

# Phylogenetic position of a threatened stag beetle, *Lucanus datunensis* (Coleoptera: Lucanidae) in Taiwan and implications for conservation

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**Abstract** Among nine endemic *Lucanus* beetles in Taiwan, *L. datunensis* is the island's smallest and most threatened species. It currently exists as only one population located in tall grasslands of Mt. Datun in the Yangmingshan National Park. Given the isolated population, unique subtropical grassland, and the threats resulting from human activities, *L. datunensis* raises immediate conservation concern for its long-term survival. Phylogenies reconstructed from combined mitochondrial cytochrome oxidase subunit 1 (1310 bps) and nuclear wingless (436 bps) genes were resolved and placed *L. datunensis* as a phylogenetically distinct species sister to *L. fortunei* from China. All 13 examined individuals of *L. datunensis* shared just one mitochondrial haplotype suggesting extremely low mitochondrial DNA diversity and a small effective population size. *L. datunensis* and morphologically closest *L. miwai* were distantly related and appear to have evolved in parallel the life history traits of a small body size and diurnal mate-searching behavior. We hypothesize that these habitat-associated characters are convergent adaptations that have evolved in response to shifts from forests to grasslands.

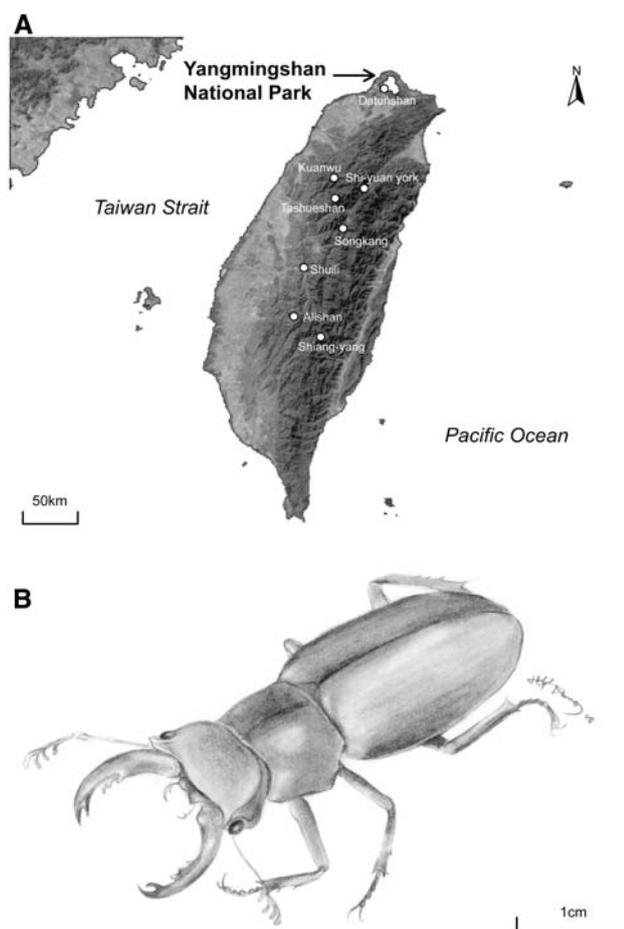
**Keywords** Yangmingshan National Park · *Cox1* · *Wingless* · Convergent adaptation · Subtropical grassland

## Introduction

The stag beetle family, Lucanidae Latreille 1804, contains over one thousand described species worldwide, and exhibits its highest species diversity in the Oriental region (Krajcik 2001; Smith 2006). Males of many lucanid species possess curving and greatly enlarged mandibles that often are used in male–male competition for access to females (Clark 1977; Kawano 1992). For their charismatic and greatly exaggerated mandibles, the stag beetles are among the most enthusiastically collected insect groups by amateur collectors and insect vendors (Goka et al. 2004; New 2005). Mass capturing for commercial purposes and destruction of suitable habitats by human activities inevitably cause the regional and global decline of stag beetle populations, and may threaten their long-term survival (Speight 1989; Berg et al. 1994; Kotze and O'Hara 2003).

*Lucanus datunensis* Hashimoto 1984 is an endemic stag beetle species of Taiwan, and occurs exclusively at one single locality, Mt. Datun (Datunshan) within the Yangmingshan National Park (Fig. 1). With an average body size of males ranging from 25 to 38 mm (females, 23–27 mm), *L. datunensis* has the smallest body and mandible size among the nine endemic *Lucanus* species in Taiwan (Chang 2006). The tall grassland habitat of *L. datunensis* is located between approximately 800 and 1,100 m, where the vegetation is dominated by dwarf bamboo, *Pseudosasa usawai* and Japanese silvergrass, *Miscanthus floridulus* (Poaceae) (Chou and Li 1991). During the monsoon season from May to July, the diurnal males are found actively flying from one plant to another above the grassland possibly searching for females (Chang 2006). Although the habitat of *L. datunensis* is located within a protected natural area, this subtropical grassland is subject to high recreational use and is susceptible to disturbance from frequent geothermal, seismic

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**Fig. 1** **a** The map of Taiwan showing the location of the Yangmingshan National Park and the collecting sites of endemic *Lucanus* species, and **b** a male of the threatened *Lucanus datunensis* (drawing by Hsuan-Yu Peng)

activities of volcanic Mt. Datun (Lin et al. 2005a, b; Konstantinou et al. 2007), and to habitat reduction from recently decreased bamboo distribution caused by mass blooming (Liu et al. 2008). Given its isolated population, unique grassland habitats, high degree of endemism, and the threats resulting from human activities, *L. datunensis* is a stag beetle of immediate concern for its long-term survival. Conservation efforts are frequently motivated by the premise that the threatened species is a distinct evolutionary entity. Before introducing any conservation management strategies, knowledge of phylogenetic relationships among morphologically similar stag beetles is essential to determine whether *L. datunensis* is indeed evolutionarily distinctive among endemic stag beetles in Taiwan and Asian mainland.

Compared with the isolated habitat of *L. datunensis*, the other two morphologically similar *Lucanus* species, *L. miwai* Kurosawa, 1996 and *L. swinhoei* Parry, 1874 have a wider distribution, and are found in mid-elevational forests in north and central Taiwan. Of all endemic *Lucanus* species in

Taiwan, males of both *L. datunensis* and *L. miwai* can be recognized as having yellowish spots on their femora and tibiae. However, no distinct morphological character was found to distinguish between these two species. On the other hand, males of *L. swinhoei* can be separated from *L. datunensis* and *L. miwai* by the presence of an enlarged posterior process of the head and a larger body size. Like *L. datunensis*, males of *L. miwai* are also diurnal and actively fly above tall grasslands by the edge of forests between April and May.

In this study, we used both mitochondrial and nuclear DNA sequences to reconstruct phylogenies among Taiwanese *Lucanus* stag beetles and related Asian species. Our aims were: (1) to investigate if individuals of *L. datunensis* form a genetically distinct lineage from related *Lucanus* species; (2) if *L. datunensis* is closely related to *L. miwai* and other endemic *Lucanus* species in the region; (3) discuss the adaptive significance of habitat-associated life history traits in *L. datunensis* and *L. miwai*.

## Methods

### Taxon sampling and DNA sequencing

Adult males of *L. datunensis* were captured using insect nets along a hiking trail leading to the top of Mt. Datun (25°10'26.54" N, 121°31'18.41" E) in June of 2007. Field-collected beetles were immediately preserved in 95% ethanol, followed by long-term storage at  $-80^{\circ}\text{C}$  freezers. Beetles of other lucanid species were either collected at various localities in Taiwan, or obtained through an insect supplier (<http://www.insect-sale.com>) (Table 1). Genomic DNAs were extracted from thoracic muscle using the MasterPure<sup>TM</sup> DNA Purification Kit (EPICENTRE<sup>®</sup> Biotechnologies, Madison, WI) following the protocol listed in the manual. A DNA fragment of approximately 1.3 kbps in the mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene was amplified using a *Lucanus*-specific primer set, *Lucanus*-COI-J-1751 (5'-GA GCTCCTGATATAGCTTTTCC-3') and *Lucanus*-TL2-N-3014 (5'-CCAATGCACTAATCTGCCATATTA-3') (modified from Simon et al. 1994). A nearly 500-bp fragment of the nuclear *wingless* (*wg*) gene was amplified using two primer sets, Wg1a/Wg2a (Brower and DeSalle 1998) and Wg1a/*Lucanus*-Wg2a (5'-TTGCACCTTTTCGACGATGGC GATCTC-3'). PCR products were extracted using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan), and then cloned into competent cells (dH-5 $\alpha$ ) (Protech, Taipei, Taiwan) using T&A cloning kit (RBC, Taipei, Taiwan). DNA sequencing was performed on an ABI PRISM<sup>TM</sup> 377 automatic sequencer (Perkin Elmer, USA) by the Mission Biotech, Taiwan. DNA sequences used in this study were deposited in GenBank (Accession nos. FJ606539–FJ606581, FJ606620, FJ606679).

**Table 1** Collecting information for stag beetles included in the present study

Genus	Species	Locality	Country	Collector	Date
<i>Lucanus</i>	<i>cervus cervus</i>	Basses Pyrenees	France	Insect-Sale	2007
	<i>datunensis</i>	Datunshan, Taipei	Taiwan	C.-Y. Kuan	2007/06
	<i>formosanus</i>	Alishan, Chiayi	Taiwan	J.-P. Huang	2005/07
	<i>fortunei</i>	San-Ming, Fujian	China	Insect-Sale	2005
	<i>fryi</i>	Chiang Mai	Thailand	Insect-Sale	2005
	<i>hayashii</i>	NA	Myanmar	Insect-Sale	2005
	<i>hermani</i>	Wu Yi Shan, Fujian	China	Insect-Sale	2006
	<i>kanoi kanoi</i>	Songkang, Nantou	Taiwan	C.-A. Chen	2007
	<i>kanoi piceus</i>	Shi-Yuan York, Yilan	Taiwan	C.-A. Chen	2007
	<i>kurosawai</i>	Kuanwu, Shinchu	Taiwan	L.-J. Wang	2005
	<i>laetus</i>	Yunnan	China	Insect-Sale	2006
	<i>laminifer laminifer</i>	Wiang Papao	Thailand	Insect-Sale	2005
	<i>maculifemoratus taiwanus</i>	Alishan, Chiayi	Taiwan	J.-P. Huang	2006/07
	<i>miwai</i>	Songkang, Nantou	Taiwan	C.-A. Chen	2006
	<i>ogakii</i>	Shiang-Yang, Taitung	Taiwan	C.-A. Chen	2007
	<i>planeti</i>	Yunnan	China	Insect-Sale	2006
	<i>sericeus</i>	Chiang Mai	Thailand	Insect-Sale	2005
	<i>swinhoei</i>	Songkang, Nantou	Taiwan	C.-A. Chen	2007
	<i>szetschuanicus szetschuanicus</i>	Leshan, Sichuan	China	Insect-Sale	2005
	<i>tibetanus isakii</i>	Kachin	Myanmar	Insect-Sale	2005
<i>Dorcus</i>	<i>schenklingi</i>	Shuili, Nantou	Taiwan	J.-P. Huang	2005/09
<i>Neolucanus</i>	<i>doro doro</i>	Shuili, Nantou	Taiwan	J.-P. Huang	2006/11
<i>Prismognathus</i>	<i>formosanus</i>	Tashueshan, Taichung	Taiwan	J.-P. Huang	2005/09
<i>Prosopocoilus</i>	<i>astacoides blanchardi</i>	Taoyuan, Kaoshiung	Taiwan	J.-P. Huang	2005/06

### Sequence alignment and phylogenetic analyses

Individual DNA sequences were aligned using the Clustal W method in MegAlign (DNASTAR, Madison, USA). *Prosopocoilus astacoides blanchardi* (Parry 1873) was used as an outgroup taxon for phylogenetic analyses. We used PAUP\* program (version 4.0b10, Swofford 2002) to search for the most parsimonious (MP) trees. Heuristic tree searches were done for each gene and the combined data with equally weighted characters. Parsimony branch supports were calculated using non-parametric bootstrapping (Felsenstein 1985) of 1,000 iterations, each with 10 stepwise random sequence additions and tree-bisection and reconnection (TBR) branch swapping. For Bayesian phylogenetic analyses, the best fitted models of nucleotide substitution for each gene were determined separately in MODELTEST (ver. 3.7, Posada and Crandall 1998) using Bayesian Information Criterion (BIC) (Posada and Buckley 2004; Alfaro and Huelsenbeck 2006) [GTR + I +  $\Gamma$  (lnL = -11,801.634, BIC = 23,675.045) for *cox1*; TrN +  $\Gamma$  model (lnL = -1,531.760, BIC = 3,099.987) for *wg*].

We calculated the Bayesian Posterior Probabilities (BPP) of the resulting trees using MrBayes (ver. 3.1.2, Huelsenbeck and Ronquist 2001). Two independent Bayesian analyses

with random starting trees were done. In each analysis, two independent runs were performed simultaneously and each run containing four Markov Chains with the default heating values of 1, 1.1, 1.2 and 1.3. The MCMC searches were performed for  $1 \times 10^7$  generations with Markov chains being sampled every 100 generations. MCMC searches were terminated after the average split frequencies of two runs fell below the value of 0.01 and the Convergence Diagnostic Potential Scale Reduction Factor (PSRF) reached the value of 1 (Gelman and Rubin 1992). The initial 20,000 sampled trees were discarded as burnin. The remaining 80,000 trees were imported into PAUP\* to compute a 50% majority rule tree.

### Results and discussion

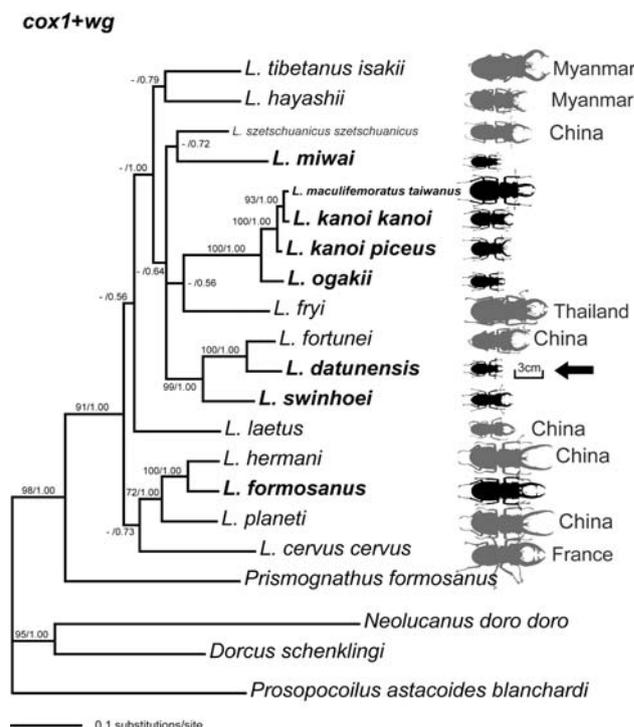
A total of 1310 bps of *cox1* were obtained for 13 individuals of *L. datunensis*. All 13 examined *L. datunensis* individuals exhibit a uniform mitochondrial haplotype (mtDNA haplotype diversity,  $h = 0$ ). Given its low level of mtDNA diversity, our results imply that the effective population size ( $N_e$ ) of this isolated *L. datunensis* population is probably very low. Threatened or endangered

species with small population sizes regularly suffer from inbreeding, reduction in fitness, accumulation of deleterious mutations, and loss of genetic diversity (Frankham et al. 2002). Loss of genetic diversity due to sustained restriction of population size is one of the immediate concerns for conservation genetics of endangered species. Further studies are needed to more precisely quantify the level of genetic diversity and the effective population size in this isolated population, and to investigate whether the lack of genetic variability is likely a threat to the persistence of *L. datunensis*.

A sequence alignment of 1310 (*cox1*) and 436 (*wg*) nucleotide sites was obtained for 24 taxa. The PCR amplification of *wg* DNA fragment was unsuccessful for three *Lucanus* species (*kurosawai*, *laminifer laminifer*, and *sericeus*). Of a total of 624 and 112 variable sites, 462 and 59 were parsimony informative characters in *cox1* and *wg* respectively. Equal weights parsimony analyses of *cox1* and *wg* found two and 258 equally parsimonious trees respectively (tree length = 2,610 and 180 steps, trees not shown). The relationships among all species were resolved based on a combined data set (Fig. 2). The monophyly of all eight endemic *Lucanus* species of Taiwan was not supported; rather, the tree suggested that the Taiwanese *Lucanus* formed four separated lineages nested within the *Lucanus* species of China and Southeast Asia. *L. formosanus* was the basal lineage of all endemic *Lucanus* in Taiwan and closely related to *L. hermani* of Fujian province of China. The phylogeny also revealed a sister relationship between *L. datunensis* and *L. fortunei* from Fujian province of China. In addition, *L. swinhoei* was sister to *L. datunensis* + *L. fortunei*, whereas *L. miwai* being morphologically the closest to *L. datunensis*, was clustered with *L. szetschuanicus szetschuanicus* from Sichuan province of China.

Phylogenies reconstructed from analyses of *cox1* and *wg* strongly support that *L. datunensis* is a phylogenetically distinct lineage among all endemic *Lucanus* species of Taiwan, and firmly place *L. datunensis* as a close relative of Chinese *L. fortunei* and Taiwanese *L. swinhoei*, but distantly related to the ecologically and morphologically similar *L. miwai* in Taiwan. This finding provides the first evidence to show the existence of substantial genetic divergence (i.e., branch length) in both mitochondrial and nuclear genes between *L. datunensis* and *L. miwai*, as well as among endemic *Lucanus* species in Taiwan. Therefore, *L. datunensis* should be recognized as a separate phylogenetic species and considered a priority of conservation management for its species status, unique grassland habitat, restricted distribution, and high human disturbance.

Based on the reconstructed phylogenies, two distantly related but morphologically similar species, *L. datunensis* and *L. miwai* appear to have evolved in parallel the life history traits of small body size and diurnal mate-searching



**Fig. 2** ML phylograms resulted from the Bayesian phylogenetic analyses based on combined *cox1* and *wg* genes. Numbers near the nodes are support values of parsimony bootstrap (left) and Bayesian posterior probability (right). Nodes without support values are those have values below 50%. Endemic *Lucanus* species of Taiwan are labeled in bold. The images of stag beetles were scaled to reflect the relative body sizes among species

behavior in grassland habitats. Whereas the closely related species of *L. datunensis* and *L. miwai*, such as *L. szetschuanicus szetschuanicus* and *L. swinhoei* and *L. fortunei* respectively, in general are larger, nocturnal, forest-dwelling stag beetles, in which most males are found to feed on plant saps in the daytime and only attracted to light in the evening (Knell et al. 2003; Chang 2006). Therefore, the life history characteristics observed in *L. datunensis* and *L. miwai* are likely to represent a derived (apomorphic) condition not found in the common ancestors of these beetles. We hypothesize that diurnal mate-searching flights and small body size in these two species are convergent adaptations that have evolved in response to a habitat shift from forests to grasslands. The active diurnal mate-searching flight above tall grasslands in these two species may have the selective advantages over a more widespread behavior of male–male competition for resources and associated females as observed in most forest-dwelling *Lucanus* (Harvey and Gange 2006; Rink and Sinsch 2007). The small body size of *L. datunensis* and *L. miwai* possibly represents an evolutionary consequence of feeding on the decayed bamboo and silvergrass, which may have a lower nutritional content than that of decayed woods in the forests (Silver and Miya 2001; Bienkowski et al. 2006). Under

these adaptive scenarios, protection and restoration of the grassland habitat consisted of the dwarf bamboo and Japanese silvergrass in Yangmingshan National Park would become the first priority for conservation planning of the threaten *L. datunensis*.

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