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Aequorea taiwanensis n. sp. (Hydrozoa, Leptomedusae) and mtCOI sequence analysis for the genus *Aequorea*

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Abstract

Aequorea taiwanensis, a new hydrozoan species from the Taiwan Strait was described using morphological and molecular characteristics. Both morphological and mitochondrial cytochrome oxidase subunit I (mtCOI) data supported *A. taiwanensis* n. sp. as a valid species. Sequence divergence and genetic distance of *A. taiwanensis* n. sp., *A. papillata* and *A. conica* were analysed based on the mtCOI gene sequences. The mtCOI sequences from these three species of the genus *Aequorea* showed high variation frequency, with sequence divergences ranging from 9.10% to 11.9%, and pairwise genetic distances ranging from 0.097 to 0.130. MtCOI sequence analysis provided diagnostic molecular systematic characteristics for accurate identification and discrimination of the species of *Aequorea* or their populations, and will be used to resolve evolutionary relationships among them. It was suggested that 10%–20% pairwise mtCOI sequence differences indicated the species-level divergence among congeneric species in the Hydromedusae.

Key words *Aequorea*, new species *Aequorea taiwanensis* n. sp., mtCOI sequence analysis

1 Introduction

Although molecular systematics has been used to study high taxonomic level relationships among the Cnidaria (Collins et al., 2006; Collins and Daly, 2005; Collins, 2002; Bridge et al., 1995), neither the phylogenetic relationships between nor with the lower taxonomic, e.g., intrageneric levels have been clearly understood. These relationships are still the subject of controversies and debates, especially since the molecular systematics sometimes conflicts with the morphological systematics (Hemmrich et al., 2007; Govindarajan et al., 2006; Collins et al., 2004). Despite the pitfalls of molecular phylogenetics, we must remember that the correct identification of species in the Cnidaria is a major problem. A sufficient number of ambiguous morphological characteristics, the dearth of distinguishable and important morphological characteristics in many taxa, ontogenetic variation, and the morphological simplicity and plasticity make morphological homoplasy likely to be common in the Cnidaria. Moreover, these problems have been aggravated because of the technical limitations in observing, sampling and culturing Cnidaria species. Thus, evolutionary relationships and even species identification in the Cnidaria are difficult or impossible to assess accurately using traditional morphological methods.

The partial mtCOI sequence is easy to amplify and sequence, and has proved to be an efficient, useful even indispensable tool in many studies of geographic populations (Dawson, 2005a; Govindarajan et al., 2005) or closely related species (Dawson, 2005b; Holland and Dawson, 2004; Dawson and Jacobs, 2001) in the Cnidaria, where other characteristics, like morphological data, are limited or hard to interpret. Although molecular analyses brought an impressive perspective to research in the Cnidaria, morphological analyses still play an important role in taxonomy. For example, Dawson (2003) demonstrated that many cryptic species can be distinguished by means of a quantitative and objective investigation of morphological variations. Therefore, we suggest that integrated molecular and morphological analyses may offer the most robust approach to resolving many outstanding issues regarding the systematics of Cnidaria.

In this paper, we present a new Hydromedusae species which we assign to the genus *Aequorea* based on both morphological and mtCOI gene sequence analyses, and we confirm that (1) *A. taiwanensis* n. sp. is a valid species, (2) assess a method (mtCOI sequencing) as a diagnostic molecular systematic characteristic for accurate identification and discrimination within the genus *Aequorea* or their populations, and (3) resolve evolutionary relationships

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among species of the *Aequorea*.

2 Materials and methods

2.1 Taxon sampling

The specimens used in this study, *A. taiwanensis* n. sp., *A. papillata* and *A. conica*, were collect-

ed from the Taiwan Strait or Xiamen Harbor and were all mature medusae. A summary of the materials used in this study is provided in Table 1, with sample location, collection date, code and sample numbers given. *Aequorea* species were identified, photographed and then preserved in 90% ethanol.

Table 1 Sample location and sample numbers of three species of *Aequorea*

Species	Sample location	Sample date	Code	Sample numbers
<i>A. taiwanensis</i> n. sp.	Taiwan Strait	Jun 2006	A. tw	3
<i>A. papillata</i>	Taiwan Strait	Jul 2005	A. pap	5
<i>A. conica</i>	Xiamen Harbor	Jun 2006	A. con	3

2.2 DNA extraction, amplification and sequencing

The DNA genome was extracted from the ethanol-preserved tissues with phenol-chloroform-isoamyl alcohol (25:24:1). The resultant DNA was dissolved in TE solution and stored at -20°C in a dark environment.

Polymerase chain reaction (PCR) was performed using a PTC-220 thermocycler (MJ Research Co. USA) and mtCOI was amplified using universal COI primers (Folmer et al., 1994): LCO-1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO-2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). Amplification reactions were carried out in a total volume of 25 μL (10 \times buffer 2.5 μL , MgCl₂ 1.5 μL , dNTP 0.4 μL , LCO-1490 0.20 μL , HCO-2198 0.20 μL , *Taq* DNA polymerase 0.15 μL , genomic DNA 2.0 μL and double-distilled H₂O 18.05 μL) under the following temperature profile: 94 $^{\circ}\text{C}$ pre-denatured for 5 min, followed by 35 cycles (94 $^{\circ}\text{C}$ denatured for 40 s, 47 $^{\circ}\text{C}$ annealed for 50 s, and 72 $^{\circ}\text{C}$ extension for 80 s), finally fragments were elongated at 72 $^{\circ}\text{C}$ for 10 min, and the reaction was terminated by cooling to 4 $^{\circ}\text{C}$.

PCR products were electrophoresed in 1.0% TBE agarose gels, stained with ethidium bromide and visualized under UV light. Sequencing was completed in the Shanghai Biotasia Biotechnologies Co., Ltd.

2.3 DNA sequence alignment and phylogenetic analysis

The sequence data were aligned with CLUSTAL X1.81 (Thompson et al., 1997) and then corrected by eye. Pairwise sequence differences (PSD) were calculated in DNAMAN. Base composition and genetic distance were calculated using MEGA (Kumar et al., 2004). Phylogenetic relationships were reconstructed using the neighbor-joining (N-J) (Kimura two-parameter substitution model), the outgroup was *Eugymnanea inquilina* (Hydromedusae, Leptomedusae, Eirenilae, GenBank accession number AY789915),

and the bootstrap test was based on 1 000 replicates.

3 Results

3.1 *Aequorea taiwanensis* n. sp.

Type material: Holotype (AOB-HL251), paratype (AOB-HL252). Collected from the Taiwan Strait (21 $^{\circ}$ 40'–23 $^{\circ}$ 51' N, 116 $^{\circ}$ 47'–118 $^{\circ}$ 56' E) by the first author in June 2006. The type specimens are deposited in the Department of Oceanography, Xiamen University.

Eymology: This new species was named after its sample location, Taiwan Strait.

Diagnosis: Lens-shaped, usually 90–102 radial canals, gonads linear, extending along almost the whole length of radial canals. Tentacles with elongated conical bases narrowing suddenly distally, with a short adaxial excretory papillae. With 3–5 small marginal bulbs between marginal tentacles with adaxial excretory papillae. With one statocyst between successive radial canals.

Description: Usually 25 mm wide. Umbrella flatter than a hemisphere, lens-shaped, with solid jelly, jelly thicker in centre, thinning gradually and evenly towards umbrella margin; manubrium broad and large, about one-half diameter of umbrella, lateral walls very extensive, with transparent lines radiating from ends of radial canals to mouth-lips, mouth-lips elongated, slender-shaped, usually approximately as numerous as radial canals. Radial canals straight and narrow, usually 90–102 in number. Ring canal narrow; gonads linear, extending along almost the whole length of radial canals. With 12 marginal tentacles, with elongated conical bases narrowing suddenly distally, with a short adaxial excretory papillae and scattered black pigments. With 3–5 small marginal bulbs between marginal tentacles with adaxial excretory papillae, but without black pigments. With one statocyst between successive radial canals, each with 1–2 concretions, veil narrow (see Fig. 1).

This new species has a very broad manubrium

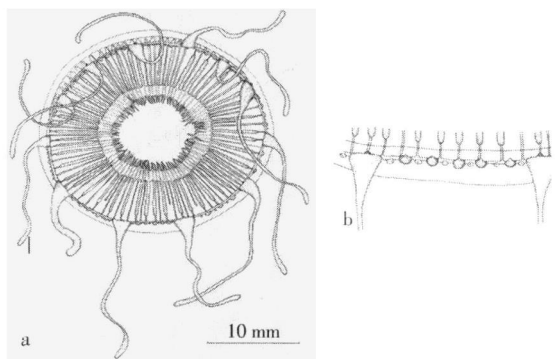


Fig 1 *Aequorea taiwanensis* sp nov. a Oral view and b bell margin

without papillae with many simple radial canals; bulbs with excretory papillae; subumbrella without rows of gelatinous papillae. So it belongs to the family Aequoreidae Eschscholtz, 1982, genus *Aequorea* Péron and Lesueur, 1810.

3.2 mtCOI gene sequences and base composition

Five hundred and thirty-eight base pairs of mtCOI gene fragments were sequenced and aligned for three individuals of *A. taiwanensis* n sp., five individuals of *A. papillata* and three individuals of *A. conica*. Sequences were logged onto the GenBank database with accession numbers EU 012498 to EU 012503, no other *Aequorea* mtCOI sequences in GenBank. Base composition of the mtCOI sequences of these three species were parallel (Table 2), with A + T obviously higher than G + C, and this result was consistent with other hydromedusae taxa (Hemmerich et al., 2007; Govindarajan et al., 2006; Väinölä and Oksavirta, 2001). The three sequences of *A. taiwanensis* n sp. were identical, the five sequences of *A. papillata* have two variable sites including three haplotypes, and the three sequences of *A. conica* have one variable site including two haplotypes. Of these sequences, 85 characters were variable, and these characters were all parsimony-informative sites including 43 transitions and 42 transversions.

Table 2 Base composition of mtCOI gene fragment sequences of three species of *Aequorea*

Species	A (%)	T (%)	G (%)	C (%)	A + T (%)	Total /bp
<i>A. taiwanensis</i>	39.6	26.8	15.1	18.6	66.4	538
<i>A. papillata</i>	39.0	26.3	15.3	19.4	65.3	538
<i>A. conica</i>	39.7	25.5	16.2	18.6	65.2	538

3.3 mtCOI sequence divergence and genetic distance

Mean percentage sequence divergence between the three *Aequorea* species varied from 9.10% to 11.9%; sequences of *A. papillata* and *A. conica* differed from each other by 11.3%—11.7%; and sequences of the new species *A. taiwanensis* and the above two *Aequorea* species differed respectively by 11.7%—11.9% and 9.10%—9.30% (Table 3). Pairwise Kimura two-parameter

genetic distance comparisons in the three species of *Aequorea* were between 0.097 and 0.130 (see Table 4). The maximum genetic distance observed, 0.130, was between *A. taiwanensis* and *A. papillata*, and a relatively high distance was also observed between *A. papillata* and *A. conica* (0.128). The minimum was found between *A. taiwanensis* and *A. conica* (0.097), and it seemed that the new species and *A. conica* had a closer relationship.

Table 3 Divergence percentage (%) of mtCOI gene fragments of three species of *Aequorea*

Species	1	2	3	4	5	6	7	8	9	10	11
1 <i>A. taiwanensis</i> 1											
2 <i>A. taiwanensis</i> 2	0.00										
3 <i>A. taiwanensis</i> 3	0.00	0.00									
4 <i>A. papillata</i> 1	11.7	11.7	11.7								
5 <i>A. papillata</i> 2	11.9	11.9	11.9	0.20							
6 <i>A. papillata</i> 3	11.7	11.7	11.7	0.00	0.20						
7 <i>A. papillata</i> 4	11.9	11.9	11.9	0.20	0.00	0.20					
8 <i>A. papillata</i> 5	11.7	11.7	11.7	0.20	0.40	0.20	0.40				
9 <i>A. conica</i> 1	9.10	9.10	9.10	11.5	11.7	11.5	11.7	11.7			
10 <i>A. conica</i> 2	9.30	9.30	9.30	11.3	11.5	11.3	11.5	11.5	0.002		
11 <i>A. conica</i> 3	9.30	9.30	9.30	11.3	11.5	11.3	11.5	11.5	0.002	0.000	

Table 4 Pairwise genetic distance between the three species of *Aequorea* (below diagonal) transitions plus transversions above diagonal standard error)

Species	1	2	3	4	5	6	7	8	9	10	11
1 <i>A. taiwanensis</i> 1		0.000	0.000	0.017	0.017	0.017	0.017	0.017	0.014	0.014	0.014
2 <i>A. taiwanensis</i> 2	0.00		0.000	0.017	0.017	0.017	0.017	0.017	0.014	0.014	0.014
3 <i>A. taiwanensis</i> 3	0.000	0.000		0.017	0.017	0.017	0.017	0.017	0.014	0.014	0.014
4 <i>A. papillata</i> 1	0.128	0.128	0.128		0.002	0.000	0.002	0.002	0.016	0.016	0.016
5 <i>A. papillata</i> 2	0.130	0.130	0.130	0.002		0.002	0.000	0.003	0.017	0.016	0.016
6 <i>A. papillata</i> 3	0.128	0.128	0.128	0.000	0.002		0.002	0.002	0.016	0.016	0.016
7 <i>A. papillata</i> 4	0.130	0.130	0.130	0.002	0.000	0.002		0.003	0.017	0.016	0.016
8 <i>A. papillata</i> 5	0.128	0.128	0.128	0.002	0.004	0.002	0.004		0.017	0.016	0.016
9 <i>A. conica</i> 1	0.097	0.097	0.097	0.126	0.128	0.126	0.128	0.128		0.002	0.002
10 <i>A. conica</i> 2	0.099	0.099	0.099	0.123	0.126	0.123	0.126	0.126	0.002		0.000
11 <i>A. conica</i> 3	0.099	0.099	0.099	0.123	0.126	0.123	0.126	0.126	0.002	0.000	

3.4 Phylogenetic analyses

The mCOI phylogram shown in Fig. 2 supported the placement of *A. taiwanensis* n. sp. within the genus

Aequorea. *A. taiwanensis* clustered as a sister species to *A. conica*, then combined with *A. papillata*, but they were strongly supported (> 95% bootstrap) to be reciprocally monophyletic.

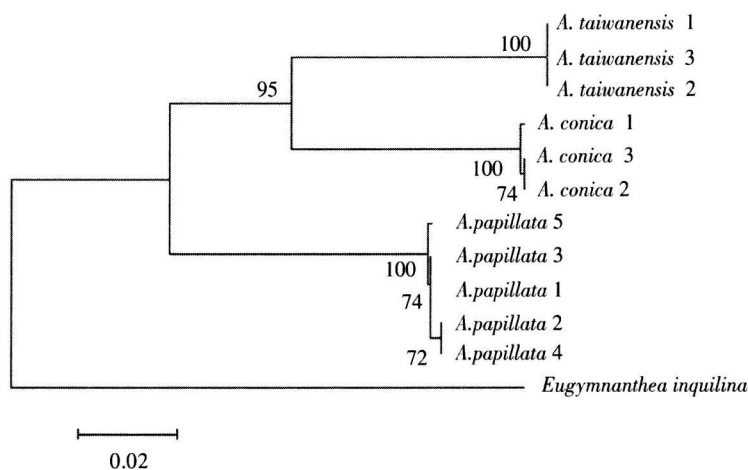


Fig. 2 N-J phylogenetic tree of three species of *Aequorea* based on the mCOI sequence. Bootstrap numbers are the result of the search with 1 000 replicates.

4 Discussion

In recent years, more and more studies have indicated that cryptic species are pervasive in the marine holoplankton. Chen (2006) suggested that all nominal species, especially those cosmopolitan species, have a large-scale range of distribution must be reexamined using the genetic approach. Additionally, Medusozoa taxonomists have to face the difficulties not faced by many other taxonomists, including the fact that morphological characteristics are limited in number and are likely to be highly labile. Species

of *Aequorea* are distributed from the neritic sea to the ocean, and their natural temporal and spatial patchiness means that morphological variation in the species of *Aequorea* is often overlooked, and this can make it difficult to identify differences between species. Preliminary molecular data (COI and ITS1) from *A. aequorea* [unpublished data, cited in Dawson (2003)] also indicate the relationship between cryptic species and fallible taxonomy.

Until recent years, the genus *Aequorea* contained only 18 valid species (Bouillon and Boero 2000). *A. taiwanensis* n. sp. can easily be distin-

guished from the others, but similar to *A. papillata* (Huang and Xu, 1994) (Table 5). The most striking difference between *A. taiwanensis* n. sp. and *A. papillata* is the form of tentacle bulb. This is elongated with a conical base narrowing suddenly distally in *A. taiwanensis* n. sp. but with lateral extensions and with the end obtuse and spherical in *A. papillata*. Knowlton (1993) suggests that sibling species are morphologically indistinguishable but pseudosibling

species are distinguishable once the appropriate characteristics are identified. For the new species *A. taiwanensis*, the appropriate characteristics used for the identification of *A. equorea* species are distinguishable. While the hypothesis is that the difference between these two species is the result of phenotypic plasticity, mtCOI sequence data unambiguously identify them as different species.

Table 5 Comparison of morphological characteristics among five species of *Aequorea*

	<i>A. taiwanensis</i> n. sp.	<i>A. aequorea</i>	<i>A. papillata</i>	<i>A. conica</i>	<i>A. australis</i>
Umbrella	lens shaped	saucer shaped	lens shaped	conical	lower than a hemisphere
Tentacles	12 tentacles with elongated conical bases narrowing suddenly distally with a short adaxial excretory papillae	at least half the length of radial canals; tentacle bulbs elongated conical excretory pores on short papillae	9–14 tentacles; tentacle bulbs with lateral extensions; the ends of which are obtuse spherical with adaxial excretory papillae	26–30 tentacles; no excretory papillae	16–40 tentacles; tentacle bulbs elongated conical with distinct excretory papillae
Radial canals	90–102	60–80, sometimes fewer or up to 160	64–81	16	16–50
Gonads	gonads along at most whole length of radial canals	gonads along at most whole length of radial canals	gonads along at most whole length of radial canals	gonads along proximal half of radial canals	gonads about half as long as radial canals
Statocysts	one statocyst between successive radial canals	5–10 statocysts between successive radial canals	6–14 statocysts between tentacles	about twice as many as tentacles	about the same number as tentacle bulbs
Marginal bulbs	3–5 marginal bulbs between tentacles with adaxial excretory papillae without black pigments	small bulbs few, scattered	5–14 marginal bulbs between tentacles with adaxial excretory papillae with black pigments	same number as tentacles	2–9 small bulbs between tentacles with distinct excretory papillae
Author	this paper	Kranp (1968)	Huang and Xu (1994)	Kranp (1968)	Kranp (1968)

Controversy persists as to how much molecular variation is needed in order to define species. Dawson and Jacobs (2001) proposed that 10%–20% sequence divergence in COI may be a suitable benchmark of distinct species for most of marine invertebrate taxa. However, no statistical data supporting this standard can be used to indicate the species-level divergence in the Hydromedusae. On the basis of our survey, we found that the pairwise COI sequence divergence observed among congeneric species in the Hydromedusae was commonly in the range of 10%–20%, the same as in Scyphomedusae species, although the lower limits in some species were less than 10% (see Table 6). Thus, we propose that a 10%–20% sequence difference could be taken as a benchmark of distinct species in the Hydromedusae, noting that the

observed 9.10%–11.9% divergence in *Aequorea* fell within the range sufficient for species recognition. These sequence differences are considerably higher than those seen between subspecies of *Mastigias* from marine lakes, Palau, Micronesia (< 3% in COI; Dawson, 2005c); or between different geographic populations of *Catostylus mosaius* (mean 3.61% in COI; Dawson, 2005a); or *Obelia geniculata* (< 6% in COI; Govindarajan et al., 2005). Additionally, the reciprocal monophyly observed in our phylogeny is consistent with the existence of two different species according to the phylogenetic species concept, and seems to be well supported by the high bootstrap values. Consequently, we propose that the COI sequence data support recognition of *A. taiwanensis* as a valid species, a result consistent with the prior study on morphology.

Table 6 Molecular divergence values for various Hydrozoa and Scyphozoa taxa based on the mtCOI gene sequence

Taxon	Divergence(%)	Author
<i>Hydra</i>	0.9–16.8	Hennrich et al. (2007)
<i>Clytia</i>	4–16.7	Govindarajan et al. (2006)
<i>Obelia</i>	6.8–13.5	Govindarajan et al. (2006, 2005)
<i>Bonniella</i>	7.7–10.6	Govindarajan et al. (2006)
<i>Laomedea</i>	15	Govindarajan et al. (2006)
<i>Orthopyxis</i>	8.7–19.8	Govindarajan et al. (2006)
<i>Campanularia</i>	15	Govindarajan et al. (2006)
<i>Forskalia</i> ¹⁾	11.7–22.5	Dunn et al. (2005)
<i>Mastigias</i>	6–9	Dawson(2005d)
<i>Cyanea</i>	11.8–15.3	Dawson(2005b)
<i>Cassiopea</i>	10.2–23.4	Holland and Dawson(2004)
<i>Aurelia</i>	13–24	Dawson and Jacobs (2001)

Notes: 1) mtCOI gene sequences of *Forskalia* come from GenBank AY 937368–AY 937370.

Another interesting result observed from our study was that the morphological difference between *A. taiwanensis* n. sp. and *A. conica* was greater than that recorded between *A. taiwanensis* and *A. papillata*, whereas molecular differences were the opposite. This phenomenon has also been presented in other taxa studies. More and more examples have suggested that morphological similarity may mask considerable molecular difference, and that conversely, morphological divergence does not necessarily imply molecular divergence. In the light of molecular data, workers found that many cryptic species exist in *A. aurita*, although they are morphologically indistinguishable (Schroth et al., 2002; Dawson and Jacobs, 2001). Preliminary analyses of *Mastigias* from Palau indicate considerable morphological, behavioral and ecological diversity, but essentially no molecular differences in either COI or ITS1 (Dawson, 2005c, d). Although we cannot conclude that *A. taiwanensis* n. sp. was closer to *A. conica* than to *A. papillata* just in the light of one fragment of gene sequence, molecular data provide important and useful information to evaluate independently the utility of morphological data in systematic studies. Thus, there is need to add molecular data in order to make diosyncratic decisions regarding what constitutes morphological characteristics that are independent, reliable, or sufficient to delineate *Aequorea* species. Our future studies are expected to integrate other DNA sequence data, which will provide robust informative characteristics for unravelling phylogenetic relationships in *Aequorea* or in the Hydrozoa at different taxonomic levels.

Conclusively, both the morphology and mtCOI sequence data provide the unambiguous evidence that *Aequorea taiwanensis* n. sp. is a valid species of *Aequorea*. Our study demonstrated mtCOI sequence data to be diagnostic molecular systematic characteristics for accurate identification and discrimination of the species of *Ae-*

quorea or their populations, and can be used to resolve evolutionary relationships among species of *Aequorea*. We also suggested that a 10%–20% pairwise mtCOI sequence difference indicated species-level divergence among congeneric species in the Hydrozoa.

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