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Revisiting the age, evolutionary history and species level diversity of the genus *Hydra* (Cnidaria: Hydrozoa) ☆

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ABSTRACT

The genus *Hydra* has long served as a model system in comparative immunology, developmental and evolutionary biology. Despite its relevance for fundamental research, *Hydra*'s evolutionary origins and species level diversity are not well understood. Detailed previous studies using molecular techniques identified several clades within *Hydra*, but how these are related to described species remained largely an open question. In the present study, we compiled all published sequence data for three mitochondrial and nuclear genes (COI, 16S and ITS), complemented these with some new sequence data and delimited main genetic lineages (=hypothetical species) objectively by employing two DNA barcoding approaches. Conclusions on the species status of these main lineages were based on inferences of reproductive isolation. Relevant divergence times within *Hydra* were estimated based on relaxed molecular clock analyses with four genes (COI, 16S, EF1α and 28S) and four cnidarians fossil calibration points All in all, 28 main lineages could be delimited, many more than anticipated from earlier studies. Because allopatric distributions were common, inferences of reproductive isolation often remained ambiguous but reproductive isolation was rarely refuted. Our results support three major conclusions which are central for *Hydra* research: (1) species level diversity was underestimated by molecular studies; (2) species affiliations of several crucial 'workhorses' of *Hydra* evolutionary research were wrong and (3) crown group *Hydra* originated ~200 mya. Our results demonstrate that the taxonomy of *Hydra* requires a thorough revision and that evolutionary studies need to take this into account when interspecific comparisons are made. *Hydra* originated on Pangea. Three of four extant groups evolved ~70 mya ago, possibly on the northern landmass of Laurasia. Consequently, *Hydra*'s cosmopolitan distribution is the result of transcontinental and transoceanic dispersal.

1. Introduction

Since its first discovery in 1702 and its use in experimental studies in the early 18th century (van Leeuwenhoek, 1702; Trembley, 1744), *Hydra* has been an important model organism for studies on regeneration, development, pattern formation, symbiosis and more recently also for genome evolution and innate immunity. As a result of this work, and because *Hydra* belongs to the basal animal phylum Cnidaria, studies on *Hydra* have contributed significantly to our understanding of the origin and evolution of developmental genes and pathways, the evolution of the immune system and the concept of the metaorganisms or holobiont (Bosch, 2013, 2014; David, 2012; Franzenburg et al., 2013; Fujisawa and Hayakawa, 2012; Galliot, 2012; Grimmelikhuijzen and Hauser, 2012; Holstein, 2012; Khalturin et al., 2009; Lasi et al., 2010; Meinhardt, 2012; Nebel and Bosch, 2012; Shimizu, 2012; Steele et al., 2011; Tanaka and Reddien, 2011; Technau and Steele, 2011; Watanabe et al., 2009). One of the most important tools for identifying relevant genes is the genome of *Hydra magnipapillata* (Chapman et al., 2010) and the ever-increasing transcriptome datasets of other *Hydra* and cnidarian species (http://www.compagen.org).

Despite *Hydra's* long history as a model organism for animal evolution, key features of the evolution of *Hydra* itself such as the evolutionary origins of *Hydra* and its species level diversity are still not well understood. As a consequence, the pace and timeframe of crucial evolutionary processes and novelties, like the emergence of numerous orphan genes specific for *Hydra* or some of its species (Khalturin et al., 2008, 2009), cannot be assessed. Thus, as *Hydra* species differ in morphology, development,

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physiology, and ecology (Campbell, 1983; Hemmrich et al., 2007; Koizumi, 2007), the lack of a solid species level taxonomy hampers research on species specific differences in gene expression and development (Khalturin et al., 2008; Thomsen and Bosch, 2006).

Moreover, in every animal species divergence in host genes seems positively correlated with differentiation of the microbiome (Fraune and Bosch, 2007; McFall-Ngai et al., 2013; Brucker and Bordenstein, 2013; Bosch, 2014). Parallel cladograms between the host phylogeny and the microbiome relationships is one test of a pattern termed "phylosymbiosis" (Brucker and Bordenstein, 2012, 2013). Each *Hydra* species is equipped with a unique composition of antimicrobial peptides (Franzenburg et al., 2013). Loss-of-function experiments have shown that species-specific AMPs sculpture species-specific bacterial communities by selecting for co-evolved bacterial partners (Franzenburg et al., 2013). Current lack of information on precise phylogenetic placement of the *Hydra* species, however, makes it impossible to investigate the diversity of the *Hydra* specific microbiota within a co-evolutionary framework.

Finally, unambiguous phylogenetic placement and species identification of commonly used lab strains of *Hydra* is crucial to ascertain the reproducibility of experiments (requiring the usage of identical species) and to allow intra- and interspecific comparisons.

Resolving the evolutionary origins of *Hydra* has been impeded by the absence of fossilized remains. Recent phylogenetic analyses unambiguously placed *Hydra* within the hydrozoan taxon Aplanulata (Nawrocki et al., 2013), but the age of *Hydra* and the timing of its diversifications are largely unknown. Different molecular clock approaches diverged greatly in their estimates: while the age of the *viridissima* group was estimated to be 156–174 million years based on the divergence of its symbiotic *Chlorella* (Kawaida et al., 2013), the age of crown group *Hydra* was estimated to be only ~60 million years based on assumed substitution rates for COI and 16S (Martínez et al., 2010).

The taxonomy of *Hydra* species is characterized by relatively large numbers of synonymizations and wrongly applied species names (e.g. *Hydra attenuata*; see Campbell, 1989). Well accepted and supported by molecular phylogenetic studies (Hemmrich et al., 2007; Kawaida et al., 2010; Martínez et al., 2010) is the distinction of four species groups within *Hydra* – *viridissima* group (the 'green' *Hydra* featuring symbiotic *Chlorella*), *braueri* group, *oligactis* group and *vulgaris* group – which were outlined by Schulze (1917) and Campbell (1987). Over the last centuries ~80 species of *Hydra* have been described (Jankowski et al., 2008). Many of these species have been subsequently synonymized and the taxonomic status of others is still controversial (Campbell, 1987). For example, Jankowski et al. (2008) suggested less than 15 valid species of *Hydra* whereas the World Register of Marine Species lists 40 (Schuchert, 2014).

In the last years several molecular phylogenetic studies shed light on the diversity within *Hydra* (Campbell et al., 2013; Hemmrich et al., 2007; Kawaida et al., 2010; Martínez et al., 2010; Reddy et al., 2011; Wang et al., 2012). The most detailed studies regarding the number of studied individuals (=strains) were those by Kawaida et al. (2010) and Martínez et al. (2010). However, since these two studies were published nearly simultaneously they could not take each other's data into account and later studies included small fractions of the previously published data only. Apart from a few shared lab strains, all of these studies were based on different sets of strains from different species and geographic origins, limiting comparability among studies. Furthermore, despite identifying several monophyletic clades within *Hydra* and within its four groups, few explicit conclusions regarding the validity of *Hydra* species were drawn. For example, Kawaida et al. (2010) identified three monophyletic "sub-groups" within the *vulgaris* group, but it remained unclear whether these would represent three species or three clades of several species each. Martínez et al. (2010) recovered eight morphologically identified species as monophyletic (*H. viridissima, H. hymanae, H. utahensis, H. circumcincta, H. oligactis, H. oxycnida, H. canadensis, and H. vulgaris*), of which *H. viridissima* was subdivided into several clades, *H. circumcincta* into two clades and *H. vulgaris* into five clades matching geographic regions. Again, for most of these clades their species status was not discussed, the exception being the North American *vulgaris* group species – *H. littoralis*, *H. carnea*, and *H. vulgaris* AEP – which were believed to belong to a single species. The latter would have far reaching consequences for evolutionary studies on *Hydra* as *H. carnea* and *H. vulgaris* AEP are commonly used lab strains. *Hydra vulgaris* AEP is an artificially generated strain from which all transgenic *Hydra* are derived (Wittlieb et al., 2006). The *Hydra vulgaris* AEP strain originated from crossing two different *Hydra vulgaris* strains from North America (Martin et al., 1997), though Martínez et al. (2010) stated that the parental strains resembled *H. carnea* and *H. littoralis* morphologically. Similarly, two other important 'workhorses' – *H. magnipapillata* and the European *H. vulgaris* – are genetically very similar and were assigned to the same clade (Kawaida et al., 2010; Martínez et al., 2010).

To obtain a comprehensive overview of the species diversity and the evolutionary origins of *Hydra,* we compiled all available sequence data of *Hydra* from GenBank and BOLD for those genes with the highest coverage of species and individuals (e.g. those published by Campbell et al., 2013; Hemmrich et al., 2007; Kawaida et al., 2010; Martínez et al., 2010; Reddy et al., 2011 and Wang et al., 2012). This extensive dataset was complemented with some newly sequenced strains (mainly of the *viridissima* group). Species diversity was assessed by a combination of phylogenetic and genetic distance analyses. Such analyses have become very popular tools for studies of species diversities and are commonly subsumed under the term DNA barcoding. However, whether entities delimited by such approaches indeed represent distinct species is strongly dependent on the applied species concept (Agapow et al., 2004; Schwentner et al., 2011; Tan et al., 2008). For example, following the Phylogenetic Species Concept (Mishler and Theriot, 2000), which defines species as the "smallest monophyletic groups worthy of formal recognition", all entities delimited by DNA barcoding can be treated as species, if species delimitation is based on phylogenetic analysis. The Biological Species Concept (Mayr, 1942), on the other hand, requires reproductive isolation among species. Reproductive isolation can be inferred with barcoding techniques in an integrative framework, if the respective species are consistently differentiated by mitochondrial and an independent marker system – like nuclear markers or morphology – and occur in sympatry (see also Schwentner et al., 2015).

As a first step, we identified 'main lineages' (=hypothetical species) by independently analyzing three molecular markers: mitochondrial COI (cytochrome c oxidase subunit I), mitochondrial 16S and nuclear ITS region (internal transcribed spacer; spanning ITS1, 5.8S and ITS2). Two different analytical approaches were employed for each marker: Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2011), which identifies barcoding gaps in genetic distance matrixes, and the general mixed Yule coalescent model (GMYC; Pons et al., 2006), which identifies species thresholds based on changes in branching rates in a phylogenetic tree. The results are then evaluated to identify main lineages, which are consistently delimited across markers and analytical methods. These were then assessed under the different species concepts. The age of crown group *Hydra* (monophylum comprising all extant and 'internal' extinct species) and the timing of diversification events within *Hydra* were assessed by molecular clock analyses based on four molecular markers – COI, 16S, EF1α