years have been marked by an uptake in molecular approaches to centipede systematics that have made the field more integrative and informed by novel sources of data, increasingly on a scale vastly larger than was formerly the case. But the new datasets have also brought disagreement for a few critical nodes in the centipede tree – nodes that have forced us to rethink the evolutionary trajectory of major characters, including postembryonic addition of segments and maternal care.

Inferring phylogenies: the impact of Next-Gen methods

Centipede phylogenetics exemplifies a pattern across evolutionary inference as a whole since the mid-2000s, a shift from traditional Sanger sequencing to Next-Generation approaches. To date, this shift has been witnessed mostly at relatively deep phylogenetic levels, such as the question of the interrelationships of the five extant orders of Chilopoda (see Fig. 1).
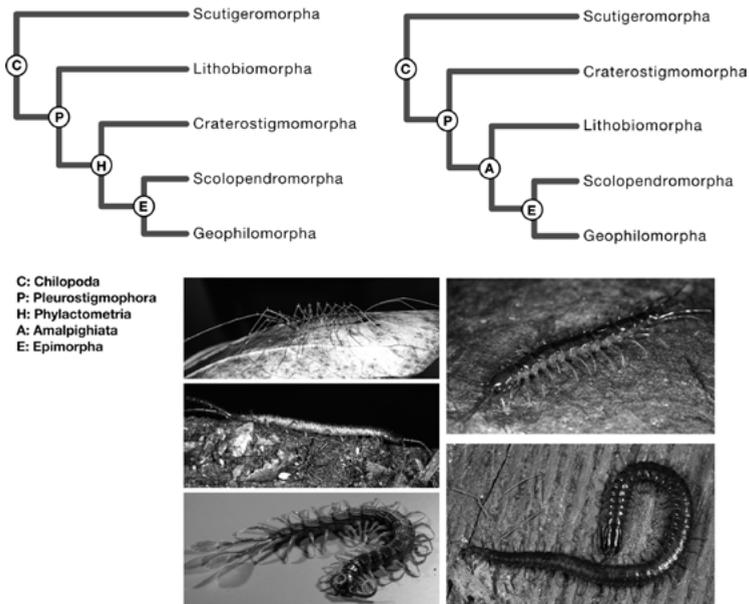
Figure 1. Relationships between the five extant centipede orders based on morphology (Phylactometria hypothesis, at left) versus transcriptomic datasets (Amalpighiata hypothesis, at right), and exemplars of Scutigeromorpha (*Sphendononema guildingii*), Craterostigmomorpha (*Craterostigmus crabilli*), Scolopendromorpha (*Alipes* sp.) (left, from top), Lithobiomorpha (*Paralamyctes levigatus*) and Geophilomorpha (*Mecistocephalus* sp.) (right, from top).
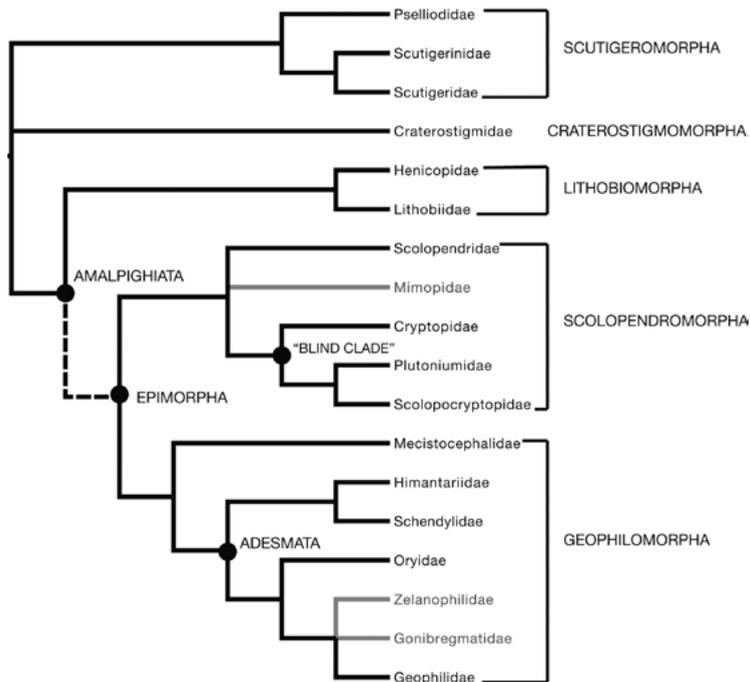
Figure 2. Summary of relationships between family-level groupings of centipedes (after Fernández *et al.*, 2016). Families lacking transcriptomic data shown in light font.

Molecular systematic studies of centipedes were launched in the late 1990s, originally targeted largely at relationships between the five orders, aiming to test such questions as whether centipedes with epimorphic development (hatching with the complete adult segment number) form a clade and the position of the Tasmanian–New Zealand Craterostigmomorpha, an order composed of just two species. These analyses used two or three nuclear protein-coding genes (Regier *et al.*, 2005), the small and large nuclear ribosomal RNAs (Edgecombe *et al.*, 1999) the latter combined with a few mitochondrial loci (Edgecombe and Giribet, 2004), or all of these markers together, with or without morphological data (Giribet and Edgecombe, 2006a). By the mid 2000s or early part of the present decade, taxonomic sampling for these Sanger-sequenced markers was dense enough within each of the four large centipede orders to allow many lower-level taxonomic hypotheses to be tested (see Fig. 2). The limits of the small set of genes used in these early analyses for resolving deep nodes have been apparent, these sometimes being weakly supported and unstable, at least in the larger clades. Nonetheless, some parts of the centipede tree were stable and well supported based on "first generation" molecular data. Scutigeromorpha divides into the Neotropical/tropical African Pselliodidae as sister group to the south-

ern African/Malagasy Scutigerinidae and the globally-distributed Scutigeridae (Edgecombe and Giribet, 2006; Butler *et al.*, 2010; Giribet and Edgecombe, 2013). Lithobiomorph monophyly is well established molecularly, the clade composed of two monophyletic families, the mostly Laurasian Lithobiidae and the mostly temperate Gondwanan Henicopidae (Edgecombe and Giribet, 2003). The latter has been especially well sampled, and taxonomic studies on Australian diversity have been accompanied by molecular data applied to phylogenetic placement of new species and Gondwanan biogeography (e.g., Giribet and Edgecombe, 2006b). Phylogenies for Geophilomorpha depict the basal split into Placodesmata (consisting of only one family, Mecistocephalidae) and Adesmata as in morphological classifications, and allow the larger clade Adesmata to be carved into subgroups (Bonato *et al.*, 2014) (Fig. 2). Scolopendromorpha divide into a blind clade and a clade whose members have the eye organized as a cluster of four ocelli, but deep nodes within species-rich groups, such as the subfamilies Scolopendrinae and Otostigminae, are often unstable and weakly supported (Vahtera *et al.*, 2012, 2013).

This last problem has partly been rectified by the greatly expanded gene samples available from transcriptomes (Fernández *et al.*, 2014; Fernández *et al.*, 2016, 2018). Phylogenetic resolution within orders is strongly supported and stable across a broad range of tree-building approaches and models. In Scolopendromorpha, the division into an ocellate clade (Scolopendridae) and a blind clade composed of the three families Cryptopidae, Scolopocryptopidae and Plutoniumidae is extremely robust (Fig. 2). Deep scutigeromorph and lithobiomorph relationships are the same as had been resolved in Sanger analyses, though to date only one or two species per family have transcriptomic data publicly available.

A reopened question, however, is the position of Craterostigmorpha. Morphological studies had settled on a sister group relationship between *Craterostigmus* and Epimorpha (=Scolopendromorpha + Geophilomorpha), and this hypothesis was formalized as the taxon Phylactometria (Edgecombe and Giribet, 2004) (Fig. 1, right). The name refers to a putative shared derived character – the mother brooding the eggs and hatchlings – in these three orders. This behavioural character is congruent with numerous other putative shared derived characters from varied organ systems. Some early molecular studies recovered support for Phylactometria (Edgecombe *et al.*, 1999) but others found novel placements for *Craterostigmus*. A recurring pattern is *Craterostigmus* as sister group to a clade composed of Lithobiomorpha and the two orders of Epimorpha (Fig. 1, left). That result was recovered in analyses of 62 nuclear protein-coding genes (Regier *et al.*, 2010) as well as with transcriptomic datasets (Fernández *et*

al., 2014; Fernández *et al.*, 2016). A putative clade composed of the three orders to which *Craterostigmus* is sister group was named Amalpighiata (Fernández *et al.*, 2014), its name signalling one of the few potentially diagnostic anatomical characters, a lack of supernumerary Malpighian tubules. *Craterostigmus* is alternatively recovered as sister group of Scutigeromorpha in a subset of analyses, a morphologically implausible grouping. Given that the morphological support for Phylactometria substantially outstrips that for Amalpighiata, it remains an open question whether *Craterostigmus* is spuriously attracted to Scutigeromorpha as a result of long branch attraction or other phylogenetic biases. However, applying substitution models intended to counter such systematic error fails to repel the "pull" of *Craterostigmus* towards the base of the centipede tree (Fernández *et al.*, 2016, 2018).

Molecular dating

Best practice for dating the tree of life using fossil-calibrated time trees is a topic of lively discussion in recent literature. Centipedes have been the subject of several molecular dating studies, some of them aimed at estimating divergence dates between deep (ordinal and familial-level) branchings, but also others that deal with shallower nodes to test biogeographic hypotheses.

The fossil record of Chilopoda is highly incomplete, but minimum divergence dates for several nodes in the crown group are constrained by Palaeozoic fossils. Incorporating these fossils under standard node calibration approaches – where prior densities for ages of nodes in a molecular tree are constrained by known fossils – recovers dated trees in which numerous family-level divergences within Chilopoda are inferred to have occurred in the Palaeozoic (Murienne *et al.*, 2010; Giribet and Edgecombe, 2013; Fernández *et al.*, 2016).

In the case of Scutigeromorpha, fossils constrain the crown-group (i.e., the clade derived from the most recent common ancestor of the group's living members) to the Late Silurian. The three extant families have ancient (Devonian to Permian) stem-groups but younger (Triassic-Jurassic) crown-group diversifications (Giribet and Edgecombe, 2013). Diversification rates show only minor shifts throughout the clade's 400 million-year history, and the dated tree is consistent with deep divergences, such as between African and Neotropical Pselliodidae and between Australian and New Caledonian Scutigeridae, probably resulting from vicariance rather than geologically recent dispersal.

Dated species-level phylogenies are increasingly being used to model vicariance and dispersal. A time tree for the scolopendrid genus *Digitipes* in the Western Ghats of peninsular India, including molecular data for numerous specimens of each species together with niche modelling, provided a basis for

evaluating how geological history may have shaped the group's diversification and distribution (Joshi and Karanth, 2012; Joshi and Karanth, 2013). Deep divergence dates and ancestral area reconstructions are consistent with the hypothesis that species inhabited refugia during Late Cretaceous vulcanism of the Deccan traps (Joshi and Karanth, 2013). Dated trees for Indian diversity of Scolopendridae more broadly show Cretaceous divergences within other genera as well (Joshi and Karanth, 2011). A dated tree using sequence data for four genes for 16 species of the Northern Hemisphere geophilomorph genus *Strigamia* recovered largely exclusive European and East Asian clades, each of which diversified over the past 30 million years (Bonato *et al.*, 2017).

Dating studies have also touched on comparative phylogeography of centipedes on continental and oceanic islands. Using likelihood mapping and neighbour nets for three loci, *Cryptops pictus*, endemic to New Caledonia (a continental island) was found to have a higher level of genetic structure and diversity than populations of *C. niuensis* from across Fiji and Vanuatu, geologically younger oceanic islands (Murienne *et al.*, 2011).

Species delimitation and integrative taxonomy

A number of studies on centipedes have used either sequences for the standard "DNA barcode" locus, cytochrome *c* oxidase subunit I (COI), or phylogenetic analysis based on COI or together with a few other widely-sampled Sanger-sequenced markers to identify species. This has the advantage in that species can be delimited based on the criterion of monophyly, which was often not the case based on morphological approaches, in which species could be distinguished with no phylogenetic context or could be non-monophyletic.

The German Barcode of Life program (www.bolgermany.de) has included analyses of a few centipede groups. Divergences within putative specimens of *Stenotaenia linearis* indicate the likely existence of cryptic species that are not clearly geographically separated from each other (Wesener *et al.*, 2015), and a similar pattern of molecularly distinct cryptic lineages is found in Italian populations of this genus (Del Latte *et al.*, 2015). COI phylogeny and pairwise distance comparisons for German populations of *Cryptops* likewise revealed a greater than expected number of species, including within long-known morphospecies (Wesener *et al.*, 2016). Notably, three geographically separate subgroups within *Cryptops parisi* signal likely cryptic species. COI barcodes have also been applied to identifying introduced species in groups that are challenging to determine to the species level morphologically, such as the lithobiomorph *Lamyctes* (Decker *et al.*, 2017) and scolopendromorph *Cryptops* (Reeves, 2017).

COI sequences and phylogenetic analyses that include them have been generated in some studies describing new species, such as for a new *Eupolybothrus* from Croatian caves (Akkari *et al.*, 2017). Molecular phylogenetics based on COI data unite two troglomorphic species of *Eupolybothrus* from the Balkans as each other's closest relative, and two different quantitative methods of molecular species delimitation allow them to be distinguished in a manner compatible with secondary sexual modifications of the males. Species descriptions have been accompanied by multi-locus sequence data (Siriwut *et al.*, 2015b; Kang *et al.*, 2017) or even transcriptomic data (Stoev *et al.*, 2013) for various centipede groups.

Cryptic species detected with molecular data have proven in some cases to be identified morphologically. In *Digitipes* from the Western Ghats, India, several clades with genetic distances indicative of species (Joshi and Karanth, 2012) were subsequently the target of morphological study, with some but not all putative new species being diagnosable based on morphological characters (Joshi and Edgecombe, 2013). Coalescent and Bayesian approaches to species delimitation allow five species of *Ethmostigmus* to be recognised in the Western Ghats, three of which form a clade in which morphological differentiation is subtle (Joshi and Edgecombe, 2018).

Similarly, trees based on COI sequences for putative material of *Lithobius* (*Monotarsobius*) *crassipes* from Germany, France and northern Spain revealed a distinct clade in Spain that was deemed a "pseudo-cryptic" species (Voigtländer *et al.*, 2017). It can be diagnosed morphologically, though based on characters (including some described using geometric morphometrics) that would likely have been dismissed as intraspecific variation under traditional species concepts, which were biased towards an assumption that species are geographic widespread and polymorphic.

The two species of *Craterostigmus*, despite each being monophyletic and distributed allopatrically in Tasmania and New Zealand, are also subtly distinguishable morphologically, but are easily diagnosed by species-specific substitutions or indels in the nuclear RNA (Edgecombe and Giribet, 2008; Giribet *et al.*, 2009; Vélez *et al.*, 2012). Geographic structure within *Craterostigmus tasmanianus* involves little variation in two mitochondrial loci within populations but considerable distance between geographically separated clusters of populations (Vélez *et al.*, 2012).

Although cryptic species are thus a common discovery in centipede analyses, in some cases molecular phylogeny is readily reconciled with traditional taxonomy based on external morphological characters. Comparisons of mitochondrial COI and 16S rRNA for populations of *Scolopendra cingulata* across

its geographic range in central/southern Europe depict clear gaps in intra- and interspecific distances (Oeyen *et al.*, 2014). In Southeast Asian species of *Scolopendra*, clades identified from molecular phylogeny inferred from three markers are compatible with groupings in geometric morphometric analyses, and species are diagnosed using traditional external taxonomic characters, with little change in nomenclature (Siriwut *et al.*, 2015a, 2016). This approach has been followed for Chinese species of *Scolopendra* (Kang *et al.*, 2017), and Southeast Asian species of *Rhysida* (Siriwut *et al.*, 2018). In the latter example, the molecular trees established that diagnostic characters of a monotypic genus, *Alluropus*, are actually secondary sexual characters of males, the females having been classified as a different genus and species.

Incongruence between morphological diagnoses and molecular phylogeny is pervasive in the scolopendromorph *Newportia*, a Neotropical radiation. Some geographically widespread morphospecies appear to be polyphyletic groups, with specimens of different species from the same geographic areas uniting with each other rather than with their putative conspecifics from other areas (Edgecombe *et al.*, 2015). This does not appear be common in centipede taxonomy. Morphospecies may underestimate species diversity (because cryptic species are undetected) but they are not usually in significant conflict with molecularly-delimited species.

Where to next?

It is not possible to accurately predict the future, but we can guess new directions for centipede systematics judging from what we are currently seeing in other fields of arthropod systematics and genomics. Centipedes have been at the forefront of phylotranscriptomics, but these have an important limitation – the requirement of fresh tissue preserved for RNA work. Even other PCR-based methods have limitations of sample availability, as they were not well suited for working with old museum samples, which often have degraded (i.e., highly fragmented DNA). However, new shotgun "reduced genomic approaches", namely target enrichment (e.g., Lemmon *et al.*, 2012) and ultraconserved elements, UCEs (e.g., Faircloth *et al.*, 2012), are amenable not only to specimens collected recently and preserved especially for DNA work, but also for old museum specimens (Sproul and Maddison, 2017). This opens the door to novel approaches to centipede taxonomy, perhaps the most exciting being the ability to incorporate genomic-level data from historic type material, circumventing the need to use morphological and geographic proximity of a fresh voucher to the types as the basis for fixing taxonomic names.

At the population-level, PCR-amplification of a few selected markers is now transitioning towards genomic subsampling. A popular technique is ddRAD-seq (double digest RAD sequencing) (Peterson *et al.*, 2012), a method for SNP discovery and genotyping, yet no study has applied it to the study of centipede populations. Additional SNP discovery methods include extending UCEs towards the least conserved areas (Starrett *et al.*, 2016), which could generate similar data to ddRADseq, but in a more repeatable manner.

Finally, from a genomics perspective, centipede genomes can now be sequenced with relative ease, although only that of *Strigamia maritima* is available (Chipman *et al.*, 2014). We expect a growth in centipede genomics, especially with the growing interest in their venom (Undheim *et al.*, 2016). The strength and stability of support for deep nodes in such challenging groups as Scolopendromorpha in transcriptome-based phylogenies (Fernández *et al.*, 2016) suggests a similar approach may also stabilize recalcitrant nodes at shallower taxonomic levels as more species are sequenced for large numbers of genes.

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