Obstacles to molecular species identification in sea anemones (Hexacorallia: Actiniaria) with COI, a COI intron, and ITS II

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SHORT COMMUNICATION

Obstacles to molecular species identification in sea anemones (Hexacorallia: Actiniaria) with COI, a COI intron, and ITS II

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Abstract DNA barcoding has been successfully applied to a very large number of taxa, but remains problematic for basal diploblasts, and debates about suitable molecular markers are ongoing. Sea anemones (Anthozoa: Hexacorallia: Actiniaria) populate most any marine environment and often play an irreplaceable role as hosts to other animals. Three genetic markers were tested to assess their utility for molecular species identification in members of the Actiniaria, namely the cytochrome oxidase subunit I (COI), a COI Intron with a Homing Endonuclease Gene (HEG), and the Internal Transcribed Spacer II (ITS II). Both the power of COI and the COI Intron to distinguish species is limited by events of very low inter-specific sequence differences and not by high intra-specific diversity. This finding implies that more comprehensive taxon sampling will not resolve this problem and other markers need to be investigated in several families. Results should discourage the use of ITS II as alternative to COI for barcoding in Actiniarians, since it shows similar limitations to COI.

Keywords Anthozoa · Tropical sea · DNA barcoding · Nuclear intron · HEG

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Introduction

DNA barcoding is an international effort to record and catalogue species-specific DNA sequence data (barcodes), by which unknown specimens can be identified, new or cryptic species discovered (e.g., Hebert et al. 2003a, b, 2004; Hajbabaei et al. 2006), and species identities confirmed (Clare et al. 2007; Hebert et al. 2004; Moritz and Cicero 2004). Anthozoans (corals, sea anemones and their kin) present a challenge for barcoding because the 5′ segment of the mitochondrial cytochrome subunit I gene (COI), which is consensually applied for barcoding (Ward et al. 2005; Hajbabaei et al. 2006), has been found to be highly conserved (e.g., Flot et al. 2013) and a clear barcoding gap is absent in many genera due to low interspecific variability (Shearer et al. 2002). However, Keshavmurthy et al. (2013) were able to identify four deeply divergent clades (species) of the coral *Stylophora pistillata* within its range with COI. This emphasizes the value of barcoding in groups where taxonomically defining characteristics are variable and/or inconsistent with genetic units, such as is in the anthozoa (e.g., Flot et al. 2008).

Research focusing on anthozoan barcoding has dealt almost exclusively with corals, taking little notice of other members of the group (Shearer et al. 2002; Hebert et al. 2003b; Shearer and Coffroth 2006, 2008; Oliverio et al. 2009). We here test the barcoding utility of a partial COI gene fragment in sea anemones and two additional markers that indicate potential for species identification: the highly polymorph nuclear Internal Transcribed Spacer II (Flot et al. 2013; Oliverio et al. 2009) and a Homing Endonuclease Gene (HEG) located within a self-splicing group I Intron within COI (Goddard and Burt 1999; Goddard et al. 2006). When present, this HEG is unique among metazoans and its invasion cycle may be sufficiently slow to provide potential for species delineation, without providing a host specific phylogenetic signal (Goddard et al. 2006). Sequences for three species of giant tropical sea

anemones, *Heteractis magnifica* (Quoi and Gaimard 1833), *Heteractis crispa* (Ehrenberg 1834), and *Entacmaea quadricolor* (Rüppel and Leuckart 1828) were generated and aligned with all available actiniarian GenBank sequences.

Materials and methods

All sea anemones for this study were collected and identified by M. Kochzius (one of the authors) and Janne Timm (Bremen University, Germany) from a total of nine locations in the Indo-Malay Archipelago, the South China Sea, and Okinawa. Samples from Japan, Borneo, and the Philippines where collected by J. Timm during workshops offered at local institutions and under their supervision and with their consent. Tentacle clippings were stored in 96 % EtOH. DNA was extracted using the CTAB extraction method, altered only by an additional Proteinase K digestion step for a minimum of 24 h at 55 °C. DNA fragments were amplified using primers and annealing temperatures listed in Table 1. PCR products were purified using Peqlab cycle pure spin columns (Peqlab, Erlangen) and subjected to a cycle PCR (Big Dye terminator Cycle Sequencing Kit (ver. 3.1; Applied Bioscience) with forward and reverse primers. The cycle PCR products were purified via ethanol precipitation. Sequencing was carried out on either an ABI Prism 310 or 3100 automated sequencer (Applied Biosystems, Weiterstadt).

The resulting forward and reverse sequences were aligned and edited in SeqMan (ver. 4.0.5, DNASTAR) and a total sequence alignment was achieved with the ClustalW algorithm (Thompson et al. 1994), as implemented in the software BioEdit (ver. 7.0.9.0) (Hall 1999) for COI and COI Intron sequences. ITS II sequences were aligned using MAFFT 7 (online version; Katoh and Standley 2013). Poorly aligned positions and divergent regions were removed with GBlocks 0.91b (Castresana 2000; Talavera and Castresana 2007) using the most relaxed criteria. Sequence divergences were calculated using the Kimura two-parameter (K2P) model of base substitution (Kimura 1980) for COI and simple pairwise differences for the COI Intron dataset. Maximum Parsimony (MP) and Neighbour-joining (NJ) (Saitou and Nei 1987) trees including bootstrap analysis (1000 replications) (Nei and Kumar 2000) were performed using MEGA4 (Kumar et al. 2004), as were the calculations of intra- and inter-specific genetic divergence (K2P genetic distances). Maximum Likelihood (ML) (1000 bootstraps) was used to construct the ITS II tree applying a GTR model of evolution with a 0.04 fixed proportion of invariable sites, five substitution rate categories and a Gamma shape of 1.27 in PhyML (online version, Guindon et al. 2010). Model selection for the ML run in PhyML was determined with MEGA 6, as were inter- and intraspecific sequence divergence, using the ML algorithms and the complete deletion option for alignment gaps. Tajima's Relative Rate tests was also carried out in MEGA 6, by comparing sequence pairs from all available species and using the *Zoanthus praelongus* sequence as an outgroup.

Results and discussion

COI

The COI alignment (462 bp length) contained 91 sequences from 12 species, eight genera and four families (Table 2). The large majority of sequences stemmed from this study, since the number of actiniarian COI sequences in GenBank is extremely limited and multiple sequences to a species are rare. Species level resolution, i.e., adequate grouping of conspecies and divergence between congenerics, using the COI gene was partially unsuccessful in the Actiniidae, as species within the genus *Urticina*, as well as species of genera *Urticinopsis* and *Entacmaea* could not be delineated (Table S1, Fig. S1, supplementary materials). Overall, 16 % of all inter-specific comparisons show no or minimal divergence (d=0.00-0.01), so that efforts to collect additional information on intraspecific variability are unnecessary in the context of a single marker approach (Fig. 1). The ability of COI to delineate species is limited by a lack of inter-specific divergence, an obstacle that cannot be overcome by more comprehensive taxon sampling and has been found in other anthozoan orders (scleractinian corals, Shearer and Coffroth 2008).

Contrary to patterns seen in higher metazoans, substitution rates in the mtDNA of cnidarians appeared to be much slower than in the nuclear DNA (Shearer et al. 2002). The slow mitochondrial sequence evolution found here corroborates

Table 1 PCR primers used to amplify COI, COI Intron, and ITS I-5.8S-ITS II

Marker	T_a(C°)	Forward Primer	Reverse Primer
COI intron	62	5′-CTCGCTATATGCTGGAAARACCC-3′	5′-CAATAAGCGAAGCGTTTTCCA GCC-3′
COI	51	5′-GGT ATG ATA GGC ACA GCT-3′	5′-GAAAGTTGTATTAAARTTCCTATCTG-3′
ITS I&5.8S&ITS II	56	5′-GAG GAA GTA AAA GTC GTA AC-3′	5′-GGT CAA GAT GGA AAG ATA G-3′

Table 2 Family designations of species, number and source of sequences that were used for all three marker analyses, COI, COI Intron, and ITS II

Species	Family	COI [1]	COI Intron [2]	ITS II [3]	Accession Number
Actinia equina	Actiniidae	–	1	1	[2] DQ831335; [3] DQ831298
Actinia fragacea	Actiniidae	–	1	–	[2] DQ831334
Actinia bermudensis	Actiniidae	–	–	1	3 JN118562
Anemonia sp.	Actiniidae	1	–	–	[1] AB441274
Anemonia viridis	Actiniidae	–	1	–	[2] DQ831333
Anthopleura balii	Actiniidae	–	–	1	[3] DQ831299
Anthopleura elegantissima	Actiniidae	2	–	–	[1] GU443180, AF480931
Aulactinia incubans	Actiniidae	–	–	3	[3] EF026587-EF026589
Aulactinia marplatensis	Actiniidae	–	–	6	[3] EF026592, EF026594, EF026595, EF026597, EF026601, EF026602
Aulactinia reynaudi	Actiniidae	–	–	5	[3] EF026593, EF026596, EF026598-EF026600
Aulactinia verrucosa	Actiniidae	–	–	2	[3] EF026590, EF026591
Aulactinia stella	Actiniidae	–	–	8	3 JQ412857-JQ412860, JQ844113-JQ844116
Bunodosoma caissarum	Actiniidae	–	–	3	3 JN118559, JN118560, JN118566
Bunodosoma cangicum	Actiniidae	–	–	4	3 JN118561, [3]JN118567-JN118569
Bunodosoma granuliferum	Actiniidae	–	–	1	3 JN118565
Bunodosoma sp.	Actiniidae	–	–	1	3 JN118557, JN118563
Condylactis sp.	Actiniidae	–	–	1	3 AB441419
Entacmaea quadricolor	Actiniidae	24	4	–	**[1] JQ839204-JQ839227; [2] JQ918745-JQ918748**
Phymactis papillosa	Actiniidae	–	–	1	3 JN118564
Urticinopsis antarctica	Actiniidae	1	–	–	[1] AJ830011
Urticina columbiana	Actiniidae	1	–	–	[1] UCU91613
Urticina crassicornis	Actiniidae	1	–	1	[1] UCU91612, [3] JQ844117
Urticina lofotensis	Actiniidae	1	–	–	[1] U91614
Urticina felina	Actiniidae	1	–	–	[1] UFU91610
Aiptasia mutabilis	Aiptasiidae	–	–	1	[3] DQ831297
Aiptasia sp.	Aiptasiidae	–	1	–	[2] DQ831341
Megalactis sp.	Actinodendronidae	–	1	–	[2] DQ831342
Edwardsiidae sp.	Edwardsiidae	–	–	8	[3] EU418268-EU418274, GQ464903
Nematostella vectensis	Edwardsiidae	2	–	–	[1] DQ538492, DQ538493
Adamsia carciniopados	Hormathiidae	–	1	1	[2] DQ831340; [3] DQ831304
Calliactis parasitica	Hormathiidae	–	1	6	[2] DQ831339, [3] DQ831303, FM161930, HQ156453-HQ156456
Calliactis polypus	Hormathiidae	–	–	165	3 HQ156276-HQ156440
Calliactis tricolor	Hormathiidae	–	–	12	3 HQ156441-HQ156452
Metridium senile	Metridiidae	3	3	1	[1] AF00023, U36783, NC000933; [2] NC000933, U36783, AF000023; [3] DQ831306
Actinothoe sphyrodeta	Sagartiidae	–	1	1	[2] DQ831338; [3] DQ831302
Cereus pedunculatus	Sagartiidae	–	1	1	[2] DQ831336; [3] DQ831300
Sagartia elegans	Sagartiidae	–	1	1	[2] DQ831337; [3] DQ831301
Sagartia troglodytes	Sagartiidae	–	–	1	3 FM161931
Heteractis crispa	Stichodactylidae	27	3	–	**[1] JQ839177-JQ839203; [2] JQ918749-JQ918751**
Heteractis magnifica	Stichodactylidae	27	63	26	**[1] JQ839150-JQ839176; [2] JQ918688- JQ918744; [3] JQ918752 JQ918766**, AF050201-AF050211
Heterodactyla sp.	Thalassianthidae	–	–	1	[3] DQ831305

Sequences by the authors are in bold

findings from other anthozoan orders, and supports the hypothesis of an ancestral slow substitution state, rather than this being a secondarily acquired feature in the anthozoa (Shearer et al. 2002; Huang et al. 2008).

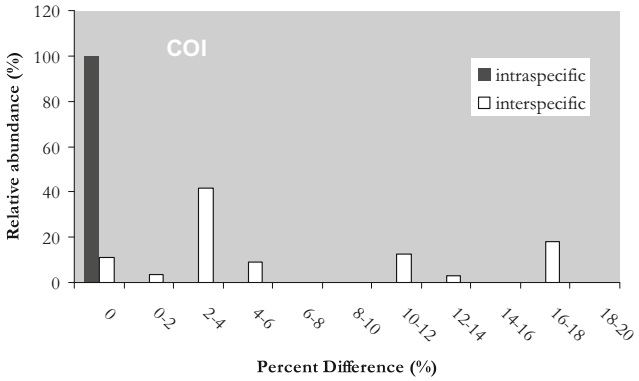


Fig. 1 Bar chart showing the proportion of pairwise distance comparisons of the COI gene for each range of sequence divergence (K2P)

COI intron

The COI Intron alignment (590 bp length) contained 84 sequences from 14 species in 12 genera embedded in seven families (Table 2). The vast majority of sequences in this alignment stemmed from *H. magnifica*, since actiniarian GenBank sequences are limited, and multiple sequences/species even less than for COI. The use of the COI Intron failed species delineation in at least three families: the Actiniidae, Hormathiidae, and Sagartiidae (Table S2, Fig S2 supplementary materials).

Where data were available, intraspecific variability was marginally higher (though still less than 1 %, Table S2), indicative of faster rates of evolution for the HEG fragment. Apart from the three instances of delineation failure, which were due to a lack of between species divergence, the COI intron produced a higher inter-specific divergence than the previous marker (COI intron: max 38 %, mean=21 %), with two thirds of all comparisons falling above the maximum divergence seen with COI (COI: max. 18 %, mean= 7 %)(Fig. 2, Table S2). As seen in COI, the range of inter-specific divergence (0.2 – 38 %) overlaps with the intra-specific variability (0 – 0.3 %) in a few instances, preventing a clear barcoding gap from forming (Fig. 2). Nevertheless, this unconventional marker may yet hold barcoding potential in some families, since intra-specific variation is very low in the species tested, while the inter-specific divergence is markedly higher. The phylogenetic signal seen in the NJ-Tree (Fig. S2, supplementary materials) should not be interpreted as such, as it may well reflect the infection pathway of the HEG in Actiniaria and not relationships among sea anemones. Most problematic appears to be the Actiniidae, as here some species cannot be discriminated using either COI or the COI Intron.

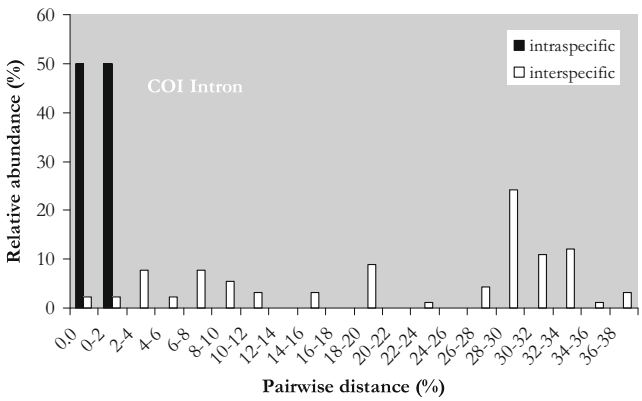


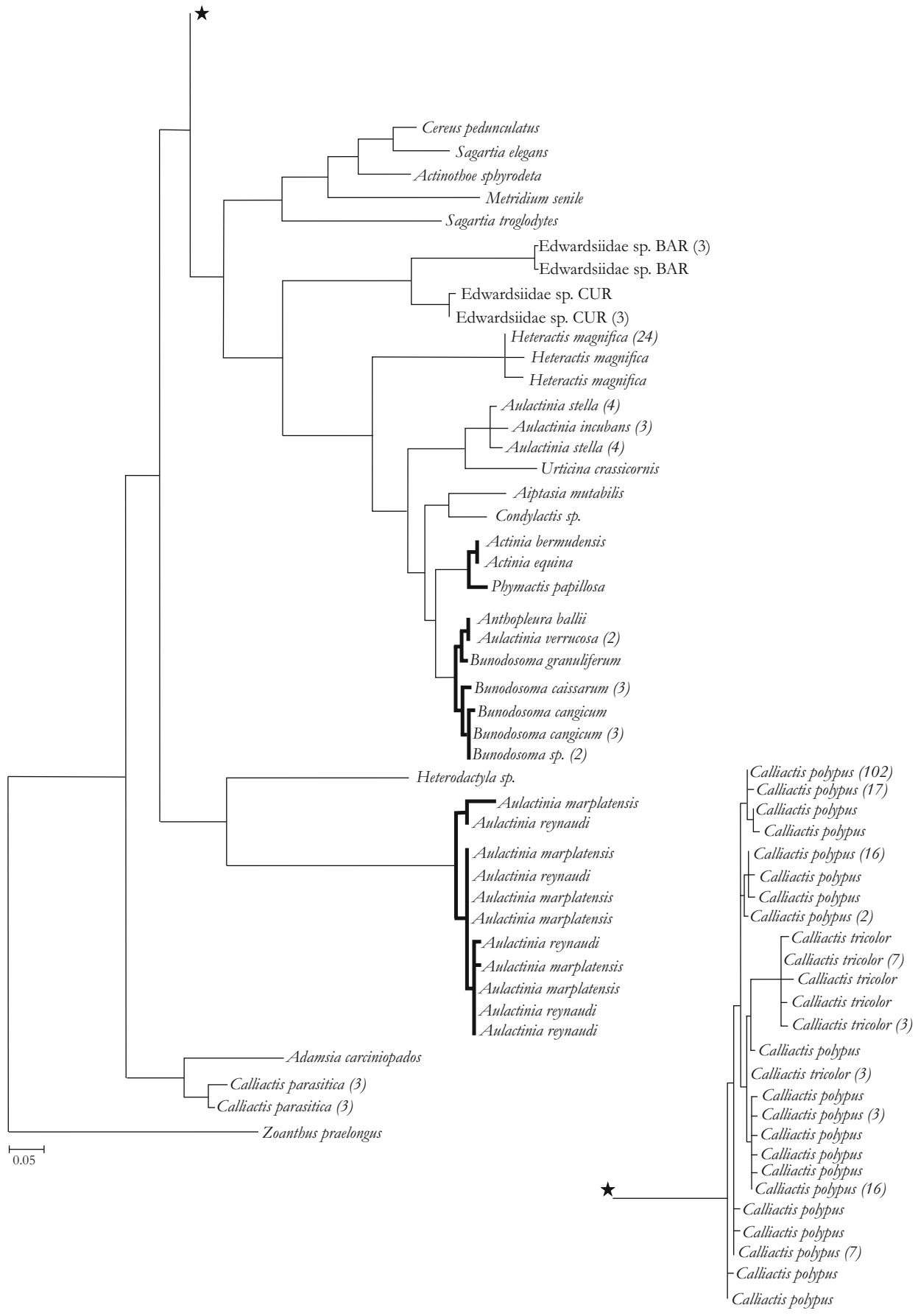
Fig. 2 Bar chart showing the proportion of pairwise comparisons of the COI Intron for each range of sequence divergence (pairwise differences)

ITS II

ITS species divergence rates from 2–11 % have been reported in various coral genera (Medina et al. 1999; Diekmann et al. 2001; Hunter et al. 1997) and as high as 45 % in Zoantharia (Anthozoa: Hexacorallia) (Reimer et al. 2007). Based on these findings, the ITS II marker was assessed here as an actiniarian barcoding marker.

The ITS II alignment contained 264 sequences (171 bp) from 28 species representing 17 genera from seven families (Table 2). The majority of sequences in this alignment stem from the public database and only sequences for *Heteractis magnifica* were contributed by the authors. Similar to the two previously discussed markers, the ability of ITS II to delineate species is limited by the lack of interspecific sequence divergence, which ranges between 0.00-65.5 %, overlapping in 13 instances with intraspecific sequence divergence (0.00-2.6 %) (Fig. 3, Table S3). These events of overlap were restricted to the family Actiniidae and included delineation failure within and between different genera. However, when ITS II was used, *Adamsia carcinopados* and *Calliactis parasitica* (within the Hormathiidae), as well as *Cereus pedunculatus* and *Actinothoe spyrodeta* (within the Sagartiidae) were well separated, which was not possible with the COI intron. A concatenated alignment of both markers, in addition to the information provided through the absence/presence of the intronic region may prove useful, though problems within the Actininiidae may still persist. Relative rate tests (Tajima 1993) with the available ITS II sequences indicated that members of the genus *Calliactis* may be evolving at a faster rate than other taxa tested here (significant χ^2=3.84-11.84, mean χ^2=5.61). This indicates that there are differences in the genetic differentiation of congenerics that would have to be investigated thoroughly before this marker could be used with any reliability.

Fig 3 ML tree with all available actinian ITS II sequences, both from this ▶ study and the public databases (171) positions). Bootstrap values below 50 are not shown. Branches marked in bold denote nodes where species level resolution could not be achieved



Conclusion

Though the data pool for this study is small, it represents the largest study on COI divergence in Actiniarians so far. Concatenation of COI Intron sequences and ITS II for an alignment based on both markers, might prove useful, as the advantages of each might produce a useful marker system in some families. The absence of an intron can also serve as an informative character. Currently the dearth of data available for this taxon does not allow such a step.

The Actiniaria is a taxonomically very challenging group, which would benefit immensely from a reliable barcoding system. In turn, the performance of the markers is gauged on how well they recover taxonomic categories, which may themselves be flawed or under discussion. The results from this study imply that the goal of finding a genetic marker applicable to the whole of the Actiniaria may prove futile, though for some families the mitochondrial and nuclear markers tested provide sufficient resolution. This should be further explored to include more comprehensive taxon sampling. For problematic groups, where the interspecific genetic variability clearly impedes species delineation, efforts should rather focus on exploring other markers or supplemental ID systems (Concepcion et al. 2008; Huang et al. 2008; Sinniger et al. 2008).

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