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Morphotypes of the common beadlet anemone *Actinia equina* (L.) are genetically distinct

Running head: mtDNA of *Actinia equina*

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Abstract

Anemones of the genus Actinia are ecologically important and familiar organisms on many rocky shores. However, this genus is taxonomically problematical and prior evidence suggests that the North Atlantic beadlet anemone, Actinia equina, may actually consist of a number of cryptic species. Previous genetic work has been largely limited to allozyme electrophoresis and there remains a dearth of genetic resources with which to study this genus. Mitochondrial DNA sequencing may help to clarify the taxonomy of Actinia. Here, the complete mitochondrial genome of the beadlet anemone Actinia equina (Cnidaria: Anthozoa: Actinaria: Actiniidae) is shown to be 20,690bp in length and to contain the standard complement of Cnidarian features including 13 protein coding genes, two rRNA genes, two tRNAs and two Group I introns, one with an in-frame truncated homing endonuclease gene open reading frame. However, amplification and sequencing of the standard mtDNA barcoding region of the cytochrome oxidase 1 gene revealed only two haplotypes, differing by a single base pair, in widely geographically separated A. equina and its congener A. prasina. COI barcoding shows that whilst A. equina and A. prasina share the common mtDNA haplotype, haplotype frequency differed significantly between A. equina with red/orange pedal discs and those with green pedal discs, consistent with the hypothesis that these morphotypes represent incipient species.
Introduction:

The Cnidarian genus *Actinia* (Cnidaria: Anthozoa: Actinaria: Actiniidae) is notably diverse, phenotypically variable and has had a fluid taxonomical history (Perrin et al., 1999). The beadlet anemone, *Actinia equina* is a common organism of North Atlantic rocky shores but has a reported range from the Kola Peninsula of northern Russia to the coast of South Africa (Manuel, 1988; Stephenson, 1935). Additionally, populations of *Actinia* in the Adriatic, Mediterranean and Black Sea (Schama et al., 2005; Schmidt, 1971) have been ascribed to *A. equina*, as have animals from the coasts of Japan (Honma et al., 2005; Yanagi et al., 1999), Korea (Song, 1984) and Hong Kong (Morton and Morton, 1983). Allozyme electrophoresis studies have suggested that *Actinia* from some geographically distinct populations represent separate species, now labelled *A. schmidtii* (Mediterranean), *A. cari* (Mediterranean), *A. sali* (Cape Verde), *A. nigropuncta* (Madeira) and an, as yet, unnamed form from South Africa (Monteiro et al., 1997; Perrin et al., 1999; Schama et al., 2005). Whether other populations of animals currently labelled as *A. equina* are misidentified cryptic species, or truly represent extensions to the known geographic range of this species, has not yet been assessed. Even within British populations of *Actinia equina*, the tremendous diversity in colour of *Actinia* individuals has led to taxonomic confusion. Allozyme electrophoresis studies have demonstrated the specific status of the strawberry anemone *A. fragacea* (Carter and Thorpe, 1981) and suggested that the green-columned form is a separate species from *A. equina*, now labelled *A. prasina* (Sole-Cava and Thorpe, 1987) although see Schama et al. (2005), but additional cryptic species perhaps also exist, particularly those morphotypes that differ in the colour of the pedal disc (grey/green versus red/pink/orange). These morphotypes show clear differences in intertidal distribution (red/pink morphs are found higher up the shore with green/grey morphs lower down the intertidal zone) and have been shown to present diagnostic allozyme genotypes at both malate dehydrogenase and hexokinase loci (Quicke and Brace, 1984; Quicke et al., 1983). In addition, they exhibit significant differences in aggression, nematocyst morphology, adhesion, and settlement patterns (Brace and Reynolds, 1989; Collins et al., 2017; Perrin et al., 1999; Quicke and Brace, 1984;
Quicke et al., 1983; Quicke et al., 1985; Watts and Thorpe, 1998). *Actinia* remains a key and common ecological species within the intertidal zone (Collins et al., 2017; Perrin et al., 1999; Schama et al., 2005) and hence accurate understanding of its taxonomy and the ability to identify species is important for understanding the functional ecology of this environment.

Due to the taxonomic confusion surrounding this genus and the difficulties in applying morphological taxonomy to such soft-bodied animals, the study of mitochondrial DNA may aid in resolution of this problematical taxon. Whilst there is an increasing number of complete mitochondrial genomes from the phylum e.g. (Beagley et al., 1998; Chi et al., 2018; Foxe et al., 2016; Zhang and Zhu, 2017), none is currently available from the genus *Actinia*. Application of mitochondrial DNA barcoding (Ratnasingham and Hebert, 2007) also seems a promising tool. However, the evolutionary rate of mtDNA in Cnidaria is considered to be low (Huang et al., 2008; Shearer et al., 2002) potentially presenting difficulties for the application of this methodology. DNA barcoding relies on the use of standard primers which for metazoans are typically those of Folmer et al. (1994) which target the cytochrome oxidase subunit I gene. However, the utility of COI, and whether other regions of the mtDNA molecule harbour more variation, remains untested.

As part of a study to generate a full reference genome for *A. equina* to facilitate investigation of the genomic basis of differentiation among beadlet anemone morphotypes, the complete mitochondrial genome of *Actinia equina* is reported here and the utility of mitochondrial DNA barcoding for studying intraspecific variation of British populations assessed.

**Materials and Methods:**

A single specimen of *Actinia equina* (with a red column and red pedal disc) was collected from Rhosneigr, Anglesey, North Wales, UK and kept in artificial seawater at 8°C for two weeks to allow it to purge of any food which may have contaminated extracted DNA. Prior to DNA extraction, the animal was inspected to ensure that there were no intra-gastrovascular cavity brooded offspring which may have introduced additional haplotypes into the extracted DNA (although evidence to
date suggests these would be clonal (Pereira et al., 2017)). It was then minced with a scalpel and
ground under liquid nitrogen. The resultant powder was added to 20ml 80 mM EDTA (pH 8.0), 100
mM Tris-HCl (pH 8.0), 0.5% SDS, 100 µg/mL proteinase K, and 40µl RNaseA (100mg/ml) and
incubated at 60°C for 3 hours. Genomic DNA was isolated from this solution by salt-chloroform
extraction (Müllenbach et al., 1989), precipitated with 0.6 volumes of isopropanol, and dissolved in
water. Extracted DNA was further purified using a Qiagen Genomic Tip 20/G following the
manufacturer’s instructions and precipitated a second time with 0.6 volumes of isopropanol.

20kb-insert PacBio sequencing libraries were produced and sequenced on 5 SMRT cells on a Pacific
Biosciences Sequel (Pacific Biosciences, Menlo Park, CA, USA) at the Centre for Genomic Research,
University of Liverpool.

Sequencing produced 3,507,426 ‘polymerase reads’ (single reads that can cover the same insert
multiple times) that were split into a total of 4,936,001 subreads (full or partial passes of the same
insert). Of these subreads, 487,629 were longer than 20 kb and 1,409,598 longer than 10 kb.

All subreads were assembled using CANU v1.7 (Koren et al., 2017) with default parameters for
PacBio data. To identify mitochondrial DNA, all assembled contigs were used to make a BLAST
database using BLAST+ v2.2.28 (Camacho et al., 2009). This was queried using BLASTn with two
published A. equina mitochondrial genes: a partial cytochrome B-like gene (cytB; GenBank accession
DQ683369.1) and a sequence containing the cytochrome oxidase subunit I gene (COI) and homing
endonuclease gene (HEG) (GenBank accession DQ831335.1). To check for errors in the final
mitochondrial genome sequence, all subreads were aligned to the sequence using bwa v0.7.12-
r1039 (Li and Durbin, 2009), using the bwa-mem algorithm with default parameters.

Initial annotation of mitochondrial genome features used MITOS (Bernt et al., 2013) with manual
annotation conducted to finalise gene models. tRNA genes identified by MITOS were further
investigated using tRNAscan (Lowe and Chan, 2016). Gene order was depicted using MTVIZ
(http://pacosy.informatik.uni-leipzig.de/mtviz) and G/C content depicted with CGView (Stothard and
Wishart, 2005). Sequence divergence between the mitochondrial genomes of A. equina and A. viridis
(accession number KY860669) was estimated for a 500 bp sliding window, moving in steps of 25 bp across the mitochondrial genome, using DNAsp v6 (Rozas et al., 2017).

**DNA barcoding**

*A. equina* (*N* = 43) and *A. prasina* (*N* = 3) were collected from a range of locations around the UK and the Isle of Man (Table 1) leaving at least 2m between samples from the same shore to avoid sampling clones. DNA was extracted from tentacle samples using a GeneJet Genomic DNA extraction kit (ThermoFisher, UK). Partial COI fragments were amplified using the primers of Folmer et al. (1994) at 0.2µM using 1x GoTaq HS mastermix (Promega) with cycling conditions of 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 1.5 mins, followed by a final extension step at 72°C for seven minutes. Alternative primers were used to amplify a 559bp section of the intergenic region between COIII and COI (5′-cgggttttcatgtctgcat-3′ and 5′-ccagggcagataactccaa-3′) and a 598bp region between COI and ND4 (5′-cccgccttgttcatact-3′ and 5′-caccataattgccagccaa-3′), designed using Primer3 (Untergasser et al., 2012). PCRs were undertaken using 1x GoTaq HS mastermix with cycling conditions of 5 min at 94°C followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and a final extension time of 72°C for 5 min with PCR products purified using a GeneJet PCR purification kit and sequenced by GATC Biotech, Konstanz, Germany. Sequences were aligned using ClustalW (Larkin et al., 2007).

**Results**

Both BLASTn searches (with default parameters) returned a single contig (41,948 bp) but each query sequence aligned to the contig more than once (which can occur when a circular sequence is assembled). The first position of the cytB sequence (positions 6,928 and 27,619 in tig00010800) was used to trim the contig into a full-length putative mitochondrial genome of 20,690 bp which was then reoriented with the first base of ND5 at base 1. 8,996 subreads could be aligned back to this sequence with a median coverage depth of 2,044.
This genome contains the standard 13 protein coding genes typical of the mitochondrial DNA, two ribosomal subunits (small and large) and, in line with other Cnidarian genomes, just two transfer RNAs (tryptophan and methionine) (Figure 1). In addition, an open reading frame (OrfA) potentially encoding a 645 amino acid protein was located between COII and ND4. As for other Cnidaria, type I introns were found within NDS (with ND1 and ND3 within the intron) and COI (containing a truncated homing endonuclease gene (HEG) ORF). The HEG of A. equina has an ORF of 343 amino acids, slightly longer than that of A. viridis (339 amino acids). This is due to a single base insertion relative to the ORF of A. viridis, with a 6 base poly-A tract at positions 17,646-17,651 at which A. viridis has a 5bp poly-A tract. The insertion was confirmed by Sanger sequencing of PCR products.

The region between ND3 and NDS displayed a partial ORF with similarities to NDS and it appears likely that this is pseudogenic. The mitochondrial genome was 61.02% A/T although this varied across the genome (Figure 1). The complete sequence has been deposited in Genbank with accession number MH545699.

Sequencing of PCR amplicons of the standard barcoding region of COI using the primers of Folmer et al. (1994) showed that beadlet anemone samples collected from England, Scotland, Wales and the Isle of Man displayed only two haplotypes (Table 1, Figure 2) which differed by just 1bp, a G-A synonymous transition at position 16,941 (Haplotype A accession number: MH636618; Haplotype B accession number: MH636619). Whilst variation was low, haplotype A was only ever seen in A. equina with red or orange pedal discs and never in animals with green pedal discs or in A. prasina and this association between haplotype and A. equina colour morph was significant ($\chi^2 = 16.43; P < 0.0001$).

Sliding window analysis comparing the complete A. equina mitochondrial genome to the complete mitochondrial genome of Anemonia viridis revealed low sequence divergence, with the exception of the intergenic regions separating COII and COI, and between COI and ND4 (Figure 3). Using primers designed to amplify these regions of maximum interspecific divergence then, as for COI, intraspecific variation among A. equina in these regions was low with a single haplotype in the intergenic
fragment between COI and ND4 (accession number MH686230) and just two haplotypes (accession numbers MH686231-MH686232), differing by two base pairs, for the fragment between COIII and COI (an A-T transversion at position 16,432 resulting in an I-L amino acid change in COIII and an intergenic C-T transition at 16,517).

Discussion

The mitochondrial genome of Actinia equina has the standard mitochondrial gene order typical of Cnidaria (Beagley et al., 1998; Chi et al., 2018; Fook et al., 2016) although at 20,690bp the mtDNA is slightly longer than that of other Cnidaria, largely due to an increased intergenic region between ND3 and the second exon of ND5. This region contains a partial pseudogene of ND5 as seen also in Bolocera tuediae (Emblem et al., 2014). As in A. viridis (Chi et al., 2018), the homing endonuclease gene of A. equina is fused in-frame with COI (Chi et al. 2018) but differs at the 3’ end due to an insertion in a poly-A tract resulting in a frameshift.

Whole mitochondrial DNA sequences have been used to study Cnidarian phylogeny e.g. (Emblem et al., 2014; Fook et al., 2016) but individual mitochondrial genes are often used in studies of phylogenetics and population studies with the cytochrome oxidase subunit I the most common target. Here, sequencing of COI from A. equina samples collected from Scotland, Wales, England and the Isle of Man, and including individuals of A. prasina deemed a separate species based on allozyme evidence (Sole-Cava and Thorpe, 1987), detected only two haplotypes differing by just one base pair. A. prasina shared the same haplotype as A. equina with a green pedal disc. It seems therefore that the low variability of COI makes this gene of little use for population genetic studies. However, the fact that haplotype A was seen only in animals with red/orange pedal discs, and never in those with green pedal discs adds weight to the argument that these may represent incipient species (Collins et al., 2017; Perrin et al., 1999; Quicke et al., 1983). This low variability of mitochondrial DNA has been seen previously: Pereira et al. (2014), studying the mitochondrial 16S gene, found only two haplotypes among 77 anemones from the coast of Portugal. It is extremely surprising that in a
species considered largely to reproduce through budding, and in which little evidence of sexual reproduction has been found (Perrin et al., 1999), there is such a dearth of variability in the mitochondrial genome across large geographic scales. In addition, the lack of difference in sequence, or haplotype frequency, between *A. equina* and *A. prasina* is surprising. However, allosyme studies of genetic distance find levels appropriate for interspecific comparisons when *A. prasina* is compared to *A. equina* with a red pedal disc, but not when compared to samples with a grey pedal disc (Schama et al., 2005) further suggesting that what is currently regarded as *A. equina* encompasses at least two cryptic species. Other mitochondrial genes may be more variable and hence of more utility for population genetics/phylogenetics. Emblem et al. (2014) demonstrated in interspecific comparisons that of the protein coding complement of the mtDNA the HEG has the highest evolutionary rate. However, previous work showed no intraspecific variation in the HEG sequence when 95 individuals of the Anthozoan *Metridium senile* were compared (Goddard et al., 2006). Intergenic regions of the mtDNA which are likely less constrained by selection pressures may be of use and we show through comparison of sequence divergence between *A. equina* and *A. viridis* across the whole molecule that two intergenic regions of the mitochondrial genome have maximum divergence and hence hold promise as phylogenetic and population genetic markers. However, we show through sequencing of *A. equina* and *A. prasina* individuals that within these species there are few variable positions within these two regions, so they are of no more utility than the COI barcoding region. Taken together, this suggests that the low inherent mutation rate of this molecule make it uninformative for understanding the complex relationships within this genus or the population genetics of the inherent species, and that nuclear DNA studies will instead need to be conducted. Nevertheless, the significant difference in haplotype frequencies between red/orange and green pedal disc anemones does add to the growing evidence from aggression, nematocyst morphology, distribution and allosyme data (Collins et al., 2017; Perrin et al., 1999; Quicke et al., 1983) that these may be incipient species. The causes of speciation in intertidal organisms have been most intensively studied in the rough periwinkle *Littorina saxatilis* where ‘crab’ and ‘wave’
morphotypes appear to be selected by the action of predator pressure (from crabs) and wave action (Butlin et al., 2014) and the genomic regions under selection are now being revealed (Westram et al., 2018). Wave action appears also to be a factor in speciation in other organisms, e.g. kelp (Augyte et al., 2018). However, the intertidal zone is characterized by a range of strong divergent selective pressures, both biotic and abiotic and such extreme stresses (as those imposed on intertidal organisms) can be sufficient to promote speciation (Lexer and Fay, 2005). The fact that anemones with red/orange pedal discs are found higher up the intertidal zone than those with green pedal discs (Perrin et al., 1999; Quicke and Brace, 1984; Quicke et al., 1983), and that differences are present in adhesion strength and preference for substratum orientation, with red pedal disc forms preferring more vertical surfaces than the green pedal disc form (Quicke and Brace, 1984; Quicke et al., 1983), indicates that ecological factors may indeed be driving speciation. In other Cnidarians ecological specialization is a driver of speciation (González et al., 2018) and this may also be the case for Actinia. The precise nature of the selective pressures on anemone populations remain to be elucidated but the availability of genetic and genomic resources (Wilding and Weedall, unpublished) for this widely-studied species will greatly aid in our efforts to understand the extent and pattern of differentiation in this ecologically important animal.

Acknowledgments

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References

Phycologia 57, 100-112.


**Figure Legends:**

**Figure 1:** Gene order in *Actinia equina* mitochondrial DNA (20,690bp). Figure produced in MTVIZ (http://pacosy.informatik.uni-leipzig.de/mtviz). %AT is shown within the gene order diagram. Grey shaded areas indicate intergenic regions.

**Figure 2:** Collection sites for *Actinia equina* samples. For sample sizes refer to Table 1. The relative frequency of the two haplotypes (Haplotype A: black; Haplotype B: light grey) is depicted in each pie chart. R = red/orange pedal discs, G = green pedal discs, P = *A. prasina*.

**Figure 3:** Sliding window analysis of the alignment of *Actinia equina* and *Anemonia viridis* mitochondrial genomes. The line shows the value of nucleotide diversity (π) in a sliding window analysis of window size 500 bp with step size 25 bp with the value plotted at its mid-point. Genes are displayed as grey...
boxes below the x-axis. Genes with introns are labelled with *.

Positions of amplified PCR products:

a) COIII-COI b) Folmer and c) COI-ND4. Figure drawn in OGDRAW (Lohse et al., 2013).
Table 1: Haplotype distribution in geographic samples of *A. equina* and *A. prasina* from England, Wales, Scotland and the Isle of Man. Only two haplotypes are found, Haplotype A (accession MH636618) and Haplotype B (MH636619), differing by 1bp.

<table>
<thead>
<tr>
<th>Location</th>
<th>Haplotype A</th>
<th>Haplotype B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millport, Isle of Cumbrae, Scotland</td>
<td>Green 0, Orange 3, Red 1</td>
<td>Green 2, Orange 0, Red 1</td>
</tr>
<tr>
<td>Peel, Isle of Man</td>
<td>Green 0, Red 1</td>
<td>Green 1, Red 0</td>
</tr>
<tr>
<td>Niarbyl, Isle of Man</td>
<td>Red 0, A. prasina 1</td>
<td>Red 1, A. prasina 0</td>
</tr>
<tr>
<td>New Brighton, Wirral, England</td>
<td>Green 0, Red 0, A. prasina 2</td>
<td>Green 2, Red 4, A. prasina 0</td>
</tr>
<tr>
<td>Llandudno, North Wales</td>
<td>Green 0, Red 2</td>
<td>Green 2, Red 0</td>
</tr>
<tr>
<td>Holyhead, Anglesey, North Wales</td>
<td>Green 0, Red 2</td>
<td>Green 3, Red 0</td>
</tr>
<tr>
<td>Rhosneigr, Anglesey, North Wales</td>
<td>Green 0, Red 2</td>
<td>Green 2, Red 0</td>
</tr>
<tr>
<td>St Brides Bay, South Wales</td>
<td>Green 0, Red 2</td>
<td>Green 4, Red 2</td>
</tr>
<tr>
<td>Marloes, South Wales</td>
<td>Green 0, Red 3</td>
<td>Green 3, Red 0</td>
</tr>
</tbody>
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