東海大學生命科學系碩士論文

論文題目：

台灣特有種短腹幽蟌的多次入侵暨更新世晚期族群擴張事件

Multiple Invasions and Late Pleistocene Demographic Expansion of the Formosan Damselfly, *Euphaea formosa* from Taiwan

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致謝

我的碩士班研究**台灣特有種短腹幽蟌的多次入侵暨更新世晚期族群擴張事件**可以完成實仰賴許多朋友及老師們的幫忙。實驗室與大學的同學及學妹們陪伴我多次環台採短腹幽蟌，其中陳明玉、陳薇云、廖鎮磐、韋家軒、王若凡對於我累積樣本數有著極大的幫助。

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摘要

我們利用台灣特有種短腹幽蟌（*Euphaea formosa*），以分子標記粒線體細胞色素氧化酶第二段（*COII*）及核醣體基因間未編碼的區間（*ITS*），來探討其現今的族群遺傳結構，與歷史上發生的地理事件和其生物特性之間的關係。結果顯示族群間有很大的遺傳交流，並且在 *COII* 的親緣分析中發現兩個不同的系群。*COII* 西部系群的遺傳結構很顯著地受地理距離所隔離，且它們進入台灣的原因可能與最近一次冰河事件有很大的關係。利用 *COII* 以貝氏天際線點圖（BSP）及不符合分佈（mismatch distribution）分析過去的族群量變化指出，最近族群量擴張的現象。此外，*COII* 廣佈系群及 *ITS* 所有個體的 Tajima’s D 與 Fu’s F_S 都為顯著的負值，這一結果與星型的網路分析兩者都代表著族群經歷過擴張事件。而這一次的擴張事件與更新世晚期有時間上的相關。推論族群擴張現象可能與當時陸地面積增加所導致棲息地增加有關。

關鍵字：蜻蛉目，*COII*，*ITS*，貝氏天際線點圖，親緣地理學，族群量變化，蜻蛉目，幽蟌科。

CONTENTS

Abstract

We used an endemic Formosan damselfly, *Euphaea formosa* (Insecta: Odonata: Euphaeidae) in Taiwan to investigate the pattern of contemporary population genetic structure using both mitochondrial cytochrome oxidase II (*COII*) and nuclear internal transcribed spacer (*ITS*) genes, and to discuss geohistorical events and life history characteristics that may have contributed to the observed patterns. Our results suggested that there was substantial gene flow among populations. Two distinct haplotype clades, one western restricted and one widespread clade, were identified based on *COII* phylogeny. The *COII* western clade, which showed a significant isolation by distance pattern, may colonize Taiwan due greatly to recent glacial events. Historical demography estimated using Bayesian skyline plot (BSP) and mismatch distribution showed a pattern of recent population expansion. Significant negative Tajima's D and Fu's F_S values coupled with star-like networks of *COII* widespread clade and *ITS* also indicate population expansion. We conclude that the colonization and demographic expansion of damselfly populations were likely the result of increased available habitats during late Pleistocene.

Keywords: *COII*, *ITS*, Bayesian skyline plot, phylogeography, historical demography, Euphaidae, Odonata.

Introduction

The subtropical islands of Taiwan, with a size of nearly 400 km long and 150 km wide, is located southeast of the Asian mainland. Similar to the majority of volcanic island chains in the western Pacific, Taiwan is often considered as a fragment or mixed island based on geographic evidences (Liu et al. 2000; Sibuet & Hsu 2004; Huang et al. 2006). Geographically the island was created by the collision of the Philippine Sea plate and Eurasian plate about 9 Mya (Sibuet & Hsu 2004).With gradually uplifting processes, the proto-Taiwan emerged above sea level around 5 Mya (Liu et al. 2000) in which the northern region appeared earlier than the southern region (Huang et al. 2006). During the Pleistocene, land bridges between Taiwan, the adjacent Asian continent, and nearby Ryukyu islands were formed and submerged frequently due to the changes in sea levels and continental ice sheets (Boggs et al. 1979; Huang et al. 2001). As a result, the fauna and flora in Taiwan were expected to experience periodic cycles of contact and isolation with that of the adjacent Asian continent and islands. Patterns of lineage diversification and population genetic structure of Taiwanese fauna and flora were, therefore, good candidates for studying the effect of these geohistorical events of the Pleistocene on insular populations.

Studies of widely-distributed Asian organisms including, the Indian rice frog, *Rana limnocharis* (Ranidae; Toda et al. 1997; Toda et al. 1998), the bamboo viper, *Trimeresurus stejnegeri* (Viperidae; Creer et al. 2001), the grass lizard, *Takydromus* sp. (Lacertidae; Lin et al. 2002), and a common cricket, *Loxoblemmus appendicularis* (Gryllidae; Yeh et al. 2004), revealed multiple invasions originated from the adjacent Asian continent and/or the Ryukyu islands when land bridges were formed during glacial periods. A population genetics study of the giant wood spider, *Nephila pilipes* (Tetragnathidae), however, demonstrated that populations within Taiwan were undifferentiated due to the highly dispersal ability in the immature stage (Lee et al., 2004). The genetically unstructured Asian populations of the wood spider were due to a contiguous distribution of rain forest existed during Quaternary glacial periods (Su et al. 2007).

Phylogeographic analyses of Taiwanese freshwater fauna, *Zacco pachycephalus*, *Acrossocheilus paradoxus*, *Varicorhinus barbatulus* (Cyprinidae) and *Rhinogobius maculafasciatus* (Gobiidae), *Candidiopotamon rathbunae* (Potamidae), showed limited migration and substantial genetic differentiation among populations (Wang et al. 1999; Wang et al. 2000; Wang et al. 2004; Cheng et al. 2005; Shih et al. 2006). These studies revealed a strong association between population genetic structures and major geographic events of Taiwan drainage system. Therefore the historical changes in riverine geology appear to be important factors of structuring current biogeographic distribution of Taiwan's freshwater fauna with a strict aquatic life history. Some low-elevation wetland fishes, however, had low population divergence and were paraphyletic to Asian mainland lineages. The pattern may be resulted from seasonal flooding and periodic connection of drainages to the Asian continent (Tzeng et al. 2006).

Given the emerging phylogeographic patterns of the terrestrial and freshwater fauna, the phylogeography and population genetic structures of more diverse aquatic insects in Taiwan are, nevertheless poorly known. Aquatic insects have unique life history characteristic of alternating between aquatic immature and terrestrial adult stage (McCafferty 1983). Dispersal abilities of aquatic insects vary greatly among taxa, sexes, and life stages, ranging from highly mobile dragonflies to relatively less dispersed mosquitoes (Bohonak & Jenkins 2003). Aquatic insects in which a variety of dispersal abilities, mating systems, and life history characteristics can, therefore provide great opportunities, as well as challenges, to investigate combined effects of geographic barriers and organisms' biology on their genetic structure and lineage divergence.

Euphaea formosa Hagen, 1869 (Insecta: Odonata: Euphaeidae) is an endemic damselfly widely occurring near streams at low to mid elevation of Taiwan. Among Taiwanese damselflies, *E. formosa* can be easily recognized by their characteristic black wing spots on the hindwings (Fig. 1.). The adults frequently occur near streams with open canopy and rapid water flow. Mature males with established territories, usually an emerged rock surface, exhibit aggressive behavior toward intruding conspecific males. Females appear periodically inside these territories and mated with territory owners. Although the dispersal ability of *E. formosa* has not been studied, observations of strong territorial behavior of males suggest limited dispersal at the adult stage.

In the present study, genetic analyses of *E. formosa* were used as an example to explore the effect of past geographic events and life history characteristics on the current phylogeographic pattern and population genetic structure of aquatic insects in Taiwan. Since the introduction of mitochondrial and chloroplast DNA to population analyses in the 1970s (Avise 1998), phylogeographic studies have devoted to these multi-copies, maternal inherited markers for investigating the association between gene genealogy and geography (Avis 2000, 2004). Recently, nuclear markers have seen increased use due to the ease of amplification and improved analytical methods (Schaal et al. 1998; Hare 2001; Zhang & Hewitt 2003). Nuclear genes, which have been shown to have less biased substitution pattern in the phylogenetic reconstruction than that of mitochondrial genes (Lin and Danforth 2004), can provide an alternative source of molecular markers for phylogeographic applications. A previous study on the phylogeny of Mediterranean damselfly *Calopteryx* revealed up to 2% sequence variations among localities for the internal transcribed spacers (*ITS1* and *ITS2*) (Weekers et al. 2001), that suggested potential utility of these two nuclear genes in resolving phylogeographic relationships of damselflies. Here we employed both mitochondrial cytochrome oxidase II (*COII*) and nuclear *ITS* genes to investigate the phylogenetics and population genetic structure of the Formosan damselfly and to discuss possible ecological factors, geohistorical events and life history characters associated with the observed pattern.

Materials and methods

Sampling and outgroups

We collected damselflies from 32 sites representing 27 major rivers and streams in Taiwan (Fig. 1). A total of 159 and 125 individuals were included in the analysis of *COII* and *ITS*, respectively (Appendix I). Voucher specimens were preserved in 95% ethanol and stored at -80°C in the insect collection of Tunghai University. Previous morphological study of *E. formosa* and related species, *E. yayeyamana* Oguma, 1913, found only in Japanese islands of Ishigaki and Iriomote, suggested that there were no distinct morphological differences between them except the size, with *E. yayeyamana* being smaller (Hayashi 1990). Therefore, the taxonomic status and the degree of genetic isolation between these two species are unclear. Six individuals of *E. yayeyamana* were included in the *COII* data set, and two of them were analyzed for *ITS*. To test the monophyly of *E. formosa* and *E. yayeyamana*, four related species, *E. refulgens*, *E. subcostalis*, *E. impar*, and *E. decorata*, from southeast Asia were used as outgroups. *ITS* sequences of *E. impar* were obtained from GenBank (AJ746322; Dumont et al. 2005).

DNA extraction and sequencing

DNA extraction was carried out using portions of the thoracic muscle and the standard CTAB protocol (Lin & Wood 2002). We used oligonucleotide primers C2-J-3102 (Jordan et al. 2003) and *Euphaea*-C2-N-3740 (a newly designed primer specific for the genus *Euphaea*, 5'-TCA TCT AGT GAG GCT TCA-3') to amplify *COII* gene. An eukaryote-specific 18SF/28SR primer set (Weekers et al. 2001) was used to amplify *ITS1*, *ITS2* and *5.8S*. PCR reactions were performed in an Ependorf Mastercycler *Gradient* (Hamburg, Germany). PCR reactions for both *COII* and *ITS* contained a total volume of 50μl which was composed of 1μl of ProTaq polymerase (2u/μl, Protech

Technology, Taiwan), 2μl of 10mM of each primer, 4μl of 1mM dNTPs, 5μl of ProTaq buffer, 35μl of ddH_2O, and 1μl of extracted DNA (100 to 400ng/μl). For *COII*, the PCR profile was as following: denaturing at 94°C for one minute, 35 cycles of amplification at 94°C for one minute follow by 53°C for one minute and 72°C for one minute, then a final extension at 72°C for 10 minutes. For *ITS*, the PCR profile was the same as *COII* except the annealing temperature was 52°C. PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan), and then cloned into competent cells (dH-5α) using T&A cloning kit (RBC, Taipei, Taiwan) to screen for multiple copies. Positive clones were confirmed by performing small amount of PCR reactions using M13F/M13R primers, those that with successfully inserted fragments had the desired fragment lengths. DNA sequencings were performed on an ABI PRISM^{TM} 377 automatic sequencer (Perkin Elmer, USA) by the Mission Biotech, Taiwan.

Sequence alignment

DNA sequences were aligned using the Clustal W method in MegAlign program of the DNAStar package (DNASTAR, Madison, USA). *COII* sequences were translated into amino acid sequences using mitochondrial genetic code of *Drosophila* in MacClade (version 4.06, Maddison & Maddison 2000) to examine for possible stop codons caused by ambiguous sequencing results or nuclear pseudogenes. Thirteen aligned *ITS* sequences containing indels of 20 to 58bps in *ITS2* region represented different nuclear copies, and were excluded from the analyses.

Phylogenetic analyses

All unique sequences were included to perform the Neighbor Joining (NJ) and

Maximum Parsimony (MP) phylogenetic analyses using PAUP* (version 4.0b10, Swofford 2001). The best-fitted model of nucleotide substitution were selected in MODELTEST (version 3.7, Posada & Crandall 1998) using the Bayesian Information Criterion (BIC) (Posada and Buckley 2004; Alfaro and Huelsenbeck 2006). Parameter values for constructing the NJ phylogram were from the results of MODELTEST. Parsimony branch support was calculated using non-parametric bootstrapping (Felsenstein 1985) for 1000 replicates of tree bisection and reconnection (TBR) branch swapping with 10 replications of random sequence addition.

Bayesian phylogenetic analyses were done using MrBayes (version 3.1.2, Huelsenbeck & Ronquist 2001). Bayesian posterior probabilities were calculated for each gene. Prior settings and nucleotide substitution models were estimated in MODELTEST. Two separate Bayesian runs each with four Markov chains were performed simultaneously. The Markov Chain Monte Carlo (MCMC) searches were run for 1×10^7 and 6×10^6 generations for COII and ITS with trees being sampled for every 100 generations. MCMC runs were terminated after the average split frequencies fell below 0.01 and the Convergence Diagnostic Potential Scale Reduction Factor (PSRF) approached 1, suggesting the convergence of the two separate runs (Gelman & Rubin 1992). One forth of the sampled trees (25,000 and 15,000) was discarded as burnin. The remaining trees were imported into PAUP* to construct a 50% majority consensus tree.

Phylogeographic analyses

We performed the Nested Clade Analysis (Templeton et al.1995) using TCS (version 1.21, Clement et al. 2000) and GeoDis (version 2.5, Posada et al. 2000) to investigate the correlation between the geography and haplotype genealogy. First, TCS was used to find a parsimony network with 95% confidence interval. Then this network was nested according to the rules of Templeton et al. 1992. GeoDis was subsequently used to calculate clade distance (D_c) and nested clade distance (D_n). Once the significant values of D_c and D_n were detected, inference keys (Templeton 2004) were used to discriminate effects of restricted gene flow, past fragmentation, and range expansion, which may cause a significant association of sequence divergence and spatial and/or temporal factors.

Population genetics analyses

The haplotype diversity (*h*), nuclear diversity (π), exact tests of population differentiation, pairwise F_{ST}, and the overall F_{ST} between and among populations were computed using DnaSP version 4.0 (Rozas & Rozas 1999; Rozas et al. 2003). We used Geographic Distance Matrix Generator (version 1.2.0, Ersts, American Museum of Natural History, NY, USA) to calculate pairwise geographic distance between sampling sites with GPS data, and then plotted pairwise F_{ST} versus natural logarithm of geographic distance using Origin (version 75, OriginLab Co., MA, USA).

Genetic differentiations among and within clades of *COII* were calculated using Analysis of Molecular Variance (AMOVA) in the ARLEQUIN (version 3.01, Excoffier et al. 2005) with 1000 permutations. Three hierarchical levels including within populations (Φ_{ST}), among populations within clades (Φ_{SC}), and among clades (Φ_{CT}) were analyzed.

Tajima's *D* and Fu's F_S were also calculated using ARLEQUIN with 1000 permutations. A positive value of Tajima's *D* and Fu's F_S indicates possible balancing selection (single locus) or population subdivision (multiple loci). Negative values suggest positive selection (single locus) or population size expansion (multiple loci).

Demographic history and divergence time

Demographic history for *COII* clades was inferred using the mismatch distribution analysis with 1000 bootstrap replications in ARLEQUIN. An unimodal distribution indicates the population underwent a recent demographic and/or spatial expansion event (Excoffier 2004; Ray et al. 2003; Rogers& Harpending 1992; Slatkin & Hudson 1991). Populations went through a demographic expansion and/or spatial expansion with constant deme will have a single peak in the distribution; whereas a multimodal distribution implies the stochastic status of a population at equilibrium. Goodness of fit between observed data and expected models was calculated using Sum of Square Deviations (SSD). We also calculated the Harpending's Raggedness index (*r*) in which a small *r* represents a smooth curve. The significance of *r* between observed and simulated distributions were tested using 1000 permutations in ARLEQUIN.

Demographic history and lineage divergence time for *COII* clades within the *E. formosa* were further analyzed under a Bayesian coalescence-based framework (Drummond et al 2005). The Bayesian Skyline Plot (BSP) (Drummond et al. 2005) calculated in the Bayesian Evolutionary Analysis Sampling Trees (BEAST, version 1.4.2, Drummond & Rambaut 2006, 2007) was used to generate posterior distributions of effective population sizes ($N_e\tau$), and to estimate the Time to the Most Recent Common Ancestor (T_{mrca}) (Drummond et al. 2002). Group size was set to 10 following the default setting. MCMC sampling was run for 1×10^8 generations with parameters sampled for every 1×10^4 generations. The first 10% of the runs were discarded as burn-in. Each analysis was run multiple-times to optimize the scale factors until no more suggestions in the log file. The skyline group size parameter delta for the final runs was set to four (1 to 2 to 4) and seven (1 to 2 to 4 to 7) for the western and widespread clade respectively. The nucleotide substitution model and prior settings of parameters were derived from the values in MODELTEST. For *COII* gene, the nucleotide substitution rate was set at 1.15 % of divergence/lineage/myrs with a strict clock based on an universal molecular clock of *COI* and *COII* genes for arthropods (1.1%-1.2% divergence/lineage/myrs, Brower 1994). The Effective Sample Size (ESS) for the posterior distribution of estimated values was determined using Tracer (version 1.4, http://tree.bio.ed.ac.uk/software/tracer/).

Results

Sequence variation

A total of 500 bps of *COII* sequences was obtained and aligned without indels or stop codons. 51 haplotypes were found in *E. formosa*, and four in *E. yayeyamana*. The nuclear *ITS1*, *5.8S*, and *ITS2* alignment contained 681 bps, in which 100 unique sequences were found in *E. formosa* and two in *E. yayeyamana*. AT content was 71% and 46% for *COII* and *ITS*. Pairwise sequences divergence of *COII* ranged from 0 to 8.2 % within *E. formosa*, and 4.8 to 9.4 % between *E. formosa* and *E. yayeyamana*. For *ITS*, pairwise sequences divergence were from 0 to 1.7% within *E. formosa*, and 1.7 to 3.2% between *E. formosa* and *E. yayeyamana*. There was a region with GA repeats (0-8) in *ITS1*. Of all *COII* haplotypes, H01 and H17 had the highest frequency and were found in 71 and 33 individuals. Of all unique *ITS* sequences, I70 was the dominant genotypes found in 10 individuals.

Phylogenetic analyses

For NJ and Bayesian analyses, $\mathrm{HKY}+\mathrm{I}+\Gamma$ ($\ln L = -2276$) and $\mathrm{TVMef}+\mathrm{I}$ ($\ln L = -3225$) model were selected as best model of sequence evolution for the *COII* and *ITS*. The *COII* tree showed resolution at species level with strong supports, and suggested a monophyly in *E. formosa* with respect to *E. yayeyamana* (Fig. 2A). This phylogeny identified two distinct clades, one distributed in the western Taiwan only and the other one was widely distributed (Fig. 2A). The remaining haplotypes did not show any biogeographic structure. For the *ITS* tree, *E. formosa* was a monophyletic group and sister to *E. yayeyamana* with strong supports (Fig. 2B).

Phylogeographic analyses

Parsimony haplotype networks with 95% confidence interval were obtained for *COII* (Fig. 3A) and *ITS* (Fig. 3B). The *COII* and *ITS* network separated by up to eight and five mutational steps respectively having a probability of greater than 95% being connected into a single network. Statistically significant associations for *COII* were detected at clade level 1-6, 3-1, 3-2, and the total cladogram (Fig. 4A). Geographic associations for clade 1-6, 3-1, and 3-2 fitted the pattern expected for restricted gene flow with isolation by distance, and the total cladogram was inferred to be a result of either allopatric fragmentation (Table 1). Nested clade analyses of *ITS* suggested patterns of contiguous range expansion (Table 2), as significantly statistic associations were detected in clade level 2-18, 3-4, and the total cladogram (Fig. 4B).

Population genetics analyses

Haplotype diversities (h) of *COII* and *ITS* within 32 and 28 populations ranged from 0 to 1 and 0.75 to 1 (Appendix). Nucleotide diversities (π) of these two genes ranged from 0 to 0.028 and 0.002 to 0.009 (Appendix). Populations with the highest nucleotide diversities of *COII* were located at five river systems in the western Taiwan (TSKL, TC, TDtk, CSlhc, and FG). These populations were those that comprised individuals from two distinct *COII* clades. Populations with the highest nucleotide diversities of *ITS* (TDML, PN, NA, and XC) did not show geographic patterns. Numbers of individuals estimated as effective migrants in the widespread and western *COII* clades (Nm = 12.96, 9.93) were similar to that based on *ITS* (Nm = 9.26) (Table 3). Genetic differentiation of the widespread and western *COII* clades were also (F_{ST} = 0.037, 0.051) similar to that inferred from nuclear *ITS* (F_{ST} = 0.026). There was no significant association between pairwise F_{ST} of widespread *COII* clade and their geographic distances (P = 0.10549, R^2 = 0.00604) (Fig. 5A). The correlation was significantly positive between pairwise F_{ST} of

COII western clade and *ITS* and their geographic distances (P = 0.04742, 0.04518, R^2 = 0.40633, 0.01064) (Fig. 5B, C). The results suggested a significant pattern of isolation by distance for the *COII* western clade. AMOVA of two distinct *COII* clades suggested significant genetic differentiation between clades (Φ_{CT} = 0.19233; $P < 0.0001$), among populations within clades (Φ_{SC} = 0.06951; $P < 0.0001$), and within populations (Φ_{ST} = 0.24847; $P < 0.0001$) (Table 4). Values of Tajima's D in widespread *COII* clade (-2.4648, $P < 0.0001$) and *ITS* (-2.7167, $P < 0.0001$) were negative and significantly different from 0, suggesting that a population expansion event had occurred in the past. Tajima's D calculated for the western *COII* clade was positive (0.44954, P = 0.719), but it was not significantly different from 0. Values of Fu's F_S calculated for *COII* widespread and western lineages were all negative (-28.44075 and -11.97393; $P <$ 0.0001, 0.01) and significantly different from 0, which was an indication of population expansion. Fu's F_S of *ITS* also showed a significant negative value (-24.59255, $P <$ 0.0001) (Table 5).

Demographic history and estimation of divergence time

Mismatch distribution of widespread clade in *COII* was not converged with both sudden demographic expansion and spatial expansion with constant deme models using least square procedure (Fig. 2E). Whereas, mismatch distribution of western restricted clade in *COII* did not significantly differ from the expectation of both sudden expansion and spatial expansion models (SSD = 0.00842911, 0.00862781; P = 0.314, 0.268; r = 0.0197316; P = 0.199, 0.197) (Fig. 2C). Bayesian skyline plot (BSP) for widespread clade of *COII* (Fig. 2C) showed a rapid demographic expansion around 0.05 Mya (Fig. 2F). BSP for western lineage of *COII* suggested a slightly increase in population size through time (Fig. 2D).

The T_{mrca} between *E. formosa* and the sister, *E. yayeyamana* was estimated to be close to 4.5 Mya (mean = 4.475 Mya, 95% CI = 2.817-6.344 Mya, ESS = 6813). The estimated T_{mrca} of all sampled *COII* haplotypes of *E. formosa* was about 2.9 Mya (mean = 2.888 Mya, 95% CI = 1.701-4.239 Mya, ESS = 5035). The T_{mrca} for the western *COII* clade was estimated to be about 1.9 Mya (mean = 1.874 Mya, 95% CI = 1.027-2.824 Mya, ESS = 1.371×10^4). For the widespread *COII* clade, the T_{mrca} was around 0.8 Mya (mean = 0.778 Mya, 95% CI = 0.305-1.302 Mya, ESS = 1.144×10^4).

Discussion

Despite being similar in external morphology except size difference, our reconstructed molecular phylogenies indicated that *E. formosa* and *E. yayeyamana* are each monophyletic sister taxa with an average genetic distance of 8% (*COII*) and 2.5% (*ITS*). The results suggested that these two damselflies are distinctive phylogenetic species. Within *E. formosa*, our phylogenetic analyses identified two distinct clades in the *COII* region, one distributed island-wide and another restricted in western Taiwan. These two distinct clades were inferred to be resulted from past allopatric fragmentation. The hypothesis of multiple invasions of Asian lineages into Taiwan has been proposed for Taiwanese terrestrial and wetland fauna (Toda et al. 1998; Toda 1999; Creer et al. 2001; Lin et al. 2002; Yeh et al. 2004; Tzeng et al. 2006). Results of our genetic analyses of *E. formosa* also suggest at least two independent invasions of ancestral Asian damselfly lineages. The western *COII* clade distributed in rivers and streams corresponding to ancient drainage systems (Boggs et al. 1979) shows the highest genetic diversity among all sampled populations. The ancestral populations of this western haplotype lineage likely colonized Taiwan through the emerged land bridge connecting Taiwan and Asian mainland during glacial period of the Pleistocene. This land bridge was also considered to be the refugium of an endemic minnow in Taiwan (Wang et al. 1999). However, two haplotypes from the western clade were found in Fongkang river located at the very southern part of Taiwan that represents long distance southward dispersal from sites near ancient drainage of western Taiwan. Results from AMOVA suggested that there were significant genetic differentiation among clades and the overall gene tree identified two distinct phylogroups. These findings together suggest that the current populations of *E. formosa* constitute two genetically divergent subgroups resulted from different invasions.

COII and *ITS* gene trees all suggested shallow tree topologies with common lineage that are widespread and closely related lineages that are confined to many localities. These populations can be best characterized as restricted historical gene flow with isolation by distance (*COII*) and contiguous range expansion (*ITS*). Unique *COII* haplotypes were more abundant in western Taiwan and gradually decreased southward because populations within eastern Taiwan mostly possess only haplotype H01 and H17. This pattern of haplotype distribution indicates that the population founders of eastern Taiwan were derived from the western Taiwan through the southern region. This pattern of population expansion and colonization was similar to that found in *A. paradoxus* and *L. appendicularis* (Wang et al. 2000; Yeh et al. 2004). In corresponding to the newly emerged southern Taiwan (Huang et al. 2006), western Taiwan populations of these organisms were hypothesized to colonize the eastern Taiwan which was inaccessible due to geographic barrier of the Central Mountain Rang. Damselflies within the western clade were found in streams of more permanent rivers (Tamshui, Touchien, Tatu, Choshui, and Fongkang) with relatively constant water supply throughout the year. Therefore, given other ecological factors being equal, populations in the western region would tend to increase in population sizes. In contrast, damselfly populations in the eastern region where the rivers are extremely short were likely to experience seasonal draught during winter and early spring. Populations within these habitats would disperse more frequently and population sizes remain relatively constant.

Tree topologies of the *COII* widespread clade and *ITS* are all shallow with a very short depth and little branching structure near the root of *E. formosa*. This pattern suggested that the population went through rapid expansion. The results of significant negative values of Tajima’s *D* and Fu’s F_S for *COII* and *ITS*, together with star-like networks, suggest population underwent expansion. Mismatch distribution and BSP analyses of mitochondrial *COII* showed that the widespread clade exhibited a pattern of demographic expansion during late Pleistocene, whereas the western clade showed a pattern of sudden demographic expansion or spatial expansion with constant deme. But the effective population size of this lineage was inferred to expand in the BSP analysis. Our results strongly suggest that the demographic expansion of damselfly populations have taken place around 0.05 Mya. The demographic expansion, divergence time, and geographic origins of these damselflies are associated with an increase of available habitats in western Taiwan and the formation of land bridges between Asian mainland and Taiwan during late Pleistocene.

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Figure Legends

Figure 1. *E. formosa* (a: female, b: male) and *E. yayeyamana* (c).

Figure 2. (A) Phylogeny of *E. formosa* obtained from NJ phylogram with MP and Bayesian analyses of *COII* gene. (lnL=-2276, divergent times were estimated by BEAST). (B) Phylogeny of *E. formosa* derived from NJ phylogram with MP and Bayesian analyses of *ITS* gene. (lnL=-3225). (C) Mismatch distribution (accumulated numbers, Y-axis, versus pairwise differences, X-axis) of *COII* western clade. (D) Bayesian Skyline Plot (BSP) of *COII* western clade. (E) Mismatch of *COII* widespread clade. (F) BSP of *COII* widespread clade.

Figure 3. (A) Nested clade diagram of 95% most probable *COII* haplotypes network constructed with TCS. (Open circles were extinct haplotypes, hyphen between haplotypes indicated one step mutation. Unique sequences located within the same dash line block were those have the same mutational steps to the common interior one. Clade 4-1, 4-2 corresponded to the widespread and western clade in the *COII* phylogeny). (B) Nested clade diagram of 95% most probable *ITS* unique sequences network constructed with TCS.

Figure 4. (A) Result of nested cladistic analysis of geographical distance for the *COII* haplotypes. 0-step to 4-steps clades assignment were shown in which colored blocks were interiors. Significances detected by GeoDis program were labeled in *L or *S (*L: significant large; S*: significant small). (B) Result of nested cladistic analysis of geographical distance for the *ITS* unique sequences. 0-step to 5-steps clades assignment were shown.

Figure 5. Plotted pairwise F_{ST} (Y-axis) versus pairwise geographic distance (km, X-axis) for *COII* widespread clade (A), *COII* western clade (B), and *ITS* (C).

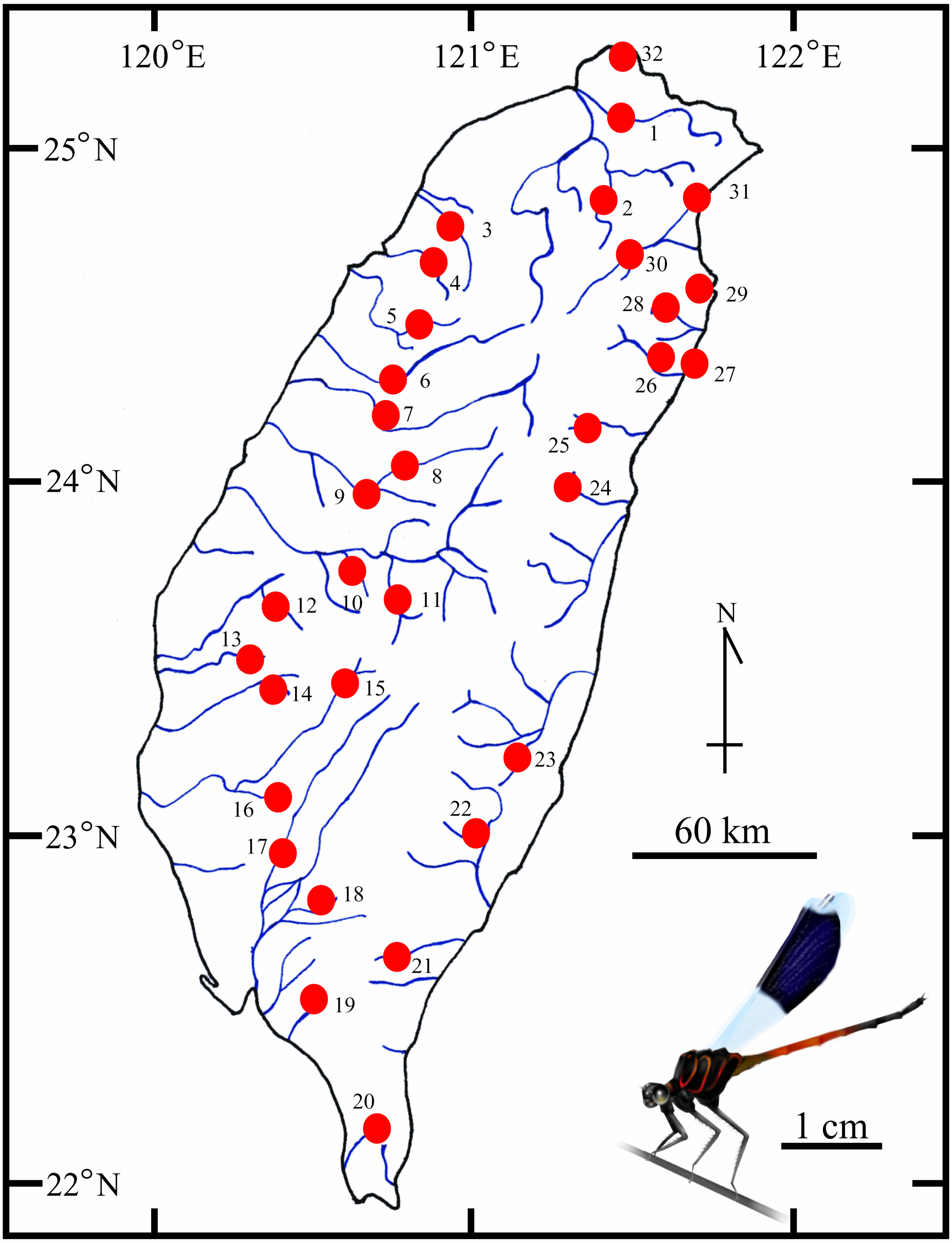
120°E
121°E
122°E
25°N
24°N
23°N
22°N
N
60 km
1 cm
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Figure 1.

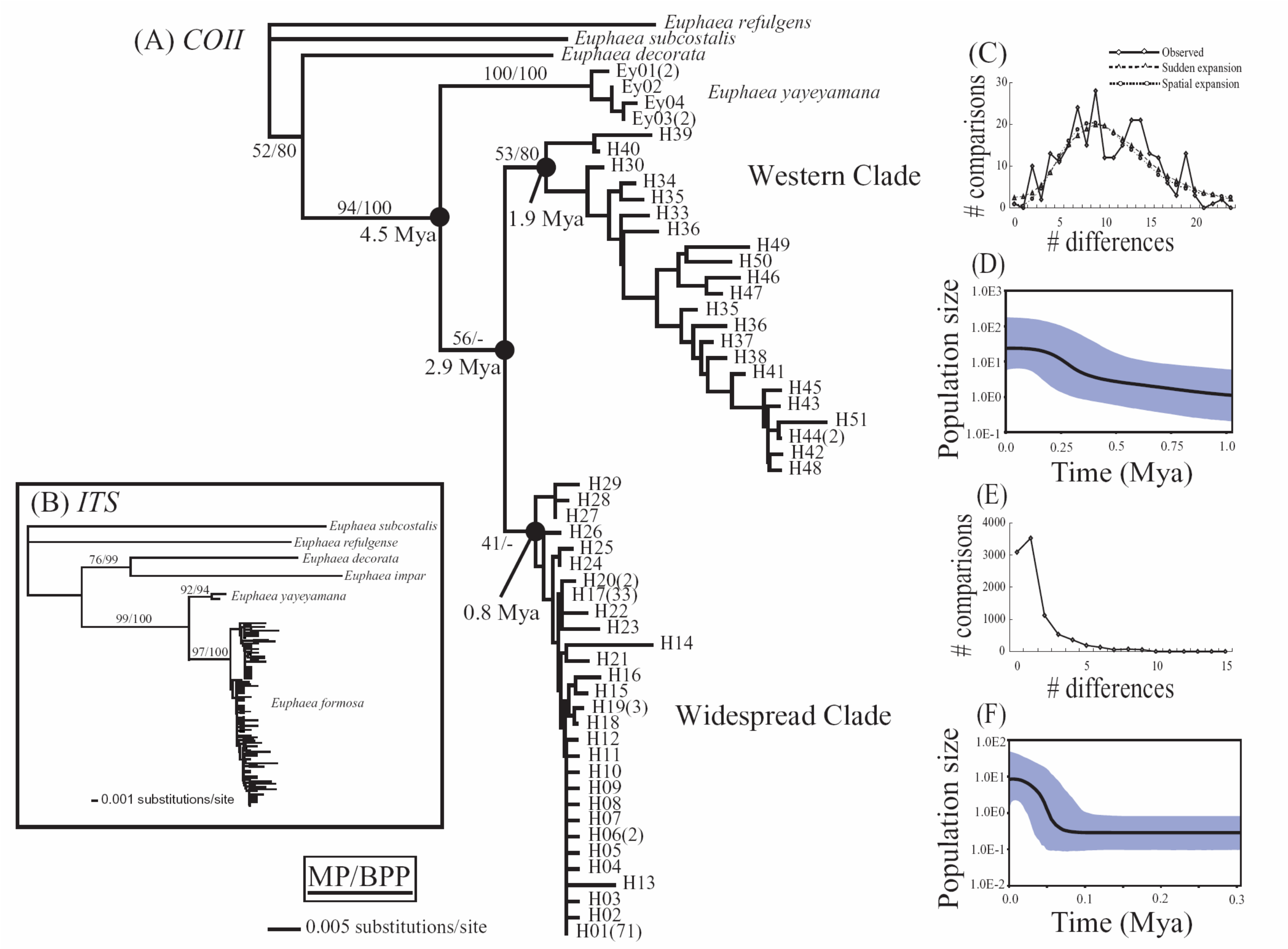
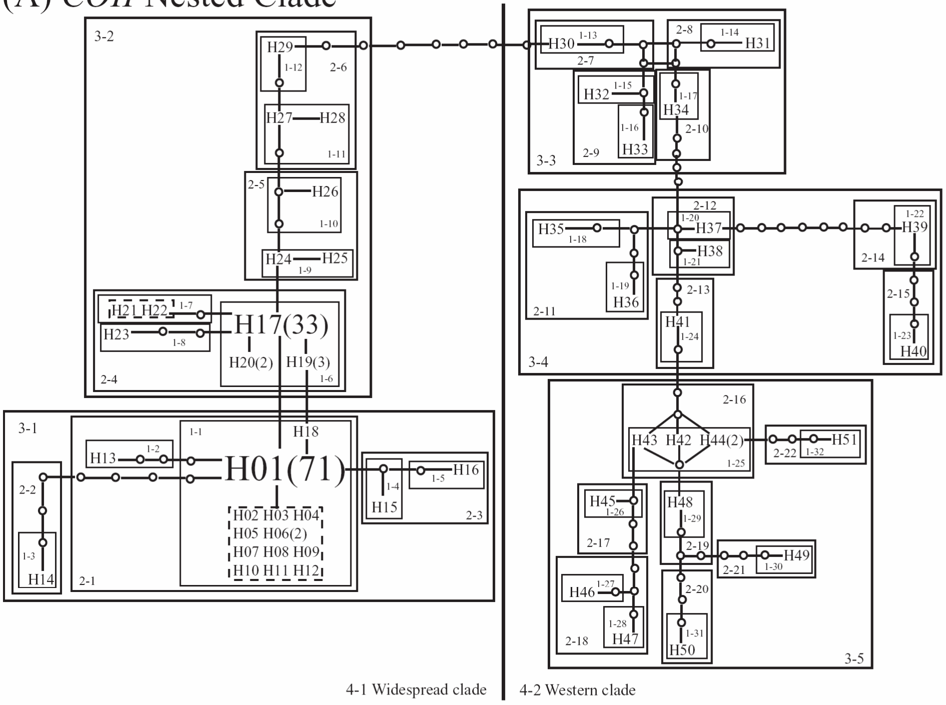

(A) COII
Euphaea refulgens
Euphaea subcostalis
Euphaea decorata
100/100
Ey01(2)
Ey02
Ey04
Ey03(2)
Euphaea yayeyamana
52/80
94/100
4.5 Mya
53/80
1.9 Mya
56/-
2.9 Mya
41/-
0.8 Mya
Western Clade
H39 H40 H30 H34 H35 H33 H36 H49 H50 H46 H47 H35 H36 H37 H38 H41 H45 H43 H51 H44(2) H42 H48
Widespread Clade
H29 H28 H27 H26 H25 H24 H20(2) H17(33) H22 H23 H14 H21 H16 H15 H19(3) H18 H12 H11 H10 H09 H08 H07 H06(2) H05 H04 H13 H03 H02 H01(71)
(B) ITS
Euphaea subcostalis
Euphaea refulgense
76/99
Euphaea decorata
Euphaea impar
92/94
Euphaea yayeyamana
99/100
97/100
Euphaea formosa
0.001 substitutions/site
MP/BPP
0.005 substitutions/site
(C)
Observed
Sudden expansion
Spatial expansion
comparisons
differences
(D)
Population size
Time (Mya)
(E)
comparisons
differences
(F)
Population size
Time (Mya)

Figure 2.

(A) *COII* Nested Clade



(B) *ITS* Nested Clade

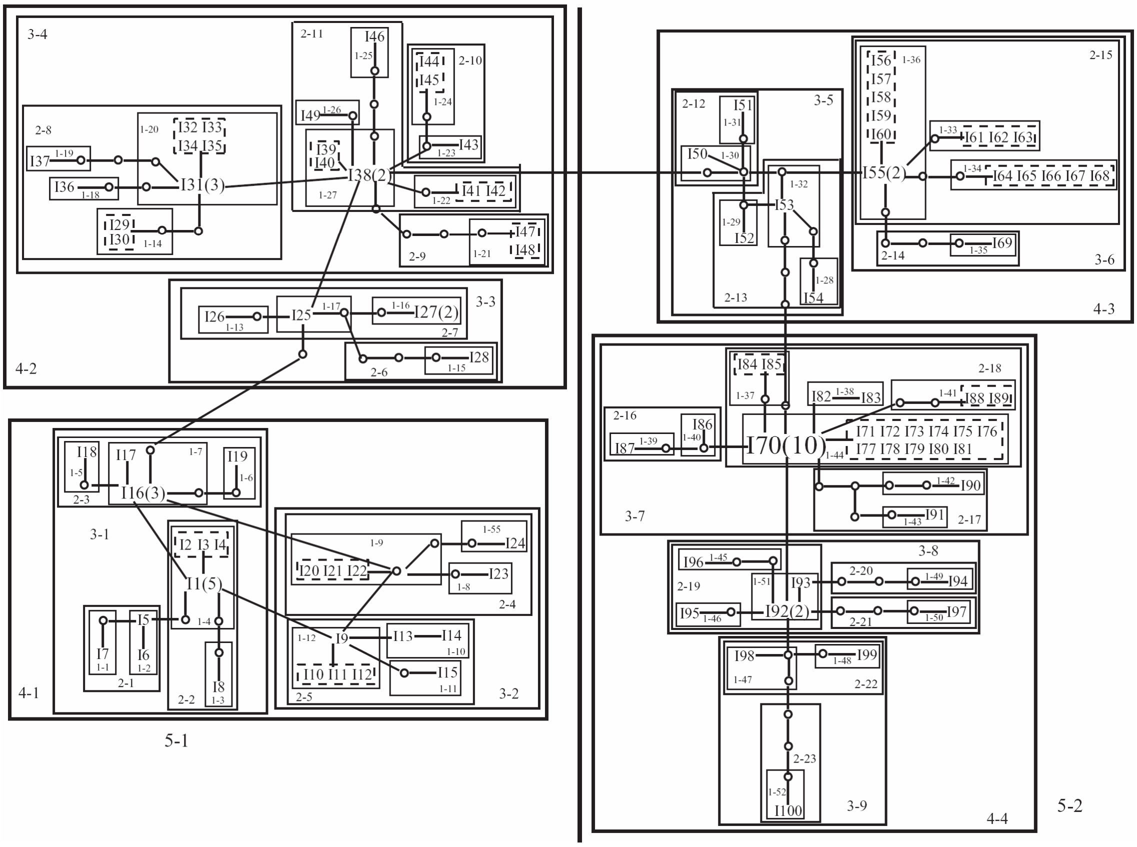
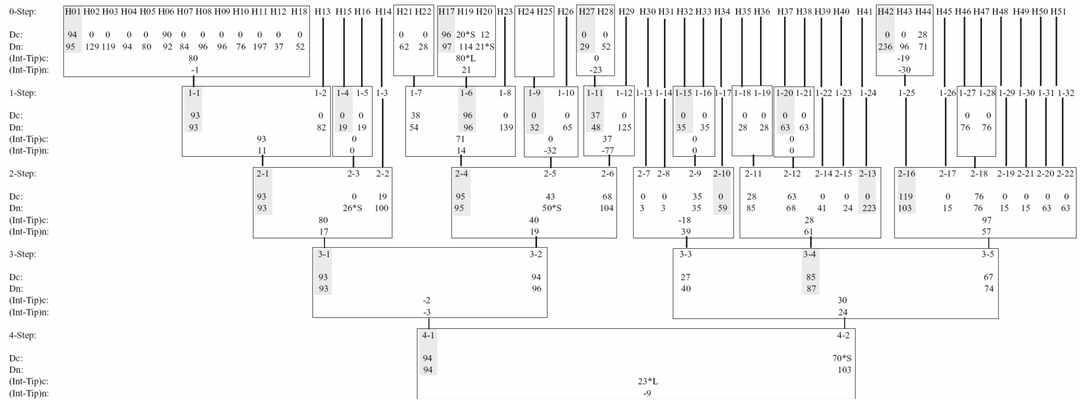


Figure 3.

(A) *COII*



(B) *ITS*

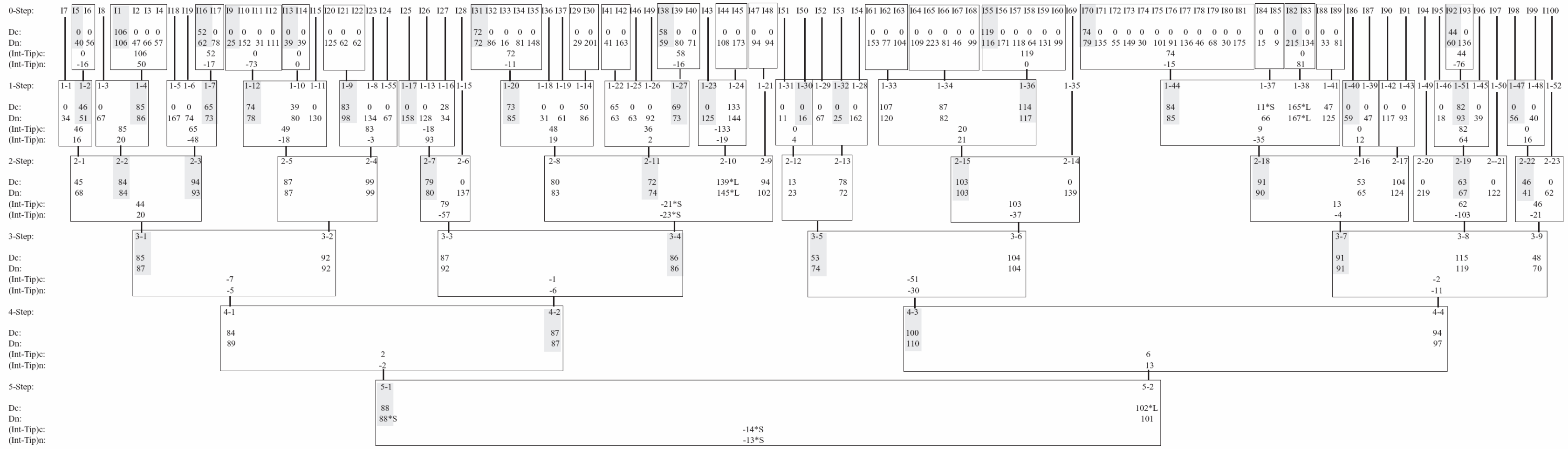
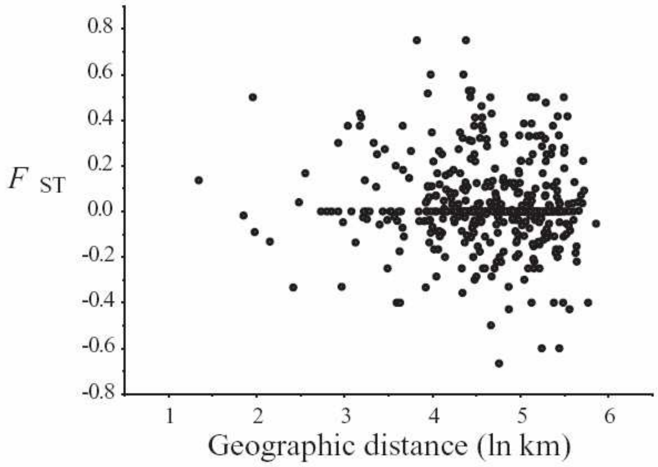
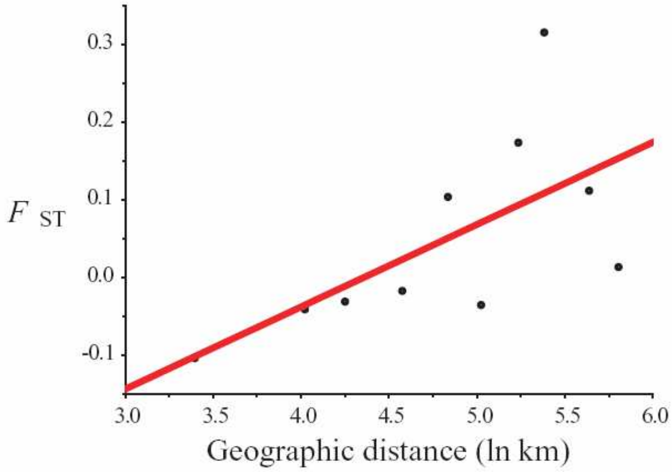


Figure 4

(A) *COII* widespread clade



(B) *COII* western lineage



(C) *ITS*

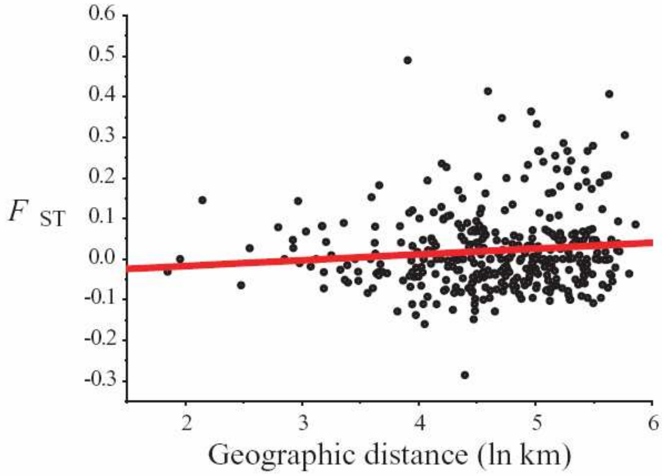


Figure 5.

Table Legends

Table 1. Inference chain on the results given in Fig. 3A and 4A.

Table 2. Inference chain on the results given in Fig. 3B and 4B.

Table 3. Estimated population indices among populations for *COII* widespread and western clades, and *ITS*. Population contained less than two individuals were excluded in this analyses.

Table 4. Analysis of molecular variance of all individuals for *COII*. Molecular variation occurred mostly at within populations level, and than among clades. Within clades among populations possessed the smallest percent of variation. Nevertheless, three hierarchical levels all showed significant fixation indices indicating differentiation pattern within the three levels.

Table 5. Tajima's D and Fu's F_S for *COII* overall individuals, widespread and western clades, and *ITS*. Significant level was labeled as * (**: $P < 0.01$, ***: $P < 0.0001$)

Table 1.

Clades	χ^2	P value	Chain of inference	Inference
1-6	39.73	0.562	1-2-3-4-NO	Restricted gene flow with isolation by distance
3-1	51.19	0.506	1-2-11-17-4-NO	Restricted gene flow with isolation by distance
3-2	46.36	0.481	1-2-11-17-4-NO	Restricted gene flow with isolation by distance
Total Cladogram	103.32	0.000	1-2-3-4-9-NO	Allopatric fragmentation

Table 2.

Clades	χ^2	P value	Chain of inference	Inference
2-18	63.75	0.540	1-2-11-12-NO	Contiguous range expansion
3-4	39.39	0.902	1-2-11-12-NO	Contiguous range expansion
Total Cladogram	33.29	0.287	1-2-11-12-NO	Contiguous range expansion

Table 3.

Molecular marker	# haplotype	h	π	Nm	F_{ST}
COII widespread	28	0.657	0.003	12.96	0.037
COII western	22	0.996	0.021	9.93	0.051
ITS	82	0.962	0.005	9.26	0.026

Table 4.

Source of variation	d.f.	Sum of squares	% of variation	Fixation index (Φ)
Among clades	1	3.823	19.23	0.19233***
Among populations within clades	35	15.274	5.61	0.06951***
Within populations	122	40.469	75.15	0.24847***

Table 5.

Molecular marker	Tajima's D	Fu's F_S
COII all	-1.08145	-20.17931***
COII widespread	-2.4648***	-28.44075***
COII western	0.44954	-11.97393**
ITS	-2.7167***	-24.59255***

Appendix I.

River Systems	Abbr.	#	*COII*	*h*	*π*	*ITS*	*h*	*π*	Localities
Danshui	TS								
Nanshih	TSNS	1	H01	0.4	0.00080	-	1	0.00337	1. 24°50'15"N-121°30'50"E
		2	H01			I56			
		3	H01			I32			
		4	H01			I92			
		5	H06			I70			
Keelung	TSKL	1	H42	1	0.02622	I70	1	0.00401	2. 25°07'30"N-121°34'35"E
		2	H35			I98			
		3	H44			I28			
		4	H17			I25			
		5	H34			I30			
		6	H50			-			
		7	H47			-			
		8	H37			I10			
		9	H51			I6			
Touchien	TC	1	H48	1	0.02789	-	1	0.00374	3. 24°43'10"N-121°15'05"E
		2	H31			I79			
		3	H33			I2			
		4	H36			I31			
		5	H30			I44			
		6	H49			-			
		7	H45			-			
		8	H44			I64			
		9	H26			-			
Chungkang	CG	1	H17	0.4	0.00080	I85	1	0.00519	4. 24°40'50"N-121°00'55"E
		2	H17			I13			
		3	H17			-			
		4	H17			-			
		5	H01			I31			
Houlung	HLong	1	H01	0.4	0.00080	I84	1	0.00483	5. 24°28'00"N-120°58'10"E
		2	H17			I57			
		3	H01			I70			
		4	H01			I5			
		5	H01			I99			
Ta-an	TA	1	H01	0	0	I92	1	0.00398	6. 24°17'30"N-120°52'30"E
		2	H01			I52			
		3	H01			I46			

		4 H01			I87			
		5 H01			-			
Tachia	TJ	1 H01	0.6	0.00120	-	1	0.00312	7. 24°14'00"N-120°50'55"E
		2 H01			I40			
		3 H17			I27			
		4 H17			I33			
		5 H17			-			
Tatu	TD							
Dakeng Scenic Area	TDtk	1 H40	0.92	0.02322	I70	1	0.00384	8. 24°11'05"N-120°53'00"E
		2 H17			-			
		3 H01			I38			
		4 H38			I36			
		5 H01			I11			
		6 H32			-			
		7 H01			-			
		8 H21			I80			
		9 H29			-			
Maolo	TDML	1 H01	0.4	0.00080	I100	1	0.00903	9. 24°02'10"N-120°38'50"E
		2 H01			I90			
		3 H01			I24			
		4 H01			I4			
		5 H17			I51			
Choshui	CS							
Lien-Hua-Chih Area	CSlhc	1 H25	0.97	0.02311	I16	1	0.00267	10. 23°55'00"N-120°52'30"E
		2 H46			I1			
		3 H01			I78			
		4 H20			I17			
		5 H17			I53			
		6 H01			I70			
		7 H39			-			
		8 H24			I50			
		9 H14			-			
Sitou Forest Recreation Area	CSst	1 H01	0.7	0.00200	I89	1	0.00623	11. 23°42'20"N-120°46'10"E
		2 H20			I8			
		3 H17			I95			
		4 H01			I9			
		5 H01			I27			
Peikang	PG	1 H17	0.67	0.00133	I96	1	0.00779	12. 23°35'10"N-120°35'20"E

		2	H01			I76			
		3	H17			-			
Putzu	PC	1	H05	1	0.00400	-			13. 23°29'30"N-120°33'00"E
		2	H17			I67			
Pachang	PJ	1	H01	1	0.00800	I31	1	0.00415	14. 23°25'50"N-120°34'25"E
		2	H06			I70			
		3	H13			-			
		4	H22			I37			
Tsengwen	TW								
Tsengwen Reservoir Scenic Area	TWd	1	H08	1	0.00400	-			15. 23°15'50"N-120°35'30"E
		2	H09			I29			
Tsailiao	TWTL	1	H02	0.7	0.00160	I1	0.75	0.00234	16. 23°00'30"N-120°25'05"E
		2	H01			I68			
		3	H17			I15			
		4	H01			-			
		5	H01			I1			
Kaoping	KP								
Laonung	KPLN	1	H01	0.83	0.00200	I55	1	0.00467	17. 22°43'10"N-120°38'00"E
		2	H17			I18			
		3	H17			I26			
		4	H19			I70			
Chishan	KPCS	1	H01	0.33	0.00067	-	1	0.00498	18. 22°58'20"N-120°29'30"E
		2	H01			I93			
		3	H17			I35			
		4	H01			I12			
		5	H01			I58			
		6	H01			I73			
Linpian	LB	1	H17	1	0.00200	I60	1	0.00467	19. 22°32'45"N-120°39'30"E
		2	H01			I62			
Fongkang	FG	1	H28	0.89	0.02644	I54	1	0.00729	20. 22°13'50"N-120°47'10"E
		2	H43			I43			
		3	H17			I69			
		4	H41			I42			
		5	H17			I45			
		6	H01			I82			
		7	H11			I63			

		8 H01			-			
		9 H01			I59			
Chihpen	CB	1 H17	1	0.00267	I88	1	0.00727	21. 22°41'55"N-120°59'05"E
		2 H01			I97			
		3 H19			-			
Peinan	PN	1 H16	1	0.00800	I39	1	0.00831	22. 22°54'05"N-121°05'05"E
		2 H01			I77			
		3 H27			-			
		4 H03			I47			
		5 H19			-			
Shiukuluan	SKL	1 H01	0.4	0.00160	I21	1	0.00498	23. 23°11'55"N-121°16'50"E
		2 H01			I75			
		3 H01			I38			
		4 H01			I22			
		5 H15			I66			
Hualien	HLien	1 H12	1	0.00200	I74	1	0.00623	24. 23°56'55"N-121°30'00"E
		2 H01			I41			
Leewu	LW	1 H01	0.67	0.00133	-			25. 24°10'10"N-121°36'10"E
		2 H18			-			
		3 H01			-			
Hoping	HP	1 H17	0.6	0.00120	I61	1	0.00415	26. 24°21'45"N-121°44'05"E
		2 H01			I72			
		3 H01			I70			
		4 H17			-			
		5 H01			-			
Chihming Waterfall	CMW	1 H01	0.67	0.00133	-			27. 24°20'20"N-121°45'50"E
		2 H01			-			
		3 H10			I70			
Nan-au	NA	1 H01	0.4	0.00080	I48	1	0.00831	28. 24°28'35"N-121°44'45"E
		2 H01			I14			
		3 H01			I49			
		4 H01			-			
		5 H07			-			
Xincheng	XC	1 H01	0	0	I81	1	0.00872	29. 24°36'55"N-121°48'00"E

		2 H01			-			
		3 H01			I91			
		4 H01			I94			
		5 H01			I65			
Lanyang	LY	1 H17	0.7	0.00200	I34	1	0.00405	30. 24°41'50"N-121°36'55"E
		2 H01			I55			
		3 H17			I3			
		4 H17			I19			
		5 H04			I1			
Derchihkou	DCK	1 H23	0.7	0.00320	I16	0.8	0.00249	31. 24°49'30"N-121°45'00"E
		2 H01			I20			
		3 H17			I86			
		4 H17			I16			
		5 H17			I7			
Shihmenkeng	SMK	1 H01	0.7	0.00080	I83	1	0.00312	32. 25°17'30"N-121°34'20"E
		2 H01			I1			
		3 H01			I70			
		4 H01			I23			
		5 H17			I71			
E. yayeyamana	Ey	1 Ey03			-			24°22'53"N-124°12'00"E
Ishigaki jimma		2 Ey01			Ey01			
		3 Ey02			-			
		4 Ey01			Ey02			
		5 Ey03			-			
		6 Ey04			-			
E. decorata								22°26'11"N-114°09'23"E
Hong Kong								
E. impar								AJ746322
Malaysia peninsular								
E. subcostalis								03°40'55"N-97°39'13"E
Sumatra								
E. refulgens								13°39'47"N-123°19'56"E
Luzon								

Appendix II. Curriculum Vitae

Name: **Huang, Jen-Pan**

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EDUCATION

College	2005	Tunghai University Life Science
GRE Score:	Verbal – 470	Quantitative – 790 AWA – 3.0

TEACHING EXPERIENCE

TA	2005	Evolution
TA	2005	Biodiversity – Entomology Section
TA	2006	Entomology
TA	2006	Biodiversity – Entomology Section

WORKING EXPERIENCE

RA	2007	Evolution and Systematics Lab, Tunghai University – Study on the Population Structure and Phylogeny of an Endemic Stag Beetle, *Lucanus datunensis*

PUBLICATIONS

1. I-Min Tso*, Chen-Pan Liao, **Ren-Pan Huang**, and En-Cheng Yang (2006) Function of being colorful in web spiders: attracting prey or camouflaging oneself? *Behavioral Ecology*, 17, 606-613.
2. I-Min Tso*, **Jen-Pan Huang**, and Chen-Pan Liao (2007) Nocturnal hunting of a brightly coloured sit-and-wait predator. *Animal Behaviour*, 74, 787-793.

PROFESSIONAL PRESENTATIONS

Poster Presentation

Title: The brightly colored orchid spider is actually a nocturnal predator
Symposium: Animal Behavior Annual Meeting 2005, Salt Lake City, Utah, USA

PROFESSIONAL SOCIETIES

Animal Behavior Society	2005
Society of Systematic Biologists	2006