

Elucidating genetic signatures of native and introduced populations of the Cycad Blue, *Chilades pandava* to Taiwan: a threat both to Sago Palm and to native *Cycas* populations worldwide

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Abstract Foreign plants are usually introduced for food or aesthetic reasons. Most of these plants are non-invasive, but can alter the evolutionary trajectory of the associated native insects or inadvertently spread potential pests. A hitherto poorly documented example is the rapid expansion of *Chilades pandava*, a *Cycas*-feeding butterfly. Since about 1990, large numbers of the Sago Palm *Cycas revoluta* were introduced into Taiwan. Invading or introduced with this hostplant, *Ch. pandava* has rapidly spread to all major parts of Taiwan. To trace the source of outbreaks, we sampled 810 specimens covering 50 Taiwanese localities and other regions using mitochondrial COII sequences. Overall haplotype diversity was high ($h = 0.791$), but only 29 haplotypes

were found. The haplotype C which dominates outbreak populations from western Taiwan was endemic to the island. This is consistent with the hypothesis of a local range expansion of *Ch. pandava*, rather than an introduction. In addition, the Taiwanese Central Mountain Ridge may constitute a primary biogeographic barrier restricting gene flow between eastern and western populations. Our study not only flags an important new invasive insect that needs to be monitored and controlled within the horticultural trade and for in situ cycad conservation, but also provides a clearly documented case of the transformation of a native tropical butterfly into a pest via introduced horticultural plants.

Keywords Population outbreak · *Chilades pandava* · *Cycas* · Range expansion · Horticultural trade · COII

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Introduction

The influence of alien plants on population genetics of native phytophagous insects

The biodiversity of phytophagous insects may be inadvertently influenced by accidental, human-mediated activities (Chapin et al. 2000; Keane and Crawley 2002; Tallamy 2004; Strauss et al. 2006).

The population size of some native insects may decrease when their native hostplants are replaced by aliens, but natives can also sometimes thrive and expand their ranges by exploiting these novel hosts (Stastny et al. 2006; Strauss et al. 2006; Carroll 2007). An extreme example has been given for the native Californian butterfly fauna which has expanded on to alien plants in urban or suburban areas and some 40% of which now entirely depend on aliens (Shapiro 2002). Invasive species can alter the evolutionary pathway of native species (Mooney and Cleland 2001). However, when introduced as agricultural or ornamental plants, most alien plants are not naturally invasive, but in large quantity, they affect native biotas by direct replacement (Reichard and White 2001). The unchecked spread of popular horticultural plants around the globe concomitant with modern globalization may be having a dramatic effect on their relatives. Oliver (2006) hypothesized that the native phytophagous insects which can utilize expanded alien plants should present two clear genetic signatures: firstly, insects expanding their geographic range should exhibit lower genetic diversity in newer portions of the insects' range than older ones. Secondly, levels of gene flow should increase among isolated populations when these have been connected, due to anthropogenically mediated range expansions of their hostplants. The invasive insects will thus exhibit relatively low population genetic differentiation, evidenced by reduced local adaptation and hybridization among formerly isolated lineages (Pannell and Dorken 2006; Vellend et al. 2007).

Target species in this study: Cycad Blue and *Cycas* plants

Cycads are one of the most popular cultivated gymnosperm groups, with ancient origins (Hu et al. 1999; Treutlein and Wink 2002). These plants have a worldwide scattered distribution and occur in many kinds of habitats, such as in the understory of tropical rain forest and seasonally dry forest, grasslands, and at high elevation habitats in eastern Africa (Norstog and Nicholls 1997; Whitelock 2002). Cycads comprise 11 genera and about 305 species (Hill et al. 2004). One of the largest genera, *Cycas* (about 90 species), is mainly distributed in mainland China, Southeast Asia, India, Australia, and South Africa (Jones 1993). *Cycas* plants have been used as herb medicines, food, and ornaments

for thousands of years (Whitelock 2002). However, cycads are now endangered as a consequence of extensive collections from the wild and of decreasing natural habitats through urbanization (Donaldson 2003). Consequently, all the extant cycads are listed under Appendix I or II of Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). Two *Cycas* species are now commonly found in Taiwan: the endemic *Cy. taitungensis* and the introduced "Sago Palm" *Cy. revoluta* (Liu et al. 1994; Shen et al. 1994). The distribution of the native cycad is restricted to southeastern Taiwan where it is considered a glacial relict (Huang et al. 2001). On the contrary, Sago Palm is planted ubiquitously, especially in urban and suburban parts of western Taiwan where native *Cycas* species do not occur.

Specialist cycad-feeding is rare among Lepidoptera. The Cycad Blue, *Chilades pandava* (Horsfield, [1829]), like some other congeneric species and American *Eumaeus* butterflies, has an obligate association with cycads (Rothschild et al. 1986; Schneider et al. 2002). *Ch. pandava* is a multivoltine lycaenid, taking only 20–30 days from egg to adult butterfly (Lee 1989). Although the caterpillar of *Ch. pandava* can feed on the young leaves or soft tissue of *Cycas* plants (Chang 1989; Corbet and Pendlebury 1992; Bascombe et al. 1999; Parsons 1999), the buds of a single *Cycas* plant are usually available only for a limited duration (Lan 1999). Therefore, when the young leaves or soft tissue of *Cycas* plant become too tough for a caterpillar of *Ch. pandava* to utilize, the adult Cycad Blue tends to disperse to find new resources for the next generation, as in many other tropical butterflies which have ephemeral hostplants (Ehrlich 1984). The natural distribution of *Ch. pandava* covers mainly tropical regions west of the Wallace Line, including Taiwan, southern mainland China, Southeast Asia, India, and Sri Lanka below 1000 meters (Hsu 2002; Igarashi and Fukuda 2000). There are four currently recognized subspecies, based upon geographic isolation and genitalic/androconial morphologies (Evans 1932; Hsu 1989; Shirôzu and Ueda 1992). Taiwanese populations are currently considered to represent an endemic subspecies *Ch. pandava peripatria*, which is morphologically different from the populations in mainland China and Southeast Asia (*Ch. p. pandava*) and the Philippines (*Ch. p. vapanda*) (Hsu 1989), and associated specifically with *Cy. taitungensis* in southeast part of

Taiwan (Hsu 1987). The fourth subspecies, *Ch. p. lanka*, is restricted to the island of Sri Lanka.

The outbreak phenomenon in Taiwan and in other regions

Despite the fact that the Cycad Blue has only recently been reported from Taiwan, the nature of its origin is controversial. The first report of the Cycad Blue dates back to 1976 (Hsu 1987). In the following years, this butterfly was only occasionally found in urban or suburban areas (e.g. Konishi 1987; Chang 1989; Lee 1989). From that time, this butterfly was rarely reported up to 1990, but the population irrupted suddenly throughout the whole island by 2000 (Lan 1999; Wu et al. unpublished data). Some have considered this butterfly as an introduced species in Taiwan, because no record was registered in previous exhaustive surveys (e.g. Shirôzu 1960; Hamano 1987). However, this butterfly was later considered to be native in Taiwan after large numbers of individuals were found to coexist with *Cy. taitungensis* in a cycad nature reserve (Hsu 1987, 1989). Moreover, three previously undocumented specimens of *Ch. pandava* were found in the collection of Jinhaku Sonan, a pioneer of butterfly studies in Taiwan, bearing labels that indicates they were collected in Taitung, southeast part of Taiwan, in 1937 (Wu et al. unpublished data). These specimens lend support to the idea that *Ch. pandava* should be treated as a species native to Taiwan, rather than of exotic origin.

In other regions, the Cycad Blue has become an invasive pest after Sago Palm or other *Cycas* species were introduced as horticultural plants. Even the recent occurrences of the Cycad Blue on Guam and Madagascar are cases of severe defoliation events on native cycads that were never observed in the past (Moore 2008; this paper). Establishing the origins of these introduced populations is urgent to help put in place control measures that could prevent invasions in parts of the world with native cycad populations, and where ornamentals are being increasing introduced.

Hypotheses

Population outbreaks of the Cycad Blue in Taiwan could originate from alien or native sources. In this study, two alternative hypotheses for how population

outbreaks developed in Taiwan are proposed (Fig. 1). Firstly, if the Cycad Blues that feed on Sago Palms are derived from native populations, outbreaks may be caused by range expansion of the native populations induced through widespread horticultural planting of Sago Palm (as Hypothesis 1 in Fig. 1). In this case, as in the typical genetic signature of range expansion proposed by Oliver (2006), we would expect genetic diversities of native Taiwanese Cycad Blue populations (No. 9 in Fig. 2) to be higher than those of the local outbreak populations (No. 1–8). Additionally, Taiwanese populations should be more closely related to each other than to those of other regions or be monophyletic. In other words, significant population structure or high genetic differentiation would be found between Taiwanese and non-Taiwanese populations. Alternatively, individual larvae and/or adults of Cycad Blue may have been directly transported with the Sago Palms to locations where they established outbreak populations (as Hypothesis 2 in Fig. 1). Under this scenario, Taiwanese outbreak populations could also exhibit low genetic variation due to founder effects or alternatively high genetic variation due to multiple colonization events (e.g. the case study in Darling et al. 2008), but the haplotype composition of outbreak populations would be different than that of Taiwanese native populations. In addition, the introduced outbreak populations should be more closely related to source regions than to the native population in Taiwan. That is, recent colonization would either obscure the genetic structuring between Taiwanese and other regions, or the level of genetic differentiation would be low. Both scenarios are also possible. Outbreak populations could result from range expansion of both native and introduced populations, with the Cycad Blue exhibiting a genetic signature combining features of both alternative hypotheses (M1 & 2 in Fig. 1).

Aims and purposes

In this study, we examined the genetic signatures of Taiwanese populations of the Cycad Blue to shed light on how a rare butterfly species can be rapidly transformed into a pest, both locally and in other parts of the world. Mitochondrial COII sequences were employed in this study using standard phylogeographic methodologies to test the above hypotheses and also to address the following specific questions:

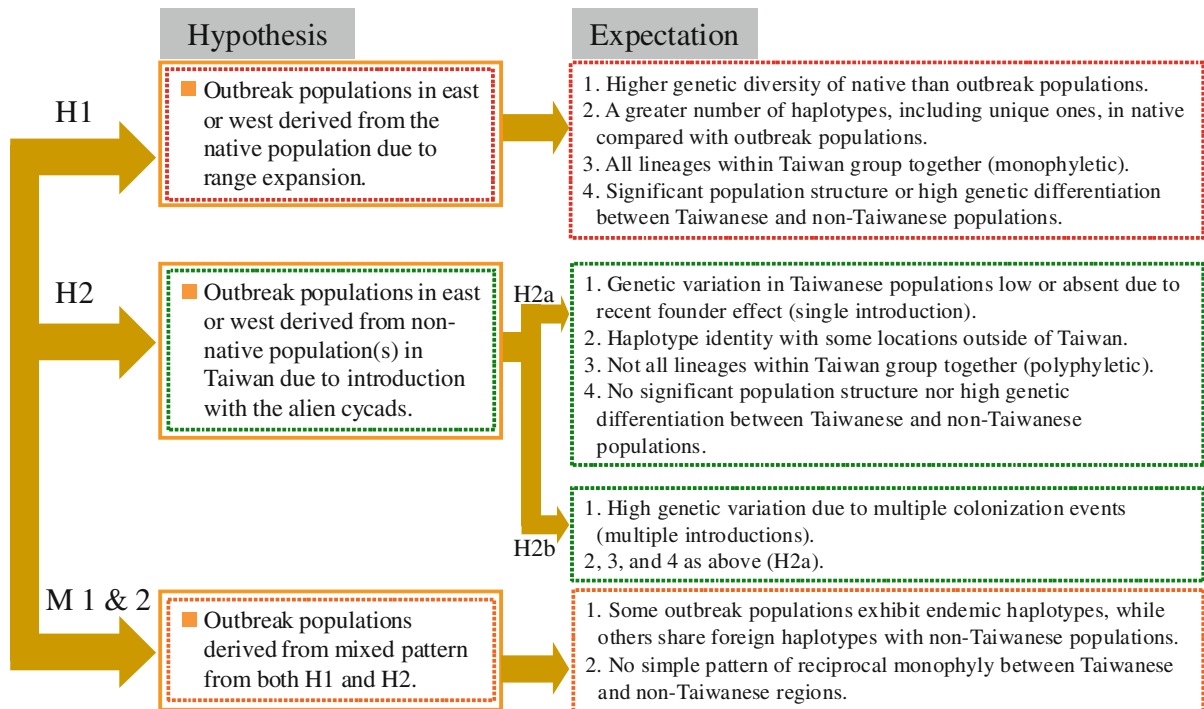


Fig. 1 Hypotheses for how population outbreaks of Cycad Blue developed in Taiwan. H1 denotes hypothesis 1, H2 hypothesis 2, and M1 & 2 a mixture of H1 and H2

- (1) Does the population structure among the three principal subspecies regions support the current subspecies status within *Ch. pandava*?
- (2) Do the outbreak populations of the Cycad Blue in Taiwan derive from native or recently introduced populations? What are the possible geographic sources of introduced populations of the Cycad Blue in various parts of the world?
- (3) Is the pattern of haplotype distribution influenced by the population dynamics of this tropical butterfly?
- (4) Have geographic barriers such as straits and mountains affected the population structure of this lycaenid?

Materials and methods

Specimens and sampling locations

About 810 individuals of *Chilades pandava* representing 50 locations from Taiwan and its neighbors were used in the study (Fig. 2; Table 1). Nine

locations were sampled in Taiwan, including six populations west of the Central Mountain Ridge (CMR) (No. 1–6, Taipei, Xinzhu, Taizhong, Jiayi, Kaohsiung and Yilan) and three populations east of the CMR (No. 7–9, Hualian, Guanshan, and Luye). The CMR is reported as a biogeographic boundary for other Taiwanese biota (e.g. Cheng et al. 2005; Chen et al. 2006; Tzeng et al. 2006). Among these sites, Luye (No. 9) is the first and only locality where *Ch. pandava* was found feeding on the native cycad, *Cy. taitungensis* (Hsu 1989). Because tropical organisms often have high dispersal ability as needed to search for temporally scattered resources and related large fluctuations in abundance (Ehrlich 1984), they should exhibit a mixed genetic composition, especially when local assembly takes place from different sources over a protracted time period. For the purposes of observing the effect of temporal variation on their genetic pattern, butterfly specimens were sampled each month from February, 2000 to October, 2002 (Table 3).

Two subspecies neighboring Taiwan were also sampled to investigate their relationship with the

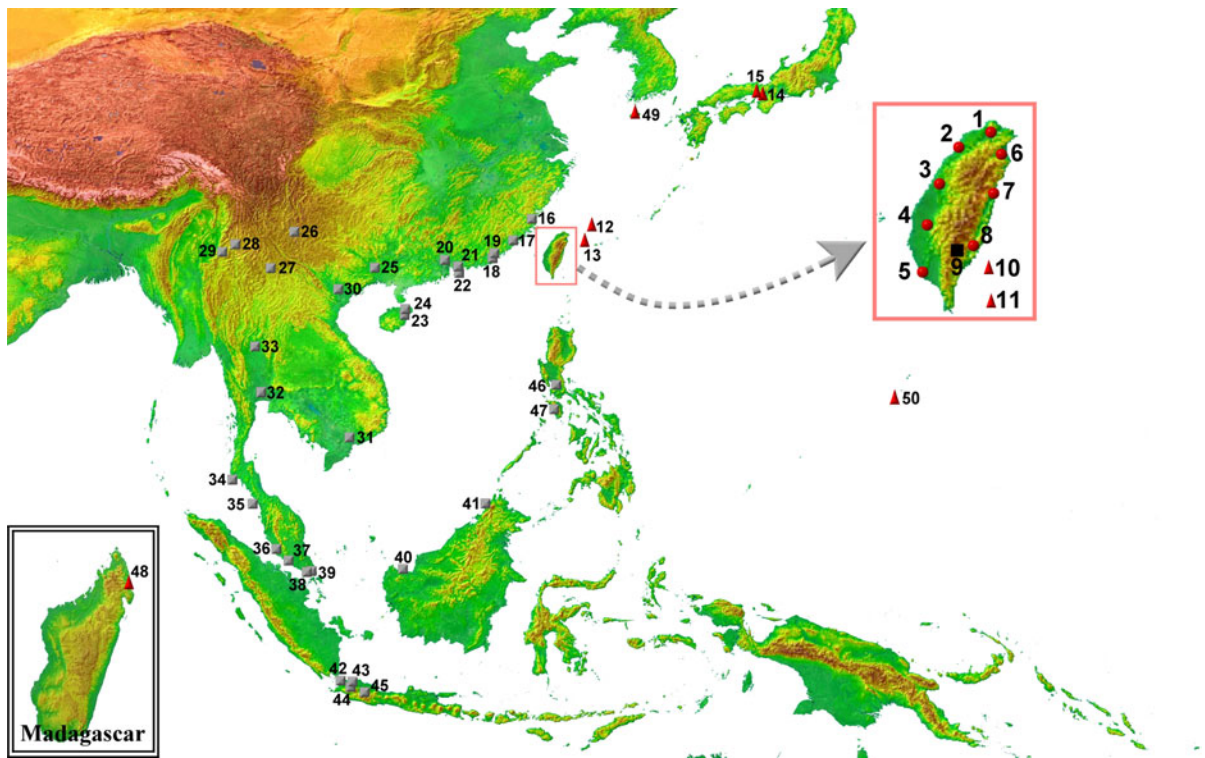


Fig. 2 Sample locations. *Red circle*: outbreak populations; *red triangle*: introduced or recently colonized populations; *Black square*: native population of Taiwan; *Gray square*: status unknown

endemic *Ch. p. peripatria*. Samples collected from regions west of Taiwan Strait belong to *Ch. p. pandava*. This subspecies has the largest distributional range, extending from mainland China to Southeast Asia and India (Igarashi and Fukuda 2000; Hsu 2002). The specimens were collected mainly in mainland China and Southeast Asia (Fig. 2, locations No. 16–45), regions expected to be outbreak sources for the Cycad Blue because of their large export trade in *Cycas* plants. Another subspecies sampled in this study is *Ch. p. vapanda*, which is endemic to the Philippines archipelago (No. 46–47). Additionally, two evidently recently introduced populations, namely Guam (No. 50) and Madagascar (No. 48), and seven recently colonized populations of *Ch. pandava*, namely Korea (No. 49; Takeuchi 2006), Japan (No. 13–15) and the islands near Taiwan (No. 10–12) were sampled to broaden the search for the origins of these recent outbreak populations. Cycad Blue occurrences from these islands near Taiwan are newly reported in this paper, which have no native cycads but do have some introduced *Cy. revoluta*.

In addition, three congeneric species, *Ch. cleotas* (Malekula Island, Vanuatu), *Ch. laius* (Kaohsiung, Taiwan), and *Ch. mindora* (Dinagat Island, Philippines) were used as the outgroups for phylogenetic analyses. All the specimens were stored at -80°C , except for a small number of museum specimens.

DNA extraction and sequencing

Genomic DNA was obtained from the thoracic muscle tissue or legs using the Purgene DNA Isolation kit (Gentra Systems, Minnesota, USA), following the extraction protocol of manufacturer. Precipitated DNAs were resuspended in 100 μL of dH_2O and we used the primer pairs: Pierre (5'-AGAGC CTCTC CTTTA ATAGA ACA-3') and Eva (5'-GAGAC CATT A CTTGC TTTCA GTCAT CT-3') to amplify the partial mitochondrial cytochrome oxidase II gene (COII) by polymerase chain reaction (PCR) (Caterino and Sperling 1999). Each PCR reaction was carried out in a final volume of 25 μL with 0.8 μL of 10 μM dNTP, 1.5 μL of 25 mM MgCl_2 , 0.5 μL of each

Table 1 Sampling locations and size (*N*)

Map symbol	Collecting locality	Collection date	Host plant	<i>N</i>	Latitude	Longitude
1	Taipei, Taiwan	2000–2002	<i>Cy. revoluta</i> , <i>Cy. taitungensis</i>	56	25° 02' N	121° 36' E
2	Xinzhu, Taiwan	2000–2002	<i>Cy. revoluta</i>	42	24° 48' N	120° 57' E
3	Taizhong, Taiwan	2000–2002	<i>Cy. revoluta</i>	25	24° 15' N	120° 43' E
4	Jiayi, Taiwan	2000–2002	<i>Cy. revoluta</i>	65	23° 29' N	120° 27' E
5	Kaohsiung, Taiwan	2000–2002	<i>Cy. revoluta</i>	93	22° 28' N	120° 16' E
6	Yilan, Taiwan	2000–2002	<i>Cy. revoluta</i> , <i>Cy. thourarsii</i>	27	24° 45' N	121° 45' E
7	Hualian, Taiwan	2000–2002	<i>Cy. revoluta</i>	54	24° 00' N	121° 36' E
8	Guanshan, Taiwan	2000–2002	<i>Cy. taitungensis</i>	63	22° 59' N	121° 10' E
9	Luye, Taiwan	2000–2002	<i>Cy. taitungensis</i> (Native)	83	22° 52' N	121° 01' E
10	Ludao Is., Taiwan	2004–2005	<i>Cy. revoluta</i>	15	22° 40' N	121° 30' E
11	Orchid Is., Taiwan	2008	<i>Cy. revoluta</i>	1	22° 00' N	121° 5' E
12	Pengjia Is., Taiwan	2008	<i>Cy. revoluta</i>	5	25° 38' N	122° 04' E
13	Yonaguni Is., Okinawa, Japan	2001	<i>Cy. revoluta</i>	4	24° 27' N	122° 56' E
14	Osaka, Honshū, Japan	2007	<i>Cy. revoluta</i>	1	34° 41' N	135° 30' E
15	Takarazuka, Honshū, Japan	2007	<i>Cy. revoluta</i>	3	34° 48' N	135° 22' E
16	Fuzhou, Fujian, China	2007	<i>Cy. elongata</i> , <i>Cy. hainanensis</i> , <i>Cy. micholitzii</i> , <i>Cy. revoluta</i> , <i>Cy. szechuanensis</i>	6	26° 02' N	119° 19' E
17	Xiamen, Fujian, China	2006	<i>Cy. revoluta</i>	6	24° 27' N	118° 05' E
18	Shantou, Guangdong, China	2006	<i>Cy. revoluta</i>	5	23° 22' N	116° 40' E
19	Chaozhou, Guangdong, China	2006	<i>Cy. revoluta</i>	4	23° 40' N	116° 37' E
20	Guangzhou, Guangdong, China	2001, 2003	<i>Cy. fairylakea</i> , <i>Cy. revoluta</i>	19	23° 11' N	113° 22' E
21	Shenzhen, Guangdong, China	2007	<i>Cy. revoluta</i>	2	22° 62' N	114° 07' E
22	Hong Kong, China	2003	<i>Cy. revoluta</i>	6	22° 15' N	114° 10' E
23	Qionghai, Hainan, China	2007	<i>Cy. taiwaniana</i> (Native)	3	19° 02' N	110° 16' E
24	Haikou, Hainan, China	2007	<i>Cy. revoluta</i> , <i>Cy. sp.</i>	7	19° 32' N	110° 10' E
25	Nanning, Guangxi, China	2005–2006	<i>Cycas sp.</i>	30	22° 84' N	108° 33' E
26	Kunming, Yunnan, China	2004	<i>Cy. revoluta</i>	10	25° 04' N	102° 73' E
27	Jinghong, Yunnan, China	2006	<i>Cy. revoluta</i> , <i>Cy. siamensis</i>	7	22° 00' N	100° 47' E
28	Mangshi, Yunnan, China	2006	<i>Cy. revoluta</i>	3	26° 21' N	98° 25' E
29	Ruili, Yunnan, China	2006	<i>Cy. revoluta</i>	2	24° 05' N	97° 48' E
30	Hanoi, Vietnam	1997	<i>Cycas sp.</i>	3	20° 59' N	105° 50' E
31	Ho Chi Minh, Vietnam	2005	<i>Cycas sp.</i>	7	10° 45' N	106° 42' E
32	Bangkok, Thailand	2005	<i>Cy. revoluta</i>	14	13° 42' N	100° 33' E
33	Phitsanulok, Thailand	2007	<i>Cy. revoluta</i>	9	17° 34' N	100° 54' E
34	Phuket, Thailand	2005	<i>Cy. sp.</i>	5	7° 85' N	98° 36' E
35	Langkawi, Malaysia	2005	<i>Cy. sp.</i>	12	6° 25' N	99° 45' E
36	Klang, Malaysia	2004	<i>Cy. revoluta</i>	11	3° 05' N	101° 30' E
37	Maleka, Malaysia	2004	<i>Cy. revoluta</i>	1	2° 11' N	102° 14' E
38	Johor Bohru, Malaysia	2003–2004	<i>Cy. sp.</i>	7	01° 27' N	103° 45' E
39	Desaru, Malaysia	2004	<i>Cy. sp.</i>	13	1° 32' N	104° 15' E
40	Kuching, Borneo, Malaysia	2004–2005	<i>Cy. revoluta</i> , <i>Cy. sp.</i>	13	1° 24' N	110° 19' E
41	Kota Kinabalu, Borneo, Malaysia	2005	<i>Cy. sp.</i>	4	6° 00' N	116° 04' E
42	Serang, Java, Indonesia	2004	<i>Cy. revoluta</i>	12	6° 23' S	105° 49' E

Table 1 continued

Map symbol	Collecting locality	Collection date	Host plant	<i>N</i>	Latitude	Longitude
43	Jakarta, Java, Indonesia	2004	<i>Cy. revoluta</i>	2	6° 10' S	106° 50' E
44	Bogor, Java, Indonesia	2004	<i>Cy. revoluta</i> , <i>Cy. sp.</i>	12	6° 36' S	106° 48' E
45	Bandung, Java, Indonesia	2004	<i>Cy. sp.</i>	9	6° 54' S	107° 36' E
46	Quezon, Luzon, Philippines	1991	<i>Cy. revoluta</i>	11	14° 38' N	121° 06' E
47	Mindoro, Philippines	1994	Not determined	1	13° 00' N	121° 00' E
48	Isle St Marie, Madagascar	2006	<i>Cy. revoluta</i> , <i>Cy. thouarsii</i>	2	17° 05' S	49° 49' E
49	Seogwipo, Jeju Is., Korea	2005	<i>Cy. revoluta</i>	2	33° 48' N	126° 57' E
50	Guam, U.S.A.	2007	<i>Cy. circinalis</i>	13	13° 64' N	144° 86' E

Native hostplants were listed, and others were horticultural hostplants

10 μ M primers, 2.5 μ L of 10 \times Taq buffer, 0.1 μ L of Amersham Taq (Amersham Biosciences, Buckinghamshire, UK), and finally we added dH₂O up to 25 μ L. PCR was carried out as the following three steps: an initial denaturation step of 94°C (2 min), followed by 35 cycles consisting of denaturation at 94°C (30 s), annealing at 55°C (30 s), extension at 72°C (1 min), and a final extension step of 72°C (7 min). Different annealing temperatures (50–58°C) were used to improve PCR quality when the above PCR conditions failed. Some museum specimens resistant to amplification via the above primer pairs could be amplified through using the internal primers Cppcox-J-3300 (5'-ATAWG AATCA AATTC AATRT TT-3') and Cppcox-N-3400 (5'-TTATT GCWTT ACCTT CWTTA CG-3'). Finally, the products were run on 1.0% agarose gels in 1 \times TBE buffer to ensure that the lengths of PCR fragments were correctly amplified. Blank controls were also run each time to check that no contamination occurred during the PCR process. PCR products were cleaned using Gel/PCR DNA Fragments Extraction kit (Geneaid, Taipei, Taiwan) when only a single DNA band was visible in a gel. DNA sequence reactions were conducted using a 96 well Gel/PCR Clean Up kit (Geneaid) on an ABI3730 DNA Analyzer (Applied Biosystems). Both directions were sequenced. Finally, the sequences were checked and assembled into contiguous arrays using Sequencher 4.5 (GeneCode, Boston, USA).

Analyses

Neutrality of the mtDNA COII gene sequence data was tested in case that some selection was hidden

(Otto 2000). A combination of Tajima's *D* (Tajima 1989), Fu & Li's *D** (Fu and Li 1993) and Fay and Wu's *H* (Fay and Wu 2000) was used, and statistical significance for these neutral tests was assessed by coalescent simulations with 10,000 replicates performed by using DNASP 4.10 (Rozas et al. 2003). General population genetics, such as the number of unique haplotypes, variable nucleotide positions and measured genetic diversities including nucleotide diversity (π) and haplotype diversity (*h*) were also described by using DNASP 4.10.

If Taiwanese outbreaks were derived from populations outside the island, genetic differentiation between subspecies would be obscured. Therefore, an exact test was employed to check the null hypothesis of random distribution of haplotypes, using the software Arlequin 3.1 (Excoffier et al. 2005). To evaluate evidence for genetic structure among different geographic regions, the pairwise *F*_{ST} between populations were evaluated as implemented in the program DNASP 4.10 (Rozas et al. 2003). The analysis of molecular variance (AMOVA) was also performed to partition total variance into variance components attributable to inter-individual, and/or inter-population differences (Excoffier et al. 1992). Three different levels of hierarchical components were included in this analysis (called Φ -statistics): Φ_{CT} , the degree of differentiation among all regions; Φ_{SC} , the degree of differentiation among local populations within regions, and Φ_{ST} , the degree of differentiation among all local populations. In this analysis, datasets A–C were grouped as below. In dataset A (the taxonomic subspecies dataset), the whole sampled populations were grouped into three groups according to their

subspecies status, including *Ch. p. peripatria* (Nos. 1–9), *Ch. p. pandava* (Nos. 16–45), and *Ch. p. vapanda* (Nos. 46–47). In dataset B (the geographical “disjunct regions” dataset), distinct regions were defined as Taiwan (Nos. 1–9), eastern mainland China (Nos. 16–22), Hainan (Nos. 23–24), central mainland China (Nos. 25, and 30), western mainland China (Nos. 26–29), Central Indochina (Nos. 31–33), north of Indochina Peninsula (Nos. 34–35), western Malaysia (Nos. 36–39), Borneo (Nos. 40–41), Java (Nos. 42–45) and Philippine region (Nos. 46–47) based on the geographical separation by mountains, straits or long distance. In dataset C (the geographical “Taiwanese populations” dataset), the grouping was similar to dataset B, except that the Taiwan region was divided into the eastern (Nos. 1–5) and western populations (Nos. 6–9) based on the barrier of the Taiwanese CMR. These three datasets were compared to each other to detect changes in the above three differentiation parameters. The significance of the components was computed using a nonparametric permutation test (10,000 permutations) as performed by Arlequin 3.1. Some new outbreak populations of the Cycad Blue (No. 10–15, 48–50) were excluded from the AMOVA because these locations were not included in the native distribution of *Ch. pandava* and it was not possible to define their group membership.

In order to infer the most basal haplotype of *Ch. pandava* and the phylogenetic origins of this species in Taiwan, all haplotypes (29 ingroups; 3 outgroup; 621 bp) were aligned and analyzed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis. Although the phylogenies based on MP, ML and Bayesian methods all showed high branch support recovering *Ch. pandava* in the same monophyletic group (MP: tree length = 99, CI = 0.869, RI = 0.797, bootstrap value = 100; ML: HKY85 model, $-\ln = -1357.68$, bootstrap value = 85; Bayesian inference: GTR + I + G model, $-\ln = -1407.50$, posterior probabilities = 0.85), the relationships among the haplotypes or three subspecies of *Ch. pandava* were unresolved (tree not shown). Because conspecific populations often have lower divergences than at interspecific level, we also performed haplotype network joining for studying closed relationships (Posada and Crandall 2001). Haplotype networks were constructed with the software TCS 1.21 based on the principle of parsimony (Clement et al. 2000). Each branch in the network was supported with

a 0.95 probability (over 0.95). This setting provided plausibility for the uncertainty of the exact cladogram when only a part of dataset was used (Templeton et al. 1992).

Results

Sequence information

A total of 810 specimens of *Chilades pandava* and 3 congeneric species were sequenced for 621 base pairs (bp) of the partial COII gene (GenBank Accession numbers FJ941955-FJ942767). All sequences could be translated into amino acids. No stop codon was found but one specimen from Mindoro (Fig. 2; No. 43) showed a 3-bp indel. In total 99 polymorphic sites were detected, most due to outgroup sequences, but 38 among populations of *Ch. pandava*. The neutral tests, Tajima’s *D* test (average value of $D = -0.065$; P value = 0.83, NS.), Fu and Li’s test (D^* test: average value of $D = -0.025$; P value = 0.79, NS.), and Fay and Wu’s *H* test (average value of $H = 0.015$; P value = 0.60, NS.), all showed that the accumulated mutations of the dataset were not seriously affected by positive selection, and the dataset was suitable for population analyses without bias from positive selection.

Gene diversities

Gene diversities of each location were listed on Table 2. Overall haplotype and nucleotide diversities of *Chilades pandava* were 0.791 and 0.00446, respectively. Although the haplotype diversities of Taiwanese populations ($h = 0.1$ – 0.5) appear lower than those from non-Taiwanese regions ($h = 0.2$ – 0.8), the statistical analysis between these two is not significant ($t = 0.989$, $P = 0.329$, $df = 37$), even though nucleotide diversities were not different between Taiwan and other locations ($\pi = 0$ – 0.003 ; $t = 0.78$, $P = 0.440$, $df = 37$). However, populations sampled from the eastern part of Taiwan ($h = 0.1$ – 0.5) had higher haplotype diversity than those from the western part ($h = 0$ – 0.1 ; $t = 2.869$, $P = 0.024$, $df = 7$). The populations from western part of CMR (No. 1–6) all showed low gene diversities, even the locations of Taizhong and Yilan became fixed over 3 years of observation (Table 3).

Table 3 Haplotype distribution among monitored locations per month

Location\Month	Apr ^a	May ^a	Jun ^a	Jul ^a	Aug ^a	Sep ^a	Oct ^a	Nov ^a	Dec ^a	Jan ^b	Feb ^b	Mar ^b	Apr ^b	May ^b	Jun ^b	Jul ^b
Taipei			C1						C1	C3		C1		C10	C6,E1	C8,E1
Xinzhu				C8											C1	C1
Taizhong		C1		C1	C1	C1							C1	C1	C1	C6
Jiayi	C5			C4	C1	C2	C4,F3	C1	C7	C4				C2	C1	C3
Kaohsiung	C13		C3	C2			C4		C4		C3	C1	C6	C5	C1	
Yilan				C25												
Hualian	B3	B1		B4	B1,A1	B5,A2	A1		A2					B3,A8	B3,A3	B1,A1
Guanshan				A2	A2					A5		A1			A3	A2,C1
Luye	A15,B1,D1			A7						A3		A2,C2	A3,D1	A2B1	C6	A5
Location\Month	Aug ^b	Sep ^b	Oct ^b	Nov ^b	Dec ^b	Jan ^c	Feb ^c	Mar ^c	Apr ^c	May ^c	Jun ^c	Jul ^c	Aug ^c	Sep ^c	Oct ^c	
Taipei			C10,E2		C2							C4	C2	C2	C2	
Xinzhu	C9,E2											C2	C2	C8	C9	
Taizhong	C1	C2					C1	C2	C1			C1	C1	C1	C2	
Jiayi	C4	C3	C1		C2	C1		C1	C3	C4		C1	C2	C5	C1	
Kaohsiung	C6	C2						C5	C4,B1	C5	C7	C1	C14	C4	C2	
Yilan		C2														
Hualian	B4								B1			B1	B2,C1	B3,A3		
Guanshan	A9,E1	A5			A1	A2	A2	A3	A6			A1	A6	A3	A8	
Luye	A4					A1	A7	A6,B1	A8,B2			A1	A1	A1	A2	

The symbols represent haplotype (letter) followed by sample size (number)

^a 2000; ^b 2001; ^c 2002

Haplotype distribution

About 29 unique COII haplotypes of *Ch. pandava* were obtained from 810 specimens (Table 2). This number of haplotypes is less than in some other widely distributed species, such as *Lampides boeticus* (29 haplotypes among only 57 specimens: Lohman et al. 2008) and *Zizina maha* (27 haplotypes among 121 specimens, Yago et al. 2008), but most of these haplotypes of *Ch. pandava* were localized. Haplotype A–D and F were only found in Taiwan. Other major haplotypes were detected in mainland China or Southeast Asia, except haplotype M and N, which were only found in the Philippine archipelago. Most locations were comprised one or two haplotypes, but some locations such as Luye, Guanshan, Nanning, and Fuzhou, possessed at least four haplotypes. In Taiwan, the haplotype distribution was asymmetrical. Haplotype A and B were mainly found in the eastern part of CMR, but haplotype C was dominant in the western part. Moreover, other rare haplotypes such as F and D were only detected at local locations, Jiayi

and Luye respectively. Only haplotype E showed a disjunct distribution. This haplotype was found both in Taiwan (No. 1, 2 & 8) and Malaysia (No. 34).

Most locations which *Ch. pandava* recently colonized possessed a single haplotype. The specimens from Ludo Is. (Fig. 2, No. 10), and Yonaguni Is. (No. 13) all possessed haplotype A, only detected in eastern Taiwan (No. 7–9). Specimens from Jeju Is. (No. 49) and Orchid Is. (No. 11) possessed haplotype C, detected both in eastern and western Taiwan. Specimens from Guam (No. 50) possessed only haplotype M, which also occurs in Luzon (No. 46, Quezon Province) in the Philippine archipelago. The specimens from Madagascar (No. 48) possessed haplotype O, found mainly in southern part of mainland China or Southeast Asia. Japanese regions had single haplotypes in each region: haplotype A was detected on Yonaguni Is. (No. 13), whilst haplotype H was found on Honshu Is. (Osaka, No. 14 and Takarazuka, No. 15). Among all recently colonized populations, only Pengjia Islet (No. 12) had two haplotypes, haplotype O and H, both detected dominantly in mainland China and Southeast Asia.

Haplotype network

The 29 haplotypes of *Ch. pandava* were used to construct a haplotype network (Fig. 3). A star-like network indicates rapid population expansion. Most unique haplotypes (colored in Fig. 3) were present on tip clades, meanwhile common haplotypes were presented in interiorly nested clades (e. g. haplotype O and H). Haplotypes belonging to the subspecies *Ch. p. pandava* and *Ch. p. peripatria* were connected together by a single step mutation, while the haplotype of *Ch. p. vapanda* exhibits a long branch connection with *Ch. p. pandava* (9 steps). In Taiwan, the haplotypes were connected together, and the first Taiwanese haplotype connected to outside haplotypes was haplotype B, only found in eastern part of Taiwan. Curiously, the dominant haplotype of the western part (Haplotype C) was a tip clade connected to haplotype A instead of to other haplotypes from mainland China and Southeast Asia or the Philippines. This relationship among haplotypes supports the hypothesis that western populations may derive from eastern populations (Hypothesis 1 of Fig. 1).

Population differentiation and population structure of *Ch. pandava*

The exact test performed in Arlequin 3.1 software showed that haplotypes of *Ch. pandava* were not

randomly distributed ($P < 0.0001$ with 10,000 steps in Markov chain). Therefore, population structure or population differentiation of the Cycad Blue should exist. High genetic differentiation of Cycad Blue populations was detected through performing a pairwise F_{ST} (Table 4). For example, in the populations of *Ch. p. pandava*, the proportion of F_{ST} values higher than 0.8 was 46.5%, in contrast to the 15.8% of F_{ST} values that were lower than 0.3, reflecting low gene flow in this widely distributed subspecies (Table 4a). High F_{ST} values were also found in Taiwan when western part populations (No. 1–6) were compared to the eastern part (Table 4b, No. 7–9). In other words, F_{ST} values calculated through comparing eastern and western populations were higher than the pairwise F_{ST} values obtained within eastern populations alone ($t = 2.07$, $P = 0.05$, $df = 19$) or within western populations alone ($t = 28.716$, $P < 0.0001$, $df = 28$). Although the F_{ST} values of Taiwan native populations (Table 4b, No. 9) compared to Taiwanese outbreak populations (No. 1–8) were not significantly different compared to non-Taiwanese regions (G1-10; $t = 1.726$, $P = 0.10$, $df = 16$, Table 4b), the pairwise F_{ST} values obtained from comparing within Taiwanese populations (No. 1–9) were significant lower than the pairwise F_{ST} values obtained from comparing Taiwanese and non-Taiwanese populations (No. 1–9 vs. G1-10 in Table 4b; $t = 9.179$, $P < 0.0001$, $df = 124$). This significant difference indicates that Taiwanese outbreak populations

Fig. 3 Haplotype network of *Chilades pandava* based on mitochondrial COII sequences, reconstructed by TCS 1.21. Each circle represents a unique haplotype and the line between haplotypes represents single step mutation. Colored circles represent restricted haplotypes: Taiwan, purple; Malaysia, blue; Java (Indonesia), yellow; Philippines, green; Vietnam, Brown; Thailand, red and mainland China, black

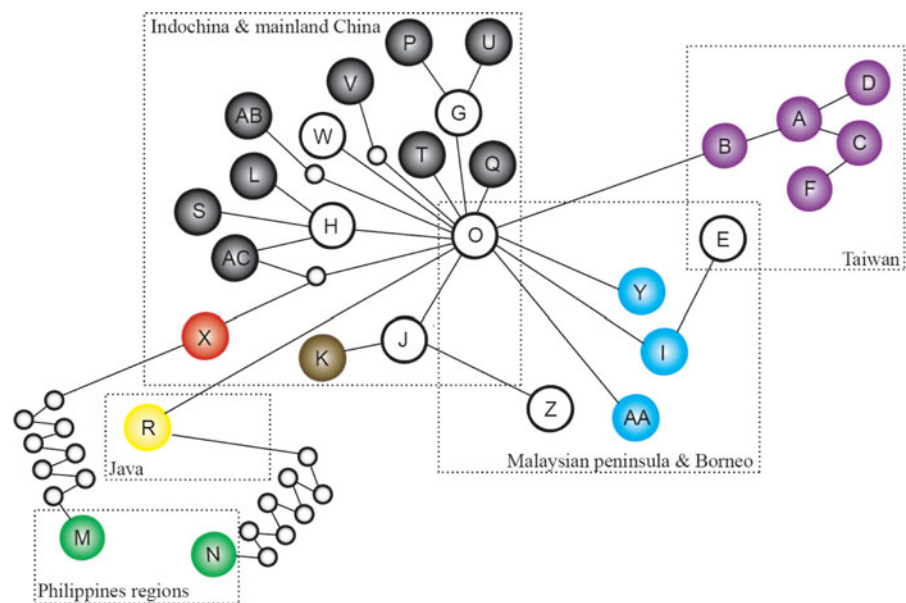


Table 4 Pairwise F_{ST} estimated by Dnasp 4.0. G1 means the group of east mainland China (No. 16–22); G2, Hainan (No. 23–24); G3, central mainland China (No. 25, 30); G4, west mainland China (No. 26–29); G5, Central Indochina (No. 31–33); G6, Phuket and Langkawi island (No. 34–35); G7, west Malaysia (36–39); G8, Borneo (40–41); G9, Java (42–45); G10, Philippines (46–47)

(A) Populations of <i>Ch. p. pandava</i>		No.16	No.17	No.18	No.19	No.20	No.21	No.22	No.23	No.24	No.25	No.26	No.27	No.28	No.29	No.30	No.31	No.32	No.33	No.34	No.35	No.36	No.38	No.39	No.40	No.41	No.42	No.43	No.44	No.45
No.17	0.240																													
No.18	0.240	1.000																												
No.19	0.578	1.000	1.000																											
No.20	0.147	0.474	0.500	0.744																										
No.21	0.578	1.000	1.000	0.000	0.744																									
No.22	0.424	0.909	0.800	0.000	0.633	0.000																								
No.23	0.835	1.000	1.000	1.000	0.868	1.000	0.966																							
No.24	0.240	1.000	0.000	1.000	0.500	1.000	0.800	1.000																						
No.25	0.021	0.228	0.388	0.699	0.173	0.699	0.579	0.843	0.388																					
No.26	0.407	0.901	0.778	0.111	0.620	0.111	0.148	0.963	0.778	0.564																				
No.27	0.051	0.625	0.000	0.700	0.282	0.700	0.528	0.897	0.000	0.166	0.506																			
No.28	0.240	1.000	0.000	1.000	0.500	1.000	0.800	1.000	0.000	0.388	0.778	0.000																		
No.29	0.240	1.000	0.000	1.000	0.500	1.000	0.800	1.000	0.000	0.388	0.778	0.000	0.000																	
No.30	0.246	0.250	0.571	0.700	0.230	0.700	0.632	0.769	0.571	0.142	0.624	0.423	0.571	0.571																
No.31	0.009	0.524	0.167	0.697	0.020	0.697	0.542	0.881	0.167	0.092	0.523	0.015	0.167	0.167	0.311															
No.32	0.022	0.154	0.769	0.898	0.300	0.898	0.785	0.944	0.769	0.045	0.774	0.384	0.769	0.769	0.237	0.276														
No.33	0.387	0.554	0.728	0.805	0.522	0.805	0.746	0.848	0.728	0.446	0.740	0.584	0.728	0.728	0.413	0.542	0.505													
No.34	0.776	1.000	1.000	1.000	0.730	1.000	0.957	1.000	1.000	0.781	0.953	0.864	1.000	1.000	0.625	0.804	0.918	0.805												
No.35	0.405	0.531	0.756	0.835	0.458	0.835	0.769	0.876	0.756	0.402	0.762	0.579	0.756	0.756	0.346	0.505	0.469	0.545	0.790											
No.36	0.216	0.000	0.917	0.957	0.434	0.957	0.866	0.971	0.917	0.204	0.858	0.579	0.917	0.917	0.234	0.480	0.108	0.523	0.957	0.490										
No.38	0.594	0.800	0.882	0.917	0.666	0.917	0.861	0.935	0.882	0.600	0.856	0.722	0.882	0.882	0.534	0.686	0.716	0.672	0.917	0.684	0.718									
No.39	0.571	0.846	0.923	0.949	0.660	0.949	0.887	0.962	0.923	0.578	0.882	0.728	0.923	0.923	0.505	0.685	0.724	0.668	0.949	0.683	0.735	0.245								
No.40	0.269	0.333	0.815	0.892	0.435	0.892	0.809	0.924	0.815	0.263	0.801	0.552	0.815	0.815	0.269	0.471	0.269	0.510	0.892	0.480	0.144	0.481	0.392							
No.41	0.655	1.000	1.000	1.000	0.737	1.000	0.941	1.000	1.000	0.665	0.937	0.800	1.000	1.000	0.571	0.762	0.851	0.728	1.000	0.756	0.900	0.333	0.000	0.583						
No.42	0.655	1.000	1.000	1.000	0.737	1.000	0.941	1.000	1.000	0.665	0.937	0.800	1.000	1.000	0.571	0.762	0.851	0.728	1.000	0.756	0.917	0.882	0.923	0.815	1.000					
No.43	0.655	1.000	1.000	1.000	0.737	1.000	0.941	1.000	1.000	0.665	0.937	0.800	1.000	1.000	0.571	0.762	0.851	0.728	1.000	0.756	0.917	0.882	0.923	0.815	1.000	0.000				
No.44	0.655	1.000	1.000	1.000	0.737	1.000	0.941	1.000	1.000	0.665	0.937	0.800	1.000	1.000	0.571	0.762	0.851	0.728	1.000	0.756	0.917	0.882	0.923	0.815	1.000	0.000	0.000			
No.45	0.655	1.000	1.000	1.000	0.737	1.000	0.941	1.000	1.000	0.665	0.937	0.800	1.000	1.000	0.571	0.762	0.851	0.728	1.000	0.756	0.917	0.882	0.923	0.815	1.000	0.000	0.000	0.000		

Table 4 continued

(B) Taiwanese populations (No. 1-9) vs. other regions

	No.01	No.02	No.03	No.04	No.05	No.06	No.07	No.08	No.09	G1	G2	G3	G4	G5	G6	G7	G8	G9
No.02	0.016																	
No.03	0.055	0.024																
No.04	0.052	0.026	0.031															
No.05	0.044	0.015	0.000	0.021														
No.06	0.055	0.024	0.000	0.031	0.000													
No.07	0.647	0.701	0.832	0.809	0.817	0.832												
No.08	0.659	0.728	0.911	0.874	0.891	0.911	0.462											
No.09	0.598	0.664	0.844	0.809	0.823	0.844	0.387	0.011										
G1	0.760	0.788	0.847	0.838	0.841	0.847	0.646	0.767	0.748									
G2	0.719	0.744	0.797	0.789	0.791	0.797	0.604	0.714	0.698	0.152								
G3	0.736	0.766	0.829	0.820	0.823	0.829	0.592	0.735	0.715	0.112	0.170							
G4	0.835	0.860	0.914	0.905	0.909	0.914	0.773	0.861	0.843	0.133	0.216	0.328						
G5	0.732	0.762	0.826	0.816	0.819	0.826	0.584	0.730	0.709	0.156	0.213	0.072	0.388					
G6	0.739	0.765	0.821	0.813	0.815	0.821	0.624	0.739	0.722	0.380	0.420	0.325	0.581	0.313				
G7	0.798	0.830	0.896	0.886	0.890	0.896	0.703	0.824	0.803	0.455	0.443	0.347	0.648	0.333	0.442			
G8	0.822	0.855	0.925	0.913	0.918	0.925	0.729	0.857	0.834	0.448	0.437	0.320	0.668	0.303	0.445	0.012		
G9	0.914	0.941	1.000	0.989	0.995	1.000	0.891	0.968	0.947	0.700	0.641	0.642	0.843	0.632	0.669	0.780	0.827	
G10	0.860	0.868	0.887	0.884	0.885	0.887	0.867	0.887	0.881	0.833	0.807	0.827	0.858	0.814	0.824	0.849	0.857	0.884

Table 5 AMOVA analyses of three groups datasets

Datasets	Source of variation	Variance components	Percentage of variation	Fixation indices
Dataset A	Among groups	1.37652 Va	70.29	$F_{CT} = 0.703 (P < 0.0001)$
	Among populations within groups	0.40672 Vb	20.77	$F_{SC} = 0.699 (P < 0.0001)$
	Within populations	0.17513 Vc	8.94	$F_{ST} = 0.911 (P < 0.0001)$
Dataset B	Among groups	1.30229 Va	72.47	$F_{CT} = 0.725 (P < 0.0001)$
	Among populations within groups	0.31951 Vb	17.78	$F_{SC} = 0.646 (P < 0.0001)$
	Within populations	0.17513 Vc	9.75	$F_{ST} = 0.902 (P < 0.0001)$
Dataset C	Among groups	1.15189 Va	78.11	$F_{CT} = 0.781 (P < 0.0001)$
	Among populations within groups	0.14765 Vb	10.01	$F_{SC} = 0.457 (P < 0.0001)$
	Within populations	0.17513 Vc	11.88	$F_{ST} = 0.881 (P < 0.0001)$

were not derived from non-Taiwanese populations, and that the native Taiwanese population is the only possible source of Taiwanese outbreak populations.

In general, significant genetic structure of *Ch. pandava* was also observed at various hierarchical levels by AMOVA (Table 5). Those significant differences also support hypothesis 1 that Taiwanese populations exhibit genetic structure in comparison with non-Taiwanese populations. In the three subspecies dataset, the variation among groups accounted for most of the variance (70.29%). When the sampled populations were grouped according to their geographical distribution, the percentage of the variation among groups increased to 72.47%. Moreover, when Taiwanese populations were divided into eastern and western populations by the CMR, the percentage of the variation among groups increased to 78.11%.

Discussion

Molecular systematics and phylogeography of Cycad Blue

Mitochondrial sequences often present little variation in widely distributed species. However, in two other recently investigated cases of polyommatine lycaenids feeding on “weedy” species, mtDNA provides good resolution on population structure and systematic status. The monobasic species *Lampides boeticus*, one of the most widely distributed Old World butterflies, has never been considered to vary geographically. However, phylogeographic analyses show this butterfly forms three distinct groups based

on mitochondrial COI and cytB genes (Lohman et al. 2008). Yago et al. (2008) address taxonomic problems in the genus *Zizina*, small butterflies often difficult to identify by wing pattern. The mitochondrial ND5 gene in combination with male genitalic morphology allows reliable identification of *Zizina* taxa. As in the above widely distributed Asian butterflies, most sampled populations of *Ch. pandava* also showed little genetic variation (only 29 haplotypes were found in 810 specimens). However, in this case genetic data supports the status of existing subspecies. *Ch. p. pandava* is the most widely distributed subspecies. This subspecies could in fact represent the source population where it feeds on several native *Cycas* populations that are distributed in south of mainland China and Indochina (Jones 1993). *Ch. p. pandava* exhibits 1–4 step mutations to *Ch. p. peripatria* endemic in Taiwan, and 9 step mutations to *Ch. p. vapanda* endemic in Philippines. Based on the mitochondrial COII gene, *Ch. p. pandava* also possesses 21 unique haplotypes, considerably more than *Ch. p. peripatria* (5 unique haplotypes) and *Ch. p. vapanda* (2 unique haplotypes). Although *Ch. p. lanka* was not surveyed in this study, this subspecies may be more closely related to *Ch. p. pandava* than to *Ch. p. peripatria* or to *Ch. p. vapanda*, based on its geographic proximity. The molecular data in combination with significant morphological differences (Hsu 1989) supports the subspecies status of *Ch. p. peripatria*.

Low genetic variation and the star-like haplotype network of the Cycad Blue (Fig. 3), is consistent with a population bottleneck after rapid range expansion, as also in the example of the highly invasive Horse

Chestnut leaf miner moth *Cameraria ohridella* in Europe (Valade et al. 2009). At the same time, Cycad Blue hostplants have suffered severe reduction through habitat destruction and collecting for the horticultural trade and for subsequent planting in urban and suburban areas (Donaldson 2003). On the one hand, CITES restrictions forbidding transport of wild cycads without permits may nevertheless have limited gene flow in the Cycad Blue, in accord with our data that show a localized, highly endemic distribution of haplotypes (Fig. 2; Table 2). On the other hand, at present, the planted range of wild cycads is much vaster than native range in Asia, presenting many opportunities for the Cycad Blue to increase its population size and range. A strongly analogous situation is found in another cycad feeding lycaenid that was formerly of conservation concern, the local race of the Atala Hairstreak butterfly (*Eumaeus atala*) in southeastern USA. This butterfly, present in the Caribbean, became extinct between 1937 and 1959 in Florida (Landolt 1984). However, after initial reestablishment in greenhouses, the Atala Hairstreak is now commonly found in southeast Florida feeding on the genera *Zamia* and *Cycas* wherever they are planted horticulturally (Hall and Butler 1995).

The origin of Taiwanese populations

Our results support the hypothesis that Taiwanese outbreak populations, especially in the western part of CMR, were mostly caused by range expansion of the native population. The hypothesis that outbreak populations were delivered by means of direct introduction along with alien cycads was rejected because the major haplotypes (haplotypes A, B, and C) represented the dominant populations which were only found in Taiwan. Western populations (haplotype C) showed a different dominant haplotype from eastern populations (haplotype A, B), supporting a hypothesis of longer coexistence of alien and native populations in Taiwan. However, the haplotype network suggests that haplotype C was derived from haplotype A, occurring only in eastern part of Taiwan (Fig. 3). In this case, the outbreak populations of western populations in Taiwan would have been maintained entirely by horticultural cycads. The Cycad Blue has thus expanded its range through a single rather than through multiple colonization events, to become widespread around the whole island. There is no evidence that the striking

biogeographic difference in haplotypes divided by the CMR (C compared with A and B) is related to a now extinct population of *Cycas* (such as *Cy. taitungensis*) native to western Taiwan, i.e. that the pattern is explained by divergence in allopatry. Taiwanese herbarium records also show that few horticultural cycads were planted in Taipei and southwestern Taiwan before 1950 (plant records at website: http://taif.tfri.gov.tw/taif_en/), and there are no records of *C. pandava* prior to 1976 in Taiwan (Hsu 1987).

Before recent anthropogenically induced outbreaks, Taiwanese populations must have been founded from neighboring regions, considering also that Taiwan is a relatively young island, formed c. 9 Ma (Sibuet and Hsu 2004). Hsu (1987) has pointed out that the source of the Taiwanese population is likely to have been either mainland China or the Philippines archipelago. According to our haplotype joining network, Taiwanese populations are more closely related to populations from mainland China than those in the Philippine archipelagos which lack a direct connection in the network. Moreover, populations in Taiwan could indeed represent a relatively old colonization. Multiple lines of evidence suggest that southeastern Taiwan constituted a Pleistocene refuge (see examples in Cheng et al. 2005 and Lee et al. 2006). The native hostplant, *Cy. taitungensis* has high genetic variance indicating a large population during interglacial stages (Huang et al. 2001), and thus sufficient resources for local survival of *Ch. pandava* over this time. Although Taiwanese populations show only one to four-step mutations from other regions (Fig. 3), the significant population structure among the three subspecies indicates a long period of isolation because none of Taiwanese endemic haplotypes (A–D, and F) was found in other native regions of the Cycad Blue.

The origin of introduced populations of the other regions

Many native species have been threatened or even extinguished when introduced species successfully establish populations in their native habitats. Therefore, an understanding of the origin, biology, and ecology of alien species could help to focus conservation efforts for native species. So far, *Chilades pandava* has already been introduced to many parts of the Old World as far apart as Korea (Takeuchi 2006), Japan (Mitsuhashi 1992; Takegami 2001; Hirai 2009), Hong

Kong in 1978 (Bascombe et al. 1999), Pacific islands including Guam in 2005 (Moore 2008), the neighboring island of Rota in 1996 (Calonje 2007; Moore 2008), Saipan in 1996 (Schreiner and Nafus 1997; Moore et al. 2005) and in the western Indian Ocean in Réunion since 2000 (Martiré and Rochat 2008; Guillermet 2009), Mauritius since 2000 (Williams 2006; Williams 2007), Madagascar since 2006 (this study), and in Florida (SHY, pers. obs. 2006). In some places, the native *Cycas* plants, *Cy. micronesica* (Guam) and *Cy. thouarsii* (Madagascar), are threatened by the Cycad Blue (this paper; Table 6). Our molecular data could provide enough information to quickly ascertain the origin of the haplotypes of different subspecies or populations, and thus provide a phytosanitary monitoring tool. For example, the most likely origin of the Guam populations is from the Philippine archipelago and that of the Korean populations, from Taiwan because the haplotypes found were shared with these

regions (Fig. 2 or Table 2). Tracing the source of Cycad Blue populations is more important in Japan because haplotypes characteristic of two subspecies were detected, *Ch. p. peripatria* found in Okinawa (No. 13) and *Ch. p. pandava* found in Honshū (No. 14–15). Besides, the increasing frequency and wide, rapid range expansion of *Ch. pandava* in Japan (Mitsuhashi 1992; Hirai 2009) increases the urgency to protect the last native region of *Cy. revoluta*, in the Ryukyu Islands of southwestern Japan (Wang et al. 1996). Nevertheless, COII sequence data was unable to establish the origin of Madagascan populations because haplotype O was found in many parts of the present range of *Ch. p. pandava*. Therefore, to improve the identification of the origin of introduced populations, more native populations should be surveyed and more sensitive genetic methods such as microsatellites (e.g. Habel et al. 2008) should be developed to more finely discriminate the origins of *Ch. pandava* outbreaks.

Table 6 Records of *Chilades pandava* on *Cycas* plants

Year	Location	<i>Cycas</i> plant	Reference
Native cycads			
1992	Okinawa, Japan	<i>Cy. revoluta</i>	Mitsuhashi (1992)
1988, 1999–2004	Taiwan	<i>Cy. taitungensis</i>	Hsu (1989); Lan (1999); this study
2005	Guam, USA	<i>Cy. micronesica</i>	Calonje (2007); Moore (2008)
2003–2004	Shenzhen, China	<i>Cy. fairylakea</i>	Gao et al. (2004); Jian et al. (2006); Tang and Oberpreiler (2006)
2006	Madagascar	<i>Cy. thouarsii</i>	S.C. Collins, pers. comm.
2008	Karnataka and Andhra Pradesh, India	<i>Cy. circinalis</i> var. <i>swamyii</i> and <i>Cy. beddomei</i>	Srivstava & de Swing (2008)
Introduced cycads			
1991	Hong Kong, China	<i>Cy. revoluta</i>	Bascombe et al. (1999)
1976–1988	Taiwan	<i>Cy. revoluta</i>	Hsu (1987); Lee (1989); Chang (1989)
1998–2001	Fuzhou, China	<i>Cy. revoluta</i>	Luo and Cai (2002)
1996, 2005	Guam and Saipan, USA	<i>Cy. revoluta</i>	Schreiner and Nafus (1997); Moore et al. (2005)
2000–2001	Reunion Is. and Mauritius Is.	<i>Cy. revoluta</i> , <i>Cy. circinalis</i> , <i>Cy. thouarsii</i>	Rochat (2008); Moore et al. (2005); Guillermet (2009); Williams (2007)
2001–2002	Guangzhou, China	<i>Cy. rumphii</i> , <i>Cy. revoluta</i>	Liu et al. (2003)
2001–2005	Guangxi, China	<i>Cy. revoluta</i>	Wei (2006)
2005	Jeju Is., Korea	<i>Cy. sp.</i>	Takeuchi (2006)
2006	Florida, USA	<i>Cy. revoluta</i>	SHY, pers. obs.
2006	Sambava, Madagascar	<i>Cy. revoluta</i>	DCL, pers. obs.
2006	Osaka and Takarazuka, Japan	<i>Cy. revoluta</i>	This study
2008	Karnataka and Andhra Pradesh, India	<i>Cy. revoluta</i> , <i>Cy. rumphii</i> , <i>Cy. pectinata</i>	Srivstava and de Swing (2008)

Indirect effects of cycad cultivation on native *Cycas* species

Introduced plants have not only the potential to enlarge the distribution of native insects, but also to increase their biomass (Tallamy 2004) and thus herbivore pressure on native plants. In Taiwan, the additional Cycad Blue food resource (*Cy. revoluta*) appears indeed to have augmented the population size of *Ch. pandava*, as apparent in increased levels of plant attack in southern monitoring sites (Lan 1999; Wu et al. unpublished data). Although our data show that western Taiwanese populations seldom disperse to the eastern side, the extra food resource provided by the introduced Sago Palm that are planted abundantly in eastern Taiwan may still greatly increase the overall population size of the Cycad Blue, threatening the survival of the rare native *Cy. taitungensis*. Adding greatly to this threat, another harmful pest, the scale insect *Aulacaspis yasumatsui*, has been introduced to Taiwan with horticultural Sago Palm since 2000 (Germain and Hodges 2007). This scale continuously sucks nutrients from the leaves, stem and primary root until the host dies (Weissling et al. 1999). It is reported that the cycad scale causes high cycad mortality in Guam (Moore et al. 2005) and in Florida (Howard et al. 1999). While our monitoring of native *Cy. taitungensis* initially showed no significant mortality from heavy attacks by *Ch. pandava* (only 4 of 162 cycads observed died between 2000 and 2004, Wu et al. unpublished data), the increased level of herbivory over many years combined with the presence of the new cycad pest *A. yasumatsui* may well jeopardise the continued survival of populations of this endemic and already endangered cycad. This is supported by data on *Cy. taitungensis* in March 2009 (LWW, pers. obs. 2009): 23 of 158 cycads being monitored had died, while the other cycads were in poor condition under the combined attack.

The role of the Central Mountain Ridge on population structure

The Central Mountain Range (CMR) of Taiwan at over 3000 meters elevation provides a primary north-south barrier considered to divide native populations of many species (examples in Peng 2006; Wang et al. 2007). Such a dominant biographic barrier that

clearly structures populations of many species is exceptional for such a small island. The maximal elevation recorded for *Ch. pandava* so far is about 700 m (Hsu 1989). As expected, CMR also serves as an effective barrier to divide the eastern and western populations of this Cycad Blue. The scattered nature of larval hostplants is commonly a significant factor in the population structure of tropical insect herbivores (Ehrlich 1984). However, haplotype distribution and population dynamics of this butterfly are clearly influenced by the CMR (Table 3; Table 5). This barrier may also be reflected in differences in emergence times in eastern and western populations: *Ch. pandava* in Yilan (Fig. 2, No. 6) emerges in September or later whilst populations in Hualian (No. 7) emerge in March (unpublished data).

Future work

Due to widespread cultivation of *Cycas* plants throughout the world (Whitelock 2002), *Chilades pandava* is rapidly becoming an undesirable alien species globally (Donaldson 2003). cursory visual inspections of *Cycas* plants before or during transport are ineffective for finding the well-concealed eggs or larvae of *Chilades pandava*. The haplotype network based on the mitochondrial COII gene has proved useful to distinguish the most likely origin of Taiwanese populations. However, inferences from a single gene may be unreliable (Gompert et al. 2006). In future work, we hope to provide more detailed genetic information that will allow us to determine the demographic histories of Cycad blues on native and introduced *Cycas* populations.

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