Temporal dynamics of the mimetic allele frequency at the *doublesex* locus, which controls polymorphic Batesian mimicry in *Papilio memnon* butterflies

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Tracking allele frequencies is essential for understanding how polymorphisms of adaptive traits are maintained. In *Papilio memnon* butterflies, which exhibit a female-limited Batesian mimicry polymorphism (wing-pattern polymorphism), two alleles at the *doublesex* (*dsx*) locus correspond to mimetic and non-mimetic forms in females; males carry both *dsx* alleles but display only the non-mimetic form. This polymorphism is thought to be maintained by a negative frequency-dependent selection. By tracking *dsx* allele frequencies in both sexes at a Taiwanese site over four years, we found that the mimetic allele persists at intermediate frequencies even when the unpalatable model papilionid butterflies (*Pachliopta* and *Atrophaneura* species) were very rare or absent. The rates of male mate choice did not differ between the two female forms; neither did insemination number nor age composition, suggesting equivalent reproductive performance of the two forms over time. Our results characterised the temporal dynamics of the mimetic allele frequency in the field for the first time and give insights into underlying processes involved in the persistence of the female-limited Batesian mimicry polymorphism.

Batesian mimicry is a textbook example of evolution by natural selection1,2. In Batesian mimicry, palatable mimics avoid predation by resembling unpalatable models1. The advantage of this mimicry is negatively frequency-dependent, decreasing when the relative abundance of Batesian mimics to unpalatable models is high because of quick learning by predators2,3. The most intriguing phenomenon regarding Batesian mimicry in butterflies is the female-limited polymorphism, in which females display both mimetic and non-mimetic forms, but males are non-mimetic4,5. Female-limited mimicry is attributed to stronger predation pressure upon females relative to males, resulting in a greater fitness advantage of mimicry only in females4-7. Moreover, butterfly colouration may be subject to sexual selection (e.g., female choice8 and male-male competition9), which may constrain the evolution of mimicry in males despite potential benefits to both sexes. Female-limited mimicry polymorphisms in butterflies are thought to be maintained by negative frequency-dependent selection (NFDS) acting on different female wing patterns, in which the advantage of mimetic forms decreases as their frequency increases10,11. To understand the mechanism of maintenance for mimicry polymorphisms, it is necessary to study the dynamics of mimic, model and predator populations while monitoring changes in the frequency of mimicry-related alleles. However, field-based population genetic studies of mimicry polymorphisms are challenging due to a lack of knowledge about the genetic basis of the polymorphism.

Recent genomic studies of *Papilio polytes*12,13 have revealed that mimetic/non-mimetic phenotypes are controlled by two alleles at the *doublesex* (*dsx*) locus, which corresponds to the hypothesised supergene that controls female phenotypes in a Mendelian fashion; the mimetic allele *H* is dominant to the non-mimetic allele *h*14-16. We demonstrated that *P. memnon* also possesses similar alleles that strictly control female phenotypes17. These findings provide a simple genotyping method for studying temporal changes in mimetic allele frequencies in...
Species were substantially less abundant. Although we collected almost all butterflies encountered, males of Papilio (an indicator of bird attack\(^7\); but see Burger & Gochfeld \(^20\) for criticism of the use of this indicator). Although the female forms suggests that they can attain similar levels of fitness if the predation pressure is equal. We did not find evidence of differential selection on the two female forms, although this result might be largely affected by insufficient sample sizes in the present data.

In P. memnon, age structure (estimated by wing wear) did not significantly differ among males, mimetic females and non-mimetic females (Table 1). The forewing lengths of females were significantly longer than those of males but did not differ between mimetic and non-mimetic forms (Fig. S2, Supplementary Information; Table S4). Forewing lengths in February–April were shorter than in other months in both sexes and both female forms (Fig. S2; Table S4). Older females contained more spermatophores in their bursa copulatrix regarding their form (Tables 2 and S5). We found no evidence of a male preference for either of the female forms (male choices for mimetic and non-mimetic forms, 27:29; binomial test; \(P = 0.042\); not significant after Bonferroni correction). Thus, we could not show differential selection on the two female forms, although this result might be largely affected by insufficient sample sizes in the present data.

We genotyped the \(d\) locus for 303 males and 99 females of P. memnon (Table S2, Supplementary Information). The genotype frequencies did not depart from the expectations of Hardy-Weinberg equilibrium at any of the sampling times (Table S2). The frequency of the \(H\) allele at each sampling time ranged between 0.23 and 0.5 for males (average, 0.35) and between 0.1 and 0.5 for females (average, 0.37) (Fig. 1D). The estimated allele frequency was controlled by NFDS, we analysed the relationship between the \(H\) allele frequency and hence favor polymorphisms, should remain constant (in fact wing wear data suggested that our samples consisted mainly of aged individuals; see below). We examined whether the \(H\) allele frequency differed between the sexes in each sampling time but found no significant case (12 sampling times; Fisher’s exact test, \(P = 0.042\); not significant after Bonferroni correction). Therefore, we could not show differential selection on the two female forms, although the frequency did fluctuate throughout the course of the study (Table S5, Supplementary Information).

To see whether the \(H\) allele frequency was controlled by NFDS, we analysed the relationship between the \(H\) allele frequencies in male and female populations at each sampling time. We assumed that the \(H\) allele frequency was identical between the sexes at the time of adult emergence but differential selection on the female forms caused changes in the \(H\) allele frequency sometime after emergence only in females; the allele frequency in males, which show no mimetic wing patterns or wing pattern polymorphisms, should remain constant (in fact wing wear data suggested that our samples consisted mainly of aged individuals; see below). We examined whether the \(H\) allele frequency differed between the sexes in each sampling time but found no significant case (12 sampling times; Fisher’s exact test, \(P = 0.042\); not significant after Bonferroni correction). Therefore, we could not show differential selection on the two female forms, although this result might be largely affected by insufficient sample sizes in the present data.

We found that the abundance of model species was relatively low throughout the year, except in early spring (Fig. 1). Although the condition of fewer models than mimics would hinder the evolution of monotypic Batesian mimicry and hence favor polymorphisms, such a condition may also diminish the protection offered by Batesian mimicry\(^15\). At our study site, the abundance of models peaked in spring (February–March) but declined to levels much lower than the abundance of mimetic P. memnon females by summer (Fig. 1C). A similar phenological trend of a model species, A. polyeuctes, has been reported in southern Taiwan\(^24\). The different phenological trends of these papilionids may be related to the difference in larval host plants; P. memnon uses wild and cultivated trees of Rutaceae, whereas the model species (A. polyeuctes and A. febanus) woody vines of Aristolochia (Aristolochiaceae). Although the reversal of the relative abundance of mimics to models could be disadvantageous for mimics, the frequency of the mimetic allele did not decrease in the absence of models during these times (Fig. 1C,D; Table S5). The advantage of mimicry may persist until later seasons due to the efficacy of predator learning in the spring. In addition, the presence of abundant alternative prey in later seasons may allow for the persistence of mimics with low model abundance. The availability of alternative prey has been shown to alter model-mimic relationships\(^25\). Alternatively, the persistence of the mimetic form despite a low abundance of models may be more fundamentally caused by the strong psychological impact of toxic models on bird foraging.
behaviour. A recent avian study revealed that birds tend to avoid prey types with variable toxicity, implying that birds will avoid mimics even when the encounter rate of highly unpalatable models is lower than that of palatable mimics. Therefore, the predation risk for mimics will remain low unless the relative abundance of mimics to models becomes very high. Our results support the previously reported evidence that model abundance does not have to be high for Batesian mimicry to persist.

In our previous study, we found that the mimetic allele frequency in *Papilio memnon* in July at nine localities throughout Taiwan ranged between 0.36 and 0.55. Together with the present results, these data indicate that the

Figure 1. Results of the field study in Hualien, eastern Taiwan. (A) *Papilio memnon* and its model species, *Atrophaneura febanus* and *Atrophaneura polyeuctes*. (B) Mean number of *P. memnon* males collected per line-transect census. (C) Mean numbers of individuals of the Batesian mimicry models *A. febanus* and *A. polyeuctes* and mimetic *P. memnon* females collected per line-transect census. Another model, *Pachliopta aristolochiae*, was collected only once, in July 2013, and is not shown. (D) Frequencies of the *doublesex* H allele (mimetic allele) in males and females of *P. memnon*. Genomic DNA extraction failed for females in July 2015; therefore, the min-max (dotted vertical line) and intermediate values of the *H* allele frequency were estimated from the numbers of mimetic and non-mimetic females collected.
The mimicry polymorphism of *P. memnon* appears to persist at moderate levels of the mimetic allele frequency both spatially and temporally in Taiwan. The precise mechanism of the allelic frequency change is unclear, but it is possibly caused by negatively frequency-dependent predation on different female phenotypes. Additional studies of predator behaviour and the mortality processes of the two female forms are needed to understand the selection pressures acting on the wing patterns of *P. memnon* butterflies.

**Methods**

**Line-transect census of wild butterflies.** We conducted 71 line-transect censuses in mixed habitats, including a pomelo (*Citrus maxima*) orchard and a wasteland at Hualien, eastern Taiwan (23°59′N, 121°32′E; elevation: 565 m) over 17 time periods from July 2013 to September 2016. At each census, a researcher (SK) walked both directions of a fixed route (1000 m) along a road for 1 h, collecting all papilionid butterflies observed. In addition, all male and female *P. memnon* butterflies seen outside of the hour-long census periods were collected.

**Genotype and allele frequencies.** Total genomic DNA was extracted from the legs or thorax of *P. memnon* using a Genomic DNA Purification Kit (Promega, Madison, WI, USA). High-Resolution Melting analysis using a real-time PCR platform was performed to identify *dsx* genotypes following Komata et al.17. The proportion of different *dsx* genotypes in each month were evaluated in terms of Hardy-Weinberg equilibrium using the R 3.0 package HardyWeinberg31. See Table S2 (Supplementary Information) for more information.

**Age structure, body size, beak mark rate and lifetime mating number.** Age was estimated based on wing wear rankings: 1, little or no wing wear; 2, moderate wing wear and 3, heavy wing wear. Variations among ages in males, mimetic females and non-mimetic females were analysed using Fisher’s exact probability test. Forewing length was measured using electronic digital callipers except for individuals with severely damaged forewings. Marks larger than 5 mm in length on butterfly wings were recorded as “beak marks” by bird attacks7. We dissected the bursa copulatrix from *P. memnon* females to count the number of spermatophores, which is an indication of the number of times the female has mated.

**Male mate choice experiment.** Experiments were conducted on 9–12 June and 15–17 July, 2014, at the same site as the line-transect censuses. A pair of mimetic and non-mimetic specimens was displayed in the foliage of pomelo trees in June, while in July three pairs were displayed in the same way at 3–4-m intervals. The distance between the two specimens was about 40 cm, and their positions were changed daily. The experiment was conducted in the morning (5:30–10:00), when males actively search out females for mating. After confirming courtship behaviour to either specimen, males were collected using an insect net to avoid double counts and for later analysis of their *dsx* genotypes.
Statistical analyses. We explored the effect of sampling season (season) on the number of *P. mennon* males collected per census using a generalised linear mixed model (GLMM) assuming Poisson distribution. Each year was divided into three sampling seasons (January–April, May–August and September–December) for all analyses. The sampling year (year) was included as a random effect. The waved length was analysed with a GLMM assuming a Gaussian distribution with season and sex/form as fixed effects, and year as a random effect.

In analyses using *dsx* mimic allele frequencies in males and females, beak mark rate, and the number of spermatophores, we performed GLMM analyses with Akaike's Information Criterion corrected for small sample size (AICc) to determine the model with the optimal set of explanatory variables. The *dsx* mimic allele frequencies in males and females were analysed with a GLMM assuming a binomial distribution with season and the number of models (total number of *A. polyeuctes, A. febanus* and *Pachliopta aristolochiae* collected in the field) as fixed effects, and year as a random effect. The beak mark rate (individuals with beak marks/total number of individuals) was analysed using a GLMM with a binomial distribution, with season, forewing length, age and sex/form as fixed effects. The number of spermatophores was analysed using a Poisson distribution with forewing length, age, and sex/form as fixed effects. The sampling time period (n = 16) was treated as a random effect for analyses of the beak mark rate and the number of spermatophores. For mate choice, the selection of mimetic/non-mimetic specimens was analysed using a binomial test, after confirming the effects of male genotype and experimental settings with a generalised linear model analysis assuming a binomial distribution with AICc to determine the model with the optimal set of explanatory variables. All analyses were performed using R packages lme4 and MuMIn.

References

1. Bates, H. W. Contributions to an insect fauna of the Amazon Valley (Lepidoptera: Heliconiidae). *Trans. Linn. Soc. (Lond.)* **23**, 495–556 (1862).
2. Ruston, G. D., Sherratt, T. N. & Speed, M. P. Avoiding Attack: the Evolutionary Ecology of Cryptis, Warning Signals and Mimicry. (Oxford University Press, 2004).
3. Huheey, J. E. Mathematical models of mimicry. *Ann. Nat.*** **131**, 522–541 (1988).
4. Wallace, A. R. On the phenomena of variation and geographical distribution as illustrated by the Papilionidae of the Malay Region. *Trans. Linn. Soc. (Lond.)*** **25**, 1–71 (1865).
5. Kunte, K. Female-limited mimetic polymorphism: a review of theories and a critique of sexual selection as balancing selection. *Anim. Behav.* **78**, 1029–1036 (2009).
6. Turner, I. R. G. Why male butterflies are non-mimetic: natural selection, sexual selection, group selection, modification and sieving. *Biol. J. Linn. Soc.* **10**, 385–432 (1978).
7. Ohsaki, N. Preferential predation of female butterflies and the evolution of Batesian mimicry. *Nature*** **378**, 173–175 (1995).
8. Belt T. The Naturalist in Nicaragua. London, UK: E. Bumpus 1874.
9. Lederhouse, R. C. & Scriber, J. M. Intrasexual selection constrains the evolution of the dorsal color pattern of male black swallowtail butterflies. *Papilio polyxenes. Evolution* **50**, 717–722 (1996).
10. Poullon, E. B. Essays on Evolution. (Clarendon, 1908).
11. Barrett, J. A. The maintenance of non-mimetic forms in a dimorphic Batesian mimic species. *Evolution*** **30**, 82–85 (1976).
12. Kunte, K. et al. *doublesex* is a mimicry superegen. *Nature*** **507**, 229–232 (2014).
13. Nishikawa, H. et al. A genetic mechanism for female-limited Batesian mimicry in Papilio butterfly. *Nat. genet.* **47**, 405–409 (2015).
14. Clarke, C. A. & Sheppard, P. M. The genetics of the mimic butterfly *Papilio polylepis*. *Phil. Trans. R. Soc. Lond, B*** **263**, 431–458 (1972).
15. Clarke, C. A., Sheppard, P. M. & Thornton, I. W. B. The genetics of the mimetic butterfly *Papilio mennon*. *Phil. Trans. R. Soc. Lond, B*** **254**, 37–89 (1968).
16. Clarke, C. A. & Sheppard, P. M. Further studies on the genetics of the mimetic butterfly *Papilio mennon*. *Phil. Trans. R. Soc. Lond, B*** **263**, 35–70 (1971).
17. Komata, S., Lin, C.-P., Ijima, T., Fujiiwara, H. & Sota, T. Identification of *doublesex* alleles associated with the female-limited Batesian mimicry polymorphism in *Papilio mennon*. *Sci. Rep.* **6**, 34782 (2016).
18. Ohsaki, N. A common mechanism explaining the evolution of female-limited and both-sex Batesian mimicry in butterflies. *J. Anim. Ecol.* **74**, 728–734 (2005).
19. Jiggins, C. D. The Ecology and Evolution of Heliconius Butterflies: A Passion for Diversity. (Oxford University Press, 2017).
20. Burger, J. & Gochfeld, M. Smooth-billed ani (*Crotophaga ani*) predation on butterflies in *Mato Grosso*, Brazil: risk decreases with increasing group size. *Behav. Ecol. Sociobiol.* **49**, 482–492 (2001).
21. Waldbauer, G. P. Asynchrony between Batesian mimics and their models. *Am. Nat.* **131**, S103–S121 (1988).
22. Pfennig, D. W., Harcombe, W. R. & Pfennig, K. S. Frequency-dependent Batesian mimicry. *Nature*** **410**, 323 (2001).
23. Mallet, J. & Joron, M. Evolution of diversity in warning color and mimicry: polymorphism, shifting balance, and speciation. *Annu. Rev. Ecol. Syst.* **30**, 201–233 (1999).
24. Umero, A. Butterflies in the Kuraru Region of Heng-chun, Taiwan (I). *Zephyrus* **5**, 239–258 (1934).
25. Kokko, H., Mappes, J. & Lindström, L. Alternative prey can change model–mimic dynamics between parasitism and mutualism. *Ecol. Lett.* **6**, 1068–1076 (2003).
26. Barnett, C. A., Bateson, M. & Rowe, C. Better the devil you know: avian predators find variation in prey toxicity aversive. *Biol. Lett.* **10**, 20140533 (2014).
27. Howarth, B., Edmunds, M. & Gilbert, F. Does the abundance of hoverfly (*Syrphidae*) mimics depend on the numbers of their hymenopteran models? *Evolution*** **58**, 367–375 (2004).
28. Ries, L. & Mullen, S. P. A rare model limits the distribution of its more common mimic: a twist on frequency-dependent Batesian mimicry. *Evolution*** **62**, 1798–1803 (2008).
29. Davis Rabosky, A. R. et al. Coral snakes predict the evolution of mimicry across New World snakes. *Nat. Commun.* **7**, 11484 (2016).
30. R Core Team. R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria, 2016). http://www.R-project.org/
31. Graffelman, J. Exploring Diallelic GeneticMarkers: The HardyWeinberg Package. *J. Stat. Soft.* **64**, 1–22 (2015).
32. Bates, D., Maechler, M., Bolker, B. M. & Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).
33. Barton, K. MuMln: multimodel inference. R package version 1.15.6. See CRAN:CRAN-R-project.org/package=MuMln (2015).

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S.K., C.L. and T.S. conceived the study; S.K. conducted experiments, field study and data analyses; S.K. and T.S. wrote the paper.

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