

DNA-Based Discrimination of Subspecies of Swallowtail Butterflies (Lepidoptera: Papilioninae) from Taiwan

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Wei-Chih Tsao and Wen-Bin Yeh (2008) DNA-based discrimination of subspecies of swallowtail butterflies (Lepidoptera: Papilioninae) from Taiwan. *Zoological Studies* 47(5): 633-643. Partial sequences of the mitochondrial cytochrome oxidase I (COI) gene of 89 individuals of 34 papilionid species from Taiwan, Hong Kong, and China were determined and compared. The uncorrected nucleotide divergence of COI increased with taxonomic distance: that among individuals within a species was 0%-4.7%, that among species of a given genus was 1.7%-11.6%, and that among genera in the same family was 6.7%-17%. In general, a low level of divergence of the COI sequence was observed among subspecies. Yet, the COI sequence divergence among subspecies of *Byasa alcinous*, *Papilio demoleus*, *Pap. helenus*, *Pap. nephelus*, and *Pazala eurous*, which exceeded 2.1%, was much greater than the average divergence observed for all 34 species. A phylogenetic analysis grouped together members of the same species or genus with high bootstrap values. The phylogenetic tree revealed a lineage of *Chilasa* and *Agehana* followed by *Papilio*, a close affinity between *Byasa* and *Atrophaneura*, and a clade comprised of *Graphium*, *Lamproptera*, *Paranticopsis*, *Pathysa*, and *Pazala*. Sequence variations and phylogenetic analysis results of papilionid COI genes showed that subspecies of *B. alcinous*, *Pap. demoleus*, *Pap. helenus*, *Pap. nephelus*, and *Paz. eurous* from different geographic regions and with wings of slightly different color intensities and spot patterns should probably constitute more than 1 species. Current undifferentiated COI data also suggested that some subspecies of *Pap. bianor*, *Pap. demoleus*, *Pap. memnon*, *Pap. nephelus*, *Pap. paris*, *Pap. polytes*, and *Pap. protenor* might therefore not be completely isolated from each other or only recently dispersed. <http://zoolstud.sinica.edu.tw/Journals/47.5/633.pdf>

Key words: Papilionidae, Swallowtail butterfly, Subspecies, Cytochrome oxidase I, COI.

Many studies have shown that taxon identification based on DNA sequences can facilitate the recognition of known species and the discovery of new species (Blaxter 2005, Hebert et al. 2003a b). DNA sequences of the mitochondrial cytochrome oxidase I (COI) gene can serve as a DNA barcode for identifying all kinds of animals (Hebert et al. 2003a b 2004a, Ward et al. 2005), especially cryptic species in tropical regions (Wilcox et al. 1997, Berkov 2002, Hebert et al. 2004b, Monaghan et al. 2005, Hajibabaei et al. 2006) and insects at different growth stages (Janzen et al. 2005). Although the barcoding region of the 5' end of COI sequences might be no better than that of the 3' end of COI

sequences (Roe and Sperling 2007), the DNA-based system has been used to identify invasive species (Sperling et al. 1995, Armstrong and Ball 2005, Scheffer et al. 2006) and has served as a non-lethal identification method in conservational biology (López et al. 2006, Rubinoff 2006). COI sequence divergence of > 2% was found in 98% of pair-wise comparisons of congeneric species for 11 animal phyla (Hebert et al. 2003b). Other studies showed that COI divergence within lepidopteran species complexes can reach 3.6% (Sperling and Hickey 1994, Sperling et al. 1996, Lee et al. 2005), and in certain cases can exceed 5% (Hebert et al. 2004b). However, Sperling et al. (1999) interpreted the 2 divergent lineages of the looper,

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Lambdina fiscellaria (Lepidoptera: Geometridae), widely distributed in North America with a COI variation of approximately 2%, as being due to genetic polymorphism instead of the presence of a cryptic species. Moreover, the overlapping ranges of intraspecific and interspecific sequence divergences of the COI gene reveal a limitation of the performance of DNA barcoding in insects (Cognato 2006, Meier et al. 2006).

No distinct COI differentiation was observed in many subspecies of *Aglaia urticae* (Lepidoptera: Nymphalidae) from the entire Palaearctic region (Vandewoestijne et al. 2004). Sequence divergences ranging 0%-1.2% were found within many species of *Papilio* widely distributed in Africa and Madagascar (Zakharov et al. 2004). Brower and Jeanstone (2004) reported a COI divergence of < 0.8% among populations of the nymphalid *Danaus plexippus* sampled from North and South America.

Butterflies are among the most popular and studied organisms in Taiwan, and their richness in diversity is reflected in the over 400 species recorded on the island. There have been substantial studies on systematics, morphology, life history, conservational biology, and ecology of butterflies, including of the Papilionidae (Hsu et al. 2004 2005a b c d 2006a b). Yet, their genetic composition and divergence are sparsely documented.

Thirty subspecies of papilionids are currently recognized in Taiwan, 25 of which are also distributed in neighboring areas and may or may not be separate evolutionary units. This study was aimed at establishing the mitochondrial COI sequences of swallowtail butterflies from Taiwan, Hong Kong, and China for taxon identification and, more importantly, to evaluate subspecies differentiation.

MATERIALS AND METHODS

Collection of materials

Sixty-eight individual papilionid butterflies from various localities throughout Taiwan, including Orchid I. (Lanyu in Chinese), along with 21 specimens of swallowtail butterflies from China and Hong Kong, were analyzed in this study. *Polyura eudamippus formosana* of the Nymphalidae and *Luehdorhia*, *Zerynthia*, and *Baronia* of other subfamilies of the Papilionidae were chosen as the

outgroups. Pertinent collecting information is given in table 1. Voucher specimens are being stored at -20°C in the Department of Entomology, Chung Hsing University, Taichung, Taiwan.

DNA extraction, amplification, and direct sequencing

The DNA of 1 butterfly leg (dried or preserved at -20°C) was extracted using a Wizard genomic DNA purification kit (modified from Yeh et al. 2004). The crude DNA dissolved in 100 µl double-distilled water (ddH₂O) was used as a template in the following polymerase chain reaction (PCR).

The primers used to amplify a portion of the mitochondrial COI gene were 5'-TGAGCTCACCAT ATATTTACTGT-3' (i.e., reversed K525.2) (Caterino et al. 2001) and 5'-TCCATTACATATAATCTGCCA TATTAG-3' (PatII) (Caterino and Sperling 1999). Amplification was carried out with 35 cycles in a final volume of 50 µl containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.1% Triton-X100, 2 units of *Taq* DNA polymerase (Protech Technology, Taipei, Taiwan), 0.2 mM of each dNTP, 10 pmoles of each primer, and 2 µl of the DNA template. The reaction used the following temperature profile: denaturation for 1 min at 95°C, annealing for 1 min at 45°C, and extension for 1 min at 72°C. The DNA was directly purified from the amplified product using a PCR purification kit (Qiagen, Hilden, Germany), or after being resolved on an agarose gel, the amplified DNA fragment was excised and extracted with a Qiaquick gel extraction kit. The resulting DNA product was sequenced using a *Taq* dye terminator cycle sequencing kit and an ABI 377A sequencer.

DNA analysis

Sequences of papilionid specimens were piled-up using the program BioEdit (Hall 1999) and aligned with 28 pertinent sequences retrieved from GenBank using the AlignX program of the Vector NTI Advance™ 10 (Invitrogen, Carlsbad, USA), followed by manual refinement. The nucleotide composition of each specimen was calculated using the MEGA3 program (Kumar et al. 2004), and pair-wise distances were estimated using uncorrected proportional distance.

The Neighbor-joining (NJ) method in MEGA3 was applied to construct a phylogenetic tree using the Kimura 2-parameter distance estimate, as the substitution patterns between transversions and transitions differed in this case. A bootstrap

analysis of 1000 replications was carried out on this NJ tree.

RESULTS

Sequence composition and divergence of papilionid COI fragments

The recorded length of the COI sequences of 117 individuals of 34 species ranged from 599 to 707 bases, with variations at 280 positions (40%). All 34 papilionid species had different COI sequences, and the sequences of individuals within each species were either identical or highly similar. However, it was interesting to note that a significant difference in the COI sequences was found in some cases between/among subspecies, such as *Pap. helenus fortunius* and *Pap. h. helenus*.

The uncorrected nucleotide divergence and its distributional frequency were separated into 3 categories: among individuals within species at 0%-4.7%, among species of a given genus at 1.7%-11.6%, and among genera in the same family at 6.7%-17%; and averages within species, genera, and families were 0.004, 0.080, and 0.127, respectively (Fig. 1). Plot analysis of total

substitutions (Tvs) vs. transversions (Tv) and transitions (Ts) revealed that the linear substitution of Tv differed from that of Ts, and substitutional saturation due to multiple hits was observed in these papilionid transitions (data not shown). Sequence divergence in each codon position increased linearly with taxonomic distance, with a substitution rate of about 5: 1: 20 (data not shown).

COI sequence divergence between/among subspecies

Of the 34 species studied, COI sequence divergences were compared for subspecies in 10 *Papilio* species, plus *Byasa alcinous* and *Graphium sarpedon*. Identical or highly similar sequences were observed between/among individuals within each subspecies. For 7 of these 12 species (marked by asterisks in figure 2), the divergence among subspecies was comparable to that within subspecies; and the same was observed for the divergence of some, but not all, subspecies of 2 other species, i.e., *Pap. demoleus* and *Pap. nephelus*.

COI sequence divergences between/among subspecies of *B. alcinous*, *Pap. helenus*, and *Pazala eurous*, as well as some subspecies of *Pap. demoleus* and *Pap. nephelus* (solid triangles

Table 1. Papilionid specimens collected from localities in Taiwan, Hong Kong, and China. Related sequences retrieved from GenBank are shown in the bottom rows

Species	Voucher no.	Locality	Accession no.	Date
<i>Agehana maraho</i> (Shiraki and Sonan)	360	Taiwan: Ilan	AB377313	13 May. 2006
<i>Atrophaneura horishana</i> (Matsumura)	586	Taiwan: Taichung	AB377319	05 Aug. 2006
<i>Byasa alcinous mansonensis</i> (Fruhstorfer)	29	Taiwan: Nantou	AB377314	05 July 2005
<i>Byasa impediens febanus</i> (Fruhstorfer)	259	Taiwan: Pingtung	AB377315	05 Apr. 2006
<i>Byasa polyeuctes termessus</i> (Fruhstorfer)	44	Taiwan: Kaohsiung	AB377316	27 June 2005
<i>Byasa polyeuctes termessus</i> (Fruhstorfer)	45	Taiwan: Kaohsiung	AB377317	27 June 2005
<i>Byasa polyeuctes termessus</i> (Fruhstorfer)	117	Taiwan: Nantou	AB377318	12 Mar. 2006
<i>Chilasa agestor matsumurae</i> (Fruhstorfer)	3	Taiwan: Taipei	AB377320	17 Apr. 2005
<i>Chilasa agestor matsumurae</i> (Fruhstorfer)	106	Taiwan: Taichung	AB377321	11 Mar. 2006
<i>Chilasa epycides melanoleucus</i> (Ney)	4	Taiwan: Taipei	AB377322	17 Apr. 2005
<i>Chilasa epycides melanoleucus</i> (Ney)	144	Taiwan: Taichung	AB377323	31 Mar. 2006
<i>Chilasa epycides melanoleucus</i> (Ney)	261	Taiwan: Pingtung	AB377324	05 Apr 2006
<i>Graphium agamemnon</i> (Linnaeus)	HK4	Hong Kong	AB377325	05 July 2006
<i>Graphium agamemnon</i> (Linnaeus)	445	Taiwan: Pingtung	AB377326	06 July 2006
<i>Graphium cloanthus kuge</i> (Fruhstorfer)	145	Taiwan: Taichung	AB377327	31 Mar. 2006
<i>Graphium doson postianus</i> (Fruhstorfer)	5	Taiwan: Taipei	AB377328	17 Apr. 2005
<i>Graphium doson postianus</i> (Fruhstorfer)	36	Taiwan: Miaoli	AB377329	09 July 2005

Table 1. (Cont.)

Species	Voucher no.	Locality	Accession no.	Date
<i>Graphium doson postianus</i> (Fruhstorfer)	58	Taiwan: Nantou	AB377330	10 July 2005
<i>Graphium doson postianus</i> (Fruhstorfer)	464	Taiwan: Hualien	AB377331	20 July 2006
<i>Graphium sarpedon connectens</i> (Fruhstorfer)	463	Taiwan: Hualien	AB377332	20 July 2006
<i>Graphium sarpedon sarpedon</i> (Linnaeus)	HK2	Hong Kong	AB377333	05 July 2006
<i>Lamproptera curius</i> (Fabricius)	HK1	Hong Kong	AB377334	05 July 2006
<i>Pachliopta aristolochiae interposita</i> (Fruhstorfer)	288	Taiwan: Taipei	AB377335	06 Apr. 2006
<i>Pachliopta aristolochiae interposita</i> (Fruhstorfer)	444	Taiwan: Pingtung	AB377336	06 July 2006
<i>Papilio bianor kotoensis</i> Sonan	140	Taiwan: Taitung	AB377337	21 Mar. 2006
<i>Papilio bianor thrasymedes</i> Fruhstorfer	10	Taiwan: Taipei	AB377338	18 Apr. 2005
<i>Papilio bianor thrasymedes</i> Fruhstorfer	14	Taiwan: Nantou	AB377339	11 June 2005
<i>Papilio bianor thrasymedes</i> Fruhstorfer	20	Taiwan: Nantou	AB377340	05 July 2005
<i>Papilio bianor thrasymedes</i> Fruhstorfer	23	Taiwan: Nantou	AB377341	03 July 2005
<i>Papilio bianor thrasymedes</i> Fruhstorfer	43	Taiwan: Kaohsiung	AB377342	27 June 2005
<i>Papilio bianor thrasymedes</i> Fruhstorfer	468	Taiwan: Hualien	AB377343	20 July 2006
<i>Papilio castor formosanus</i> Rothschild	17	Taiwan: Nantou	AB377344	11 June 2005
<i>Papilio castor formosanus</i> Rothschild	22	Taiwan: Nantou	AB377345	05 Apr. 2005
<i>Papilio castor formosanus</i> Rothschild	289	Taiwan: Taipei	AB377346	06 Apr. 2006
<i>Papilio demoleus libanius</i> (Fruhstorfer)	380	Taiwan: Taichung	AB377347	10 June 2006
<i>Papilio demoleus libanius</i> (Fruhstorfer)	NHRI1301	Taiwan: Taichung	AB377348	30 Sept. 2004
<i>Papilio dialis tatsuta</i> Murayama	41	Taiwan: Kaohsiung	AB377349	27 June 2005
<i>Papilio helenus fortunius</i> Fruhstorfer	55	Taiwan: Nantou	AB377350	10 July 2005
<i>Papilio helenus fortunius</i> Fruhstorfer	262	Taiwan: Pingtung	AB377351	05 Apr. 2006
<i>Papilio helenus helenus</i> Linnaeus	HK13	Hong Kong	AB377352	05 July 2006
<i>Papilio hermosanus</i> Rebel	24	Taiwan: Nantou	AB377353	05 July 2005
<i>Papilio hermosanus</i> Rebel	32	Taiwan: Miaoli	AB377354	09 July 2005
<i>Papilio hermosanus</i> Rebel	498	Taiwan: Hualien	AB377355	21 July 2006
<i>Papilio hopponis</i> Matsumura	39	Taiwan: Kaohsiung	AB377356	27 June 2005
<i>Papilio hopponis</i> Matsumura	40	Taiwan: Kaohsiung	AB377357	27 June 2006
<i>Papilio memnon agenor</i> Linnaeus	HK5	Hong Kong	AB377358	05 July 2006
<i>Papilio memnon agenor</i> Linnaeus	HK6	Hong Kong	AB377359	05 July 2006
<i>Papilio memnon agenor</i> Linnaeus	China6	China: Guizhou	AB377360	15 July 2006
<i>Papilio memnon heronus</i> Fruhstorfer	27	Taiwan: Taichung	AB377361	25 July 2005
<i>Papilio memnon heronus</i> Fruhstorfer	37	Taiwan: Miaoli	AB377362	09 July 2005
<i>Papilio memnon heronus</i> Fruhstorfer	42	Taiwan: Kaohsiung	AB377363	27 June 2005
<i>Papilio memnon heronus</i> Fruhstorfer	467	Taiwan: Hualien	AB377364	20 July 2006
<i>Papilio nephelus chaon</i> Westwood	China3	China: Guizhou	AB377365	15 July 2006
<i>Papilio nephelus chaon</i> Westwood	China4	China: Guizhou	AB377366	15 July 2006
<i>Papilio nephelus chaon</i> Westwood	China9	China: Guizhou	AB377367	15 July 2006
<i>Papilio nephelus chaonulus</i> Fruhstorfer	25	Taiwan: Nantou	AB377368	05 July 2005
<i>Papilio nephelus chaonulus</i> Fruhstorfer	34	Taiwan: Miaoli	AB377369	09 July 2005
<i>Papilio nephelus chaonulus</i> Fruhstorfer	496	Taiwan: Hualien	AB377370	21 July 2006
<i>Papilio paris nakaharai</i> Shirôzu	2	Taiwan: Taipei	AB377371	17 Apr. 2005
<i>Papilio paris nakaharai</i> Shirôzu	38	Taiwan: Taipei	AB377372	10 July 2005
<i>Papilio paris paris</i> Linnaeus	HK14	Hong Kong	AB377373	05 July 2006
<i>Papilio polytes pasikrates</i> (Fruhstorfer)	21	Taiwan: Nantou	AB377374	05 July 2005
<i>Papilio polytes pasikrates</i> (Fruhstorfer)	35	Taiwan: Miaoli	AB377375	09 July 2005
<i>Papilio polytes pasikrates</i> (Fruhstorfer)	438	Taiwan: Pingtung	AB377376	02 July 2006
<i>Papilio polytes pasikrates</i> (Fruhstorfer)	469	Taiwan: Hualien	AB377377	20 July 2006
<i>Papilio polytes polytes</i> Linnaeus	HK10	Hong Kong	AB377378	05 July 2006
<i>Papilio polytes polytes</i> Linnaeus	HK11	Hong Kong	AB377379	05 July 2006
<i>Papilio protenor amauro</i> (Jordan)	30	Taiwan: Nantou	AB377380	05 July 2005
<i>Papilio protenor amauro</i> (Jordan)	33	Taiwan: Miaoli	AB377381	09 July 2005

Table 1. (Cont.)

Species	Voucher no.	Locality	Accession no.	Date
<i>Papilio protenor amauro</i> (Jordan)	46	Taiwan: Nantou	AB377382	11 June 2005
<i>Papilio protenor amauro</i> (Jordan)	56	Taiwan: Nantou	AB377383	10 July 2005
<i>Papilio protenor amauro</i> (Jordan)	66	Taiwan: Pingtung	AB377384	03 Feb. 2006
<i>Papilio protenor amauro</i> (Jordan)	466	Taiwan: Hualien	AB377385	20 July 2006
<i>Papilio protenor euprotenor</i> (Fruhstorfer)	HK7	Hong Kong	AB377386	05 July 2006
<i>Papilio protenor euprotenor</i> (Fruhstorfer)	HK9	Hong Kong	AB377387	05 July 2006
<i>Papilio protenor euprotenor</i> (Fruhstorfer)	China1	China: Guizhou	AB377388	15 July 2006
<i>Papilio protenor euprotenor</i> (Fruhstorfer)	China8	China: Guizhou	AB377389	15 July 2006
<i>Papilio thaiwanus</i> Rothschild	12	Taiwan: Nantou	AB377390	11 June 2005
<i>Papilio thaiwanus</i> Rothschild	13	Taiwan: Nantou	AB377391	11 June 2005
<i>Papilio thaiwanus</i> Rothschild	465	Taiwan: Hualien	AB377392	20 July 2006
<i>Papilio xuthus koxingus</i> Linnaeus	402	Taiwan: Taichung	AB377393	17 June 2006
<i>Paranticopsis xenocles</i> Doubleday	China2	China: Guizhou	AB377394	15 July 2006
<i>Pathysa nomius hainana</i> Chou	China12	China: Hainan	AB377395	2005
<i>Pazala eurous asakurae</i> (Matsumura)	11	Taiwan: Taipei	AB377396	18 Apr. 2005
<i>Pazala eurous asakurae</i> (Matsumura)	170	Taiwan: Nantou	AB377397	01 Apr. 2006
<i>Pazala eurous eurous</i> (Leech)	China13	China: Sychuan	AB377398	2005
<i>Pazala mandarina</i> (Oberthur)	China14	China: Sychuan	AB377399	2005
<i>Pazala timur chungianus</i> (Murayama)	324	Taiwan: Taipei	AB377400	08 Apr. 2006
<i>Troides aeacus kaguya</i> (Nakahara and Esaki)	TZ1	Taiwan: Taipei	AB377401	07 July 2005
<i>Polyura eudamippus formosana</i> (Rothschild)	178	Taiwan: Taoyuan	AB453945	01 Apr. 2006
<i>Atrophaneura alcinous</i>		Japan: Okura	AF170876	
<i>Baronia brevicornis</i>		Mexico	AF170866	
<i>Chilasa epycides melanoleucus</i>		Taiwan: Taoyuan	AY457595	
<i>Graphium agamemnon</i>		SE Asia	AF170874	
<i>Luehdorfia puziloi</i>		Russia	DQ351035	
<i>Pachliopta neptunus</i>		Malaysia: Penang	AF044023	
<i>Papilio bianor</i>		Taiwan: Taipei	AY457572	
<i>Papilio demoleus libanius</i>		Taiwan	AY569057	
<i>Papilio demoleus demoleus</i>		Iran: Hormozgan	AY569053	
<i>Papilio demoleus libanius</i>		Taiwan	AY569059	
<i>Papilio demoleus libanius</i>		Taiwan	AY569060	
<i>Papilio demoleus malayanus</i>		Indonesia: Bali	AY569048	
<i>Papilio demoleus malayanus</i>		Malaysia: Penang	AY569049	
<i>Papilio demoleus malayanus</i>		Malaysia: Perak	AY569050	
<i>Papilio demoleus malayanus</i>		Malaysia: Perak	AY569051	
<i>Papilio demoleus malayanus</i>		Vietnam: Bach	AY569052	
<i>Papilio demoleus malayanus</i>		Thailand: ChingMai	AY569055	
<i>Papilio demoleus malayanus</i>		Thailand: ChingMai	AY569056	
<i>Papilio demoleus malayanus</i>		Malaysia:	AY569058	
<i>Papilio demoleus sthenelus</i>		Australia: NSW	AY569054	
<i>Papilio demoleus sthenelus</i>		Australia: NSW	AY569092	
<i>Papilio demoleus malayanus</i>		Malaysia: Penang	AF044000	
<i>Papilio helenus</i>		Japan: Gifu Pref.	AY457575	
<i>Papilio memnon agenor</i>		Japan: Gifu Pref.	AY457578	
<i>Papilio nephelus chaon</i>		Malaysia: Penang	AY457579	
<i>Papilio paris</i>		China: Guangzhou	AY457574	
<i>Papilio polytes</i>		Japan	AB192474	
<i>Papilio polytes mandane</i>		Malaysia: Penang	AY457580	
<i>Papilio protenor</i>		Japan: Aichi Pref.	AY457581	
<i>Papilio xuthus xuthus</i>		Japan: Tokyo	AF043999	
<i>Zerynthia polyxena cassandra</i>		Italy	DQ351039	

in figure 2) were much greater than the average divergence observed for all 34 species in this study. A sequence divergence of 0.038 was found between the 2 subspecies of *B. alcinous*, *B. a. mansonensis* and *B. a. japonensi*, and a divergence of 0.041-0.042 was found between *Pap. h. helenus* and *Pap. h. fortuneus*. Among the 4 subspecies of *Pap. demoleus*, divergences of 0.031-0.039 were found between *Pap. d. sthenelus* and the other 3 subspecies, with two of the latter, i.e., *Pap. d. malayanus* and *Pap. d. libanius*, having undifferentiated COI sequences. Specimens of *Paz. eurous asakurae* from Taiwan and *Paz. e. eurous* from China had a COI divergence of 0.021. While little COI sequence divergence existed between *Pap. nephelus chaon* from China and *Pap. n. chaonulus* from Taiwan, a considerably greater divergence of 0.044-0.047 was observed between *Pap. n. chaon* specimens from Malaysia and those from subspecies of the former 2 regions.

Phylogenetic analysis of papilionid COI sequences

A phylogenetic tree constructed from the NJ analysis with 1000 bootstrap replications is given in figure 3. Although the barcode analysis mainly seeks to delineate species boundaries, some phylogenetic inferences in COI sequences could still be found. In most cases, the NJ tree shows shallow intraspecific and deep interspecific divergences (Fig. 3). Members of the same species and genus were grouped together and received high bootstrap values. The dendrogram indicates a lineage of *Chilasa* and *Agehana* followed by *Papilio*, a close affinity between *Byasa* and *Atrophaneura*, and a clade comprised of *Graphium*, *Lamproptera*, *Paranticopsis*, *Pathysa*, and *Pazala*.

However, the highly divergent COI in some species lineages implies separate evolutionary

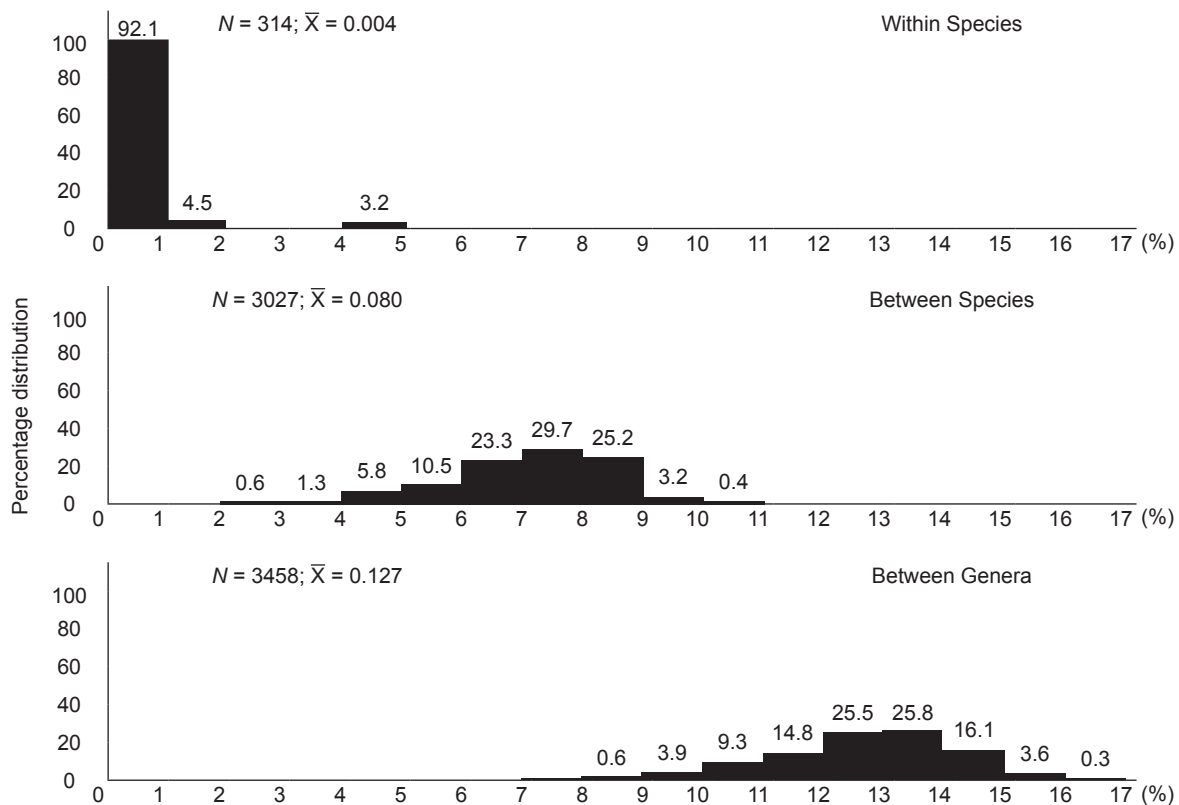


Fig. 1. Distribution of proportion divergences (percent) of cytochrome oxidase I (COI) sequences within different taxonomic categories. Frequencies of < 0.3% are not shown. Numbers of pair-wised comparisons (*N*) and the average divergences of each category are shown.

histories (the solid arrow in figure 3). Individuals of *Pap. heleus fortuneus* from Taiwan formed a highly divergent lineage from the conspecific subspecies *Pap. h. helenus* from Hong Kong and Japan. Specimens of *Pap. nephelus chaon* from Malaysia had a long branch length with its conspecific subspecies. Other differentiated subspecific branches were also found for *Pap. demoleus*, *Pap. eurous*, and *Atrophaneura alicinous*. Furthermore, in addition to the confusion at the subspecies level, there are problems in the generic recognition of specimens of *Atrophaneura alicinous* from Japan, which were classified as *B. alicinous mansonensis* in Taiwan.

The dendrogram also reveals that some subspecies are inseparable and produce a mixed cluster (dashed arrows in figure 3). For example, specimens of *Pap. protenor amaura* from Taiwan formed an intermingled cluster with its subspecies *Pap. p. euprotenor* from China and *Pap. p. protenor* from Japan; and so did the 2 subspecies of *Pap. bianor thrasymedes* and *Pap. b. kotoensis*.

DISCUSSION

No or little variation was found within the species of *Pap. groesmithi*, *Pap. morondavana*, and *Pap. demodocus* from different localities in Africa and Madagascar (Zakharov et al. 2004). COI divergences within the lepidopteran species complex of *Yponomeuta*, *Choristoneura*, *Feltia*, and *Archips* were reported to range 0%-0.9%, 0.1%-2.9%, 1.8%-3.7%, and 1.5%-2.5%, respectively (Sperling and Hickey 1994, Sperling et al. 1995 1996, Kruse and Sperling 2001). More than 98% of the 13,320 pairwise comparisons of congeneric species showed > 2% COI divergence for 11 animal phyla (Hebert et al. 2003b). In a study of 260 avian species of North America, Hebert et al. (2004a) proposed a standard threshold of 10 times the mean intraspecific variation for different congeneric species. However, DNA barcoding in 1333 COI sequences for 449 species of dipteran insects showed high intraspecific variability and low identification (Meier

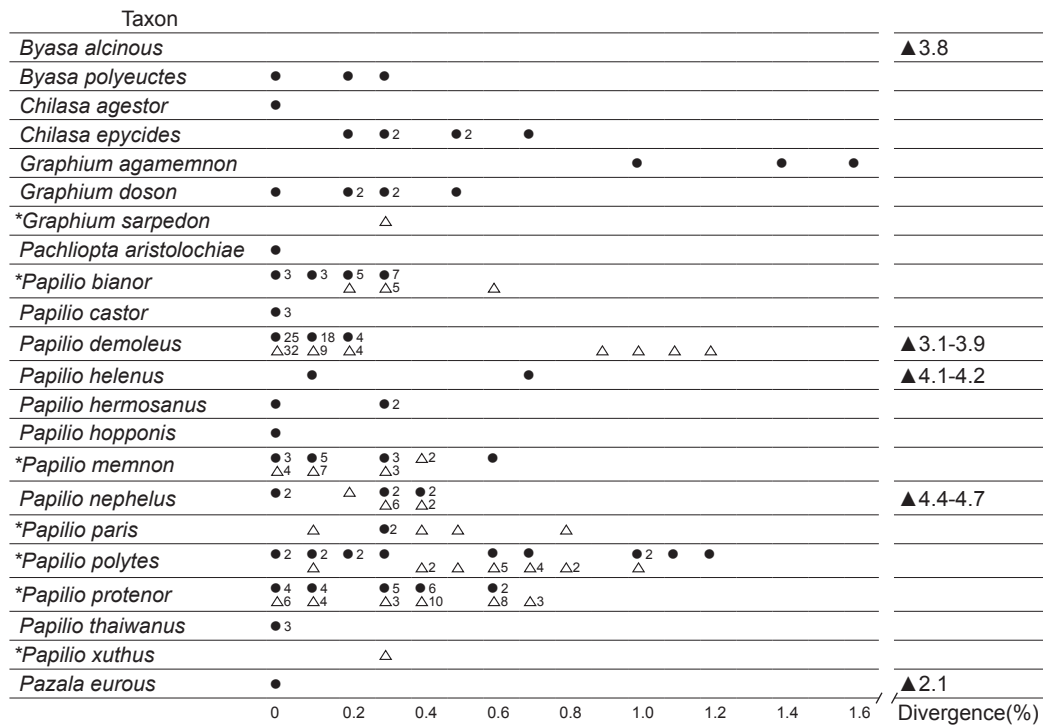


Fig. 2. Proportional divergences within subspecies (circles) or between subspecies (triangles) in a given species. The numerals beneath the signals are the number of pairwise comparisons. The solid triangle in the far right column indicates that with the greatest divergence between subspecies in a given species. The asterisk (*) indicates comparable divergences within a subspecies compared to among species.



Fig. 3. Phylogenetic tree constructed from cytochrome oxidase I (COI) sequences by the Neighbor-joining method. Bootstrap values are shown beneath the branches. Solid-line arrows indicate great differentiation between the 2 dichotomous subspecies lineages, while dotted-line arrows indicate an intermingled lineage between subspecies.

et al. 2006). Cognato (2006) also stated that the percent sequence divergence does not predict insect species boundaries. In the present work, COI divergence among conspecific individuals was generally less than that among congeneric species (Fig. 1). While little difference in COI sequences between subspecies was found in most cases, significant differences between subspecies was observed in 5 species (Fig. 3).

Alterations in lepidopteran species and subspecies recognition brought about by DNA studies have recently received extensive attention (Brower and Jeansonne 2004, Omoto et al. 2004, Vandewoestijne et al. 2004, Zakharov et al. 2004, Katoh et al. 2005, Lee et al. 2005). The present analysis showed a striking COI sequence divergence between *Pap. demoleus* subspecies from the Oriental Region and the Australian subspecies, *Pap. d. sthenelus*. In view of significant differences in both nuclear and mitochondrial DNA between these subspecies, Zakharov et al. (2004) proposed that *Pap. d. sthenelus* be raised from *Pap. demoleus* to a separate species. In addition, for *B. alcinous*, *Pap. helenus*, *Pap. nephelus*, and *Pap. eurous*, subspecies with striking genetic differentiation and yet wings of slightly different color intensities and spot patterns could all likely be constituted of more than 1 species.

The different morphs of *Pap. bianor thrasymedes* from the main island of Taiwan and *Pap. b. kotoensis* from Orchid I. (80 km off the southeastern coast of Taiwan) (Hamano 1987, Chao and Wang 1997), which showed no difference in the COI sequences (Fig. 3), could have been induced through environmental adaptations. Subspecies of the other 8 papilionids, i.e., *G. sarpedon*, *Pap. demoleus*, *Pap. memnon*, *Pap. nephelus*, *Pap. paris*, *Pap. polytes*, *Pap. protenor*, and *Pap. xuthus* from Taiwan and neighboring areas also possessed non-differentiated COI sequences (dashed line in figure 3). A lack of differentiation among COI sequences may result from gene flow between geographical populations or imply a recent migration. For example, Vandewoestijne et al. (2004) suggested that the non-differentiated COI sequence of *Aglais urticae*, which has at least 3 subspecies distributed from Europe to Japan in the Palaearctic Region, was caused by gene flow during a recent population expansion.

Sequence variations in the papilionid COI gene observed in this study illustrate that subspecies lineages can present apparently

separate evolutionary histories or unclear subspecific divisions. We thus have to ask: How many of the subspecies of widespread Oriental butterfly species, such as *Pap. protenor*, *Pap. polytes*, *Pap. memnon*, etc. distributed in Taiwan, China, Korea, Japan, and southern Asia, are really taxa of unitary evolutionary entities? The answer to this question can help refine specific and subspecific classifications and provide precise estimates of butterfly diversity in the Orient. A close genetic similarity between subspecies reported in the literature for many widely distributed butterflies, including *Aglais urticae* distributed in the Palaearctic (Vandewoestijne et al. 2004), *Papilio* spp. in Africa and Madagascar (Zakharov et al. 2004), *Pap. demoleus* in Southeast Asia (Zakharov et al. 2004), and *Danaus plexippus* throughout North and South America (Brower and Jeansonne 2004), indicates their panmictic units. On the other hand, it has been suggested that the subspecies *Pap. demoleus sthenelus* in Australia, with a distinct COI sequence in contrast to the other 3 Southeast Asian subspecies, be raised to a separate species (Zakharov et al. 2004). Nazari and Sperling (2007) also demonstrated that deep divergences in COI sequences between different subspecies or populations of 4 Parnassiinae species might indicate that they constitute more than 1 species.

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