Genetic Diversity of the Protected Jumbo Dragonfly *Anotogaster klossi* Fraser (Odonata: Cordulegastridae) in Taiwan

臺灣產保育類無霸勾蜓之遺傳多樣性

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Abstract

The jumbo dragonfly *Anotogaster klossi* Fraser is the largest and the only protected Odonata species in Taiwan, but there is little known biological information for conservation efforts. In order to understand the genetic differentiation and diversity of this species in Taiwan, we sequenced both the nuclear (ITS1) and the partial mitochondrial (COI) segments from 15 samples collected in eight localities across Taiwan. A total of two ITS1 haplotypes and eight COI haplotypes were identified, and no obvious genetic differentiation was found across the examined geographic range. Haplotype diversity (h) and nucleotide diversity (π) of the mitochondrial COI segment were 0.895 and 0.0052, respectively. The sequence variation patterns of the population showed no significant difference from neutrality. These results reveal the genetic backgrounds of *A. klossi* in Taiwan and provide useful information for its conservation.

Key words: *Anotogaster klossi*, genetic diversity, conservation, Taiwan

摘　要

無霸勾蜓為臺灣體型最大，也是唯一的保育類蜻蛉目昆蟲。雖然名列保育物種受到較多關注，然而相關基礎生物學研究卻不多。本研究為瞭解臺灣產無霸勾蜓的遺傳多樣性與族群分化，於全臺8樣點採計15樣本。細胞核(ITS1)與粒線體(COI)基因序列分析結果，區分出2個ITS1單倍型與8個COI單倍型，地理分布上無明顯遺傳分化現象。粒線體COI序列之單倍型多樣性(h)與核苷酸多樣性(π)各為0.895與0.0052，且其變異形式無明顯偏離中性假說。以上結果初步呈現臺灣產無霸勾蜓遺傳性質，可作為後續保育研究之參考。

關鍵詞：無霸勾蜓、遺傳多樣性、保育、臺灣

Introduction

The jumbo dragonfly, in the genus *Anotogaster* from Taiwan and the adjacent Japanese islands of Ishigaki and Iriomote in the Yaeyama archipelago, has long been regarded as an undescribed subspecies of *Anotogaster sieboldii* (Selys, 1854) (Asahina

1965; Lieftinck *et al*. 1984). Compared to the nominate species of *A. sieboldii* from mainland Japan, the jumbo dragonfly in Taiwan and Yaeyama islands is larger in size, and the female differs in having brown colored wing bases (Lieftinck *et al*. 1984). Lohmann (1993) described a new species *Anotogaster flaveola* from Taiwan. The type specimen was a single adult female which resembles the Taiwanese *A. sieboldii* subspecies, and it was hard to identify these two Taiwanese *Anotogaster* species due to a lack of diagnostic criteria (Wang 2000).

Molecular DNA markers have been used to revise the taxonomic status of "*A. sieboldii*", and at least three taxonomic treatments had been proposed. First, "*A. sieboldii*" could be separated into two major evolutionary lineages (Kiyoshi 2008; Osozawa *et al*. 2013). The northern lineage is composed of individuals from northern China, Korean peninsula, Japanese four main islands, and central Ryukyu islands. The southern lineage is from southern China, Taiwan, and southern Ryukyu islands (Yaeyama islands) (Kiyoshi 2008; Osozawa *et al*. 2013). Second, the northern lineage could be treated as "*A. sieboldii*". However, the southern lineage may differ from *A. sieboldii* and correspond to *A. flaveola* (Futahashi 2011). Third, *A. flaveola* was treated as synonymous with *Anotogaster klossi* Fraser, 1919, thus the southern lineage may be identical to *A. klossi* (Karube *et al*. 2012). Because of inconsistency between the molecular lineages and morphological classification (Karube 2012; Kiyoshi and Hikida 2012), the taxonomic status of the jumbo dragonfly from Taiwan still needs further investigation and clarification. Before that we adopted the third taxonomic treatment and treated the southern lineages as *A. klossi*.

The jumbo dragonfly is the largest and the only protected Odonata species in Taiwan. However, little information regarding its distribution and habitat has been described (Lieftinck *et al*. 1984; Chang and Wang 1997; Wang 2000; Trou 2006; Lin and Yang 2016). Recently, conservation genetics has become an important approach in the field of conservation biology. The approach is not only used to verify the taxonomic status of closed related species, but also provide information about the genetic diversity that helps the government in listing protected species and forming strategies to wildlife conservation (Frankham *et al*. 2002).

The nuclear internal transcribed spacers and ribosomal 5.8S gene (ITS1, 5.8S, ITS2) and mitochondrial cytochrome *c* oxidase subunit I and II gene (COI and COII) of Taiwanese *A. klossi* have been reported

(Kiyoshi 2008; Futahashi 2011; Karube *et al*. 2012; Osozawa *et al*. 2013), but there is very little sequenced data to provide useful genetic information for jumbo dragonfly conservation. In the study, we extended the research of previous studies by collecting more samples, and conducted analyses of genetic data in an attempt to gain a better understanding of the genetic structure and diversity of the species in Taiwan.

Materials and methods

Specimen collection

We obtained government permission (AC-FB-0941701233) for a total of 15 specimens collection throughout Taiwan that included two specimens each from Shilin, Touwu, Renai, Xinyi, Haiduan, Xiulin, Yuanshan, and only one from Wutai (Fig. 1, Table 1).

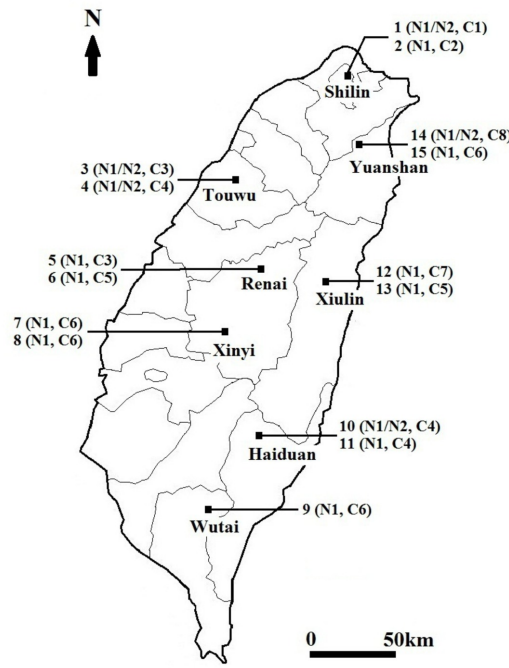

Fig. 1. The sampling sites of *Anotogaster klossi* and specimen information about specimen codes (1-15), nuclear ITS1 genotypes (N1, N1/N2), and mitochondrial COI haplotypes (C1-8).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from muscular tissue of synthorax using the protocol of the PuregeneTM DNA isolation kit type D-5000A (Gentra Sustem INC., BIOzym, Netherlands). The nuclear ITS1 and mitochondrial COI segments were amplified, sequenced and analyzed. The complete region of ITS1 was amplified by polymerase chain reaction (PCR) with the primers, 5'-TAGAGGAAGTAAAAGTCG-3' (forword, in 18S rRNA, Weekers *et al*. 2001) and 5'-CGATGATCAAGTGTCCTGCA-3' (reverse, in 5.8S rRNA, Pilgrim *et al*. 2002). The PCR reaction program was initialized at 94 °C for 1 min, followed by 30 cycles with 1

min 94 °C, 1 min 50 °C, 1.5 min 72 °C, finished at 72 °C for 10 min. The partial COI gene was amplified by PCR with the universal primer sets, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*. 1994). The PCR reaction program was initialized at 94 °C for 2 min, followed by 35 cycles with 30 s 94 °C, 30 s 45 °C, 40 s 72 °C, finished at 72 °C for 10 min. The PCR products were purified with the Micro-Elute DNA Clean/Extraction kit (Gene Mark, Taiwan) and then sequenced both directions on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, CA). At nuclear loci the genotype was either in homozygous or heterozygous form. The homologous form was visualized by only one color peak at the same nucleotide site, in contrast to the heterozygous form with multiple color peaks. Sequence alignments were done using the GeneDoc program (Nicholas *et al*. 1997) and DAMBE (Xia and Xie 2001).

Haplotype network and a Mantel test

Relationships among mitochondrial haplotypes were visualized as a network constructed using the algorithm of statistical parsimony and implemented in TCS version 1.21 (Clement *et al*. 2000). A Mantel analysis was used to examine the correlation between the spatial distributions of individuals with their genetic relatedness. Thus, a Mantel test of matrix of pairwise Kimura's (1980) two-parameter genetic distances (d) against the matrix of pairwise geographical distance was performed using the program GeoDis 2.2 (Posada *et al*. 2000).

Genetic diversity analysis and neutrality test

Genetic diversity parameters were estimated by haplotype diversity (h) and nucleotide diversity (π) (Nei 1987). Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) tests were used to determine if the pattern of sequence variation was consistent with the hypothesis of neutrality. These tests can not only detect the influence of selection on a gene but also provide potentially information about population demographic process (Ramos-Onsins and Rozas 2002). All genetic diversity and neutrality analyses was conducted with the program Arlequin ver 3.5 (Excoffier and Lischer 2010) with a setting of 1000 permutations.

Results and discussion

Fifteen Taiwanese samples were sequenced successfully for both the nuclear and mitochondrial segments. The boundaries of ITS1 were determined by comparing with the previously aligned sequence data

(Karube *et al*. 2012), and 283 to 284 base pairs (bp) of nuclear DNA sequences of ITS1 were obtained. A total of two haplotypes (N1, N2) were identified. N1 haplotype (GenBank accession number MH508248) included 283 bp and was different from N2 (GenBank accession number MH523446) with an indel of a single base pair in the 257th position. The genotype of each individual is shown in Figure 1 and Table 1. The homozygous genotype N1 existed in ten individuals, and five individuals were detected with a mixture of both N1 and N2 (N1/N2). As for COI, a total of 681 bp of the partial mitochondrial COI gene were obtained: 11 sites were variable (10 transitions, 1 transversion), and eight mitochondrial COI haplotypes were identified (C1-8: GenBank accession number MN229281-MN229288) (Table 2). The sequence divergence of among eight haplotypes ranged from 0.147 % to 1.028 % (1 to 7 bp) (Figure 1 and Table 1).

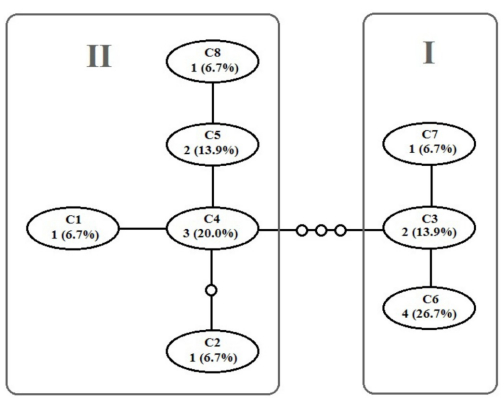

Fig. 2. The parsimonious network of mitochondrial COI haplotypes of *Anotogaster klossi*. Haplotypes are indicated by open circles with haplotypes codes (C1-8). The number of individuals (1-4) and their frequency (6.7-26.7%) are also presented.

A statistical parsimony network of mitochondrial COI segments showed a dumbbell-like haplotype network and the two clades differed by at least four substitutions (Fig. 2). The I clade was composed of seven individuals with the haplotype of C3, C6 and C7 from six sampling localities (Touwu, Renai, Xinyi, Wutai, Xiulin, and Yuanshan) (Fig. 1, Table 1). The II clade included eight individuals with C1, C2, C4, C5 and C8 from six sampling localities (Shilin, Touwu, Renai, Haiduan, Xiulin, and Yuanshan) (Fig. 1, Table 1). Apparently, both clades are widely distributed throughout the island and overlap in their broad geographic distribution. A Mantel test revealed no significant relationship between the genetic and geographical distance ($r = 0.2933$, $p = 0.371$, 1,000 permutations, one-tailed test). It implied that no obvious geographical barriers could prevent the gene flow between populations. Taiwan is a mountainous island, and the prominent Central Mountain Range running along the north-south longitudinal

Table 1. Specimen information about specimen codes, sampling sites, nuclear ITS1 genotypes, and mitochondrial COI

Specimen code	Locality	Location	Altitude (m)	Nuclear genotype	COI haplotype
1	Shilin	25.13N, 121.57E	510	N1/N2	C1
2	Shilin	25.13N, 121.57E	510	N1	C2
3	Touwu	24.56N, 120.92E	440	N1/N2	C3
4	Touwu	24.56N, 120.92E	440	N1/N2	C4
5	Renai	24.07N, 121.04E	1,140	N1	C3
6	Renai	24.07N, 121.04E	1,140	N1	C5
7	Xinyi	23.72N, 120.87E	880	N1	C6
8	Xinyi	23.72N, 120.87E	880	N1	C6
9	Wutai	22.73N, 120.74E	1,050	N1	C6
10	Haiduan	23.16N, 121.06E	710	N1/N2	C4
11	Haiduan	23.16N, 121.06E	710	N1	C4
12	Xiulin	24.00N, 121.43E	600	N1	C7
13	Xiulin	24.00N, 121.43E	600	N1	C5
14	Yuanshan	24.76N, 121.64E	380	N1/N2	C8
15	Yuanshan	24.76N, 121.64E	380	N1	C6

axis is expected to form a geographical barrier to gene flow, especially between altitudinal specialists of low or high altitude (Lin *et al*. 2012; Kuo *et al*. 2014). In contrast, it seems not to be an effective geographic barrier for altitudinal generalists or vagile organisms (Lee *et al*. 2004; Kuo *et al*. 2014). The jumbo dragonfly is a widespread species throughout the island, up to about 2000m altitude (Lieftinck *et al*. 1984; Chang and Wang 1997; Wang 2000; Trou 2006; Lin and Yang 2016). It could be considered as an

Table 2. Alignment of the mitochondrial DNA sequences of partial COI in haplotypes C1-C8. Only variable sites are reported, and dots indicate base identity with haplotype C1.

	Variable sites										
	0	0	1	2	2	3	4	4	6	6	6
Haplotypes	7	8	7	2	7	1	2	6	3	6	7
	8	7	7	8	4	3	9	2	9	3	2
C1	T	A	G	G	C	A	T	C	G	T	A
C2	C	•	•	A	•	•	•	T	•	•	•
C3	•	•	•	A	T	•	C	•	A	C	•
C4	•	•	•	A	•	•	•	•	•	•	•
C5	•	•	•	A	•	•	•	•	•	•	T
C6	•	•	•	A	T	G	C	•	A	C	•
C7	•	G	•	A	T	•	C	•	A	C	•
C8	•	•	A	A	•	•	•	•	•	•	T

altitudinal generalist and the mountain ranges cannot form barriers for the geological and genetic exchangeability of the species. Thus, the Taiwanese jumbo dragonfly may be treated as a single conservation unit based on the definition of ecological and genetic exchangeability (Crandall *et al*. 2000).

Genetic diversity analyses of mitochondrial COI segment revealed a pattern with high haplotype diversity (h=0.895±0.053, n=15) and moderate nucleotide diversity (π=0.00523±0.00050, n=15), based on the criteria of Grant and Bowen (1998). Compared with the other protected insects in Taiwan, the mitochondrial COI variation of *A. klossi* was higher than the other two extremely rare swallowtail butterflies, *Agehana maraho* (h<0.333, π<0.00022) (Lu *et al*. 2009) and *Troides aeacus formosanus* (h<0.539, π<0.00098) (Wu *et al*. 2010), but more or less closer to a rare swallowtail butterfly, *Atrophaneura horishana* (h=0.628, π=0.0102) (Liou 2011). Additionally, both Tajima's *D* (D=0.204, p=0.623) and Fu's *Fs*

(*Fs*=-1.166, p=0.230) tests were not significantly far from 0, which implied a neutrally evolving population of constant size.

For the purpose of wildlife protection, in 1989 the Taiwanese government formulated the inventory of protected species according to the Wildlife Conservation Act (Yang 1998). This inventory includes *A. klossi* and 17 other insect species. However, the definition of protected species is still subjected to scientific debates. Then, based on scientific community opinions, Chao *et al*. (2009) reexamined and revised the inventory of protected insect species according to their community status, endemism, threat, vulnerability and value. Among them, 23 insect species were listed as protected. As for *A. klossi*, it is still categorized as a Taiwanese protected species since 1989. Nevertheless, as *A. klossi* can be found in a wide range of low or middle altitude areas in Taiwan (Lieftinck *et al*. 1984; Chang and Wang 1997; Wang 2000; Trou 2006; Lin and Yang 2016), its status as a protected species has therefore been called into question. The present study is the first to examine the current state of *A. klossi* using population genetics. Due to related regulations, a very limited number of individuals were allowed to be studied. However, this number was enough to help us understand the current situation of *A. klossi*, and meanwhile also provided scientific evidence for its conservation.

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