Hybrid Origins: DNA Techniques Confirm that Papilio nandina is a Species Hybrid (Papilionidae)

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HYBRID ORIGINS: DNA TECHNIQUES CONFIRM THAT *PAPILIO NANDINA* IS A SPECIES HYBRID (PAPILIONIDAE)

Additional key words: engrailed, museum collection, mtDNA, *Papilio dardanus*, Princeps

The idea that a significant number of named species will subsequently be discovered to be species hybrids has long been accepted by botanists, even though establishing particular hybrid origins was rarely straightforward. The application of molecular techniques is rapidly changing this field, and clear-cut demonstrations of hybrid origin are now possible (e.g. Siripun & Schilling 2006). However, in a recent survey of "bad species" among butterflies it was estimated that "around 16% of the 440 European butterfly species are known to hybridize in the wild" (Descimon & Mallet 2009: p219). Although hybridisation can lead to new biological species (Kunte et al. 2011), species hybrids clearly represent a taxonomic problem that needs to be addressed by lepidopterists and, as we endeavour to demonstrate here, molecular methods can and surely will play a particularly valuable role in future investigations of putative hybrid origins.

Papilio nandina was described as a new species by Rothschild and Jordan (1901), based on two male specimens caught in East Africa. Butterflies with the *nandina* phenotype are extremely rare in nature but others have been collected since. Initially, Carcasson (1960) considered *P. nandina* to be an aberration of *Papilio phorcas ruscoei* Krüger, 1928. Then, in the 1970s, Carcasson suggested it was a hybrid between the species *Papilio dardanus* Yeats in Brown, 1776, and *P. phorcas* Cramer, 1775 (see Vane-Wright 1976; Vane-Wright et al. 1999; Clarke 1980), with the absence of females possibly explained by Haldane's rule (but see Vane-Wright & Smith 1992). Clarke & Sheppard (1975) and Clarke (1980) succeeded in crossing *P. dardanus* and *P. phorcas* using the hand pairing method (Clarke & Sheppard 1956) and found that the males produced strongly resembled *P. nandina*. It was therefore proposed that wild-caught individuals of *P. nandina* were hybrids and the existence of such a hybrid was (cautiously) given as evidence supporting the grouping of *P. dardanus* and *P. phorcas* as sister taxa.

The present study examines *Papilio nandina* from a molecular perspective. Using the butterfly collections of the Natural History Museum London, we have now extracted DNA from specimens of *P. dardanus* (Voucher BMNH746801-746802, BMNH746805-746806), *P. phorcas* (including a pinned specimen from the 'Majerus Collection'; BMNH808404, BMNH740210-740213), a wild-caught *P. nandina* (collected in 1984 in City Park, Nairobi; Gill, 1986; Figure 4 and accompanying information in Vane-Wright & Smith 1992; BMNH808400), and a 'laboratory' cross of *P. dardanus* and *P. phorcas* (pinned, from the 'Clarke/ Sheppard/ Gill Collection'; Clarke 1991; BMNH808401).

DNA was extracted from single legs according to the protocols of Thomsen et al. (2009). Amplifiable DNA was extracted from all specimens, demonstrating that usable DNA can be obtained from pinned butterfly specimens collected over 25 years ago. Individuals were sequenced for the mitochondrial gene COI (primers HCO2198 and LCO1490; Folmer et al. 1994) and the nuclear gene engrailed (primers: Pd202: 5'-agccagtacacygcaccac-3' and Pd204: 5'-tcyccgatctgmracaccgtctg-3'; 387 base pair amplicon). Sequences were submitted to GenBank (HQ636437-HQ636452).

If the wild-caught *P. nandina* is a hybrid as proposed, then we would expect the nuclear genome to be inherited 50:50 from both *P. dardanus* and *P. phorcas*, and in this respect to be indistinguishable from that of the 'laboratory' hybrid. This is exactly what is found: sequence traces reveal that the *P. nandina* individual carried a distinct *P. dardanus* and a distinct *P. phorcas* allele. Out of 46 polymorphisms revealed in the engrailed sequence, 24 are fixed in both *P. dardanus* and *P. phorcas* with the *P. nandina* individuals displaying the corresponding ambiguity, 6 show shared polymorphisms between *P. nandina* and one of the other species and 16 are uninformative (polymorphic in only one of *P. dardanus* or *P. phorcas*).

The COI fragment from the wild-caught *P. nandina* exactly matches sequences obtained in this study from *P. phorcas* and differs only at a single position from the *P. phorcas* sequence available on GenBank (AF044001; Caterino & Sperling 1999). Mitochondrial DNA is only inherited from the female parent, therefore the wild *P. nandina* specimen is a hybrid between a male of *P. dardanus* and a female *P. phorcas*.

Our results confirm that *P. nandina*, as first suggested by Carcasson, and subsequently demonstrated by Clarke & Sheppard (1975) and Clarke (1980) by breeding experiments, and by Vane-Wright & Smith (1991) on morphological grounds, is not a 'good' species, but represents a species hybrid (Vane-Wright & Smith 1992).

Given that the male parent of the one wild-caught *nandina* that we have been able to analyze must have

been *P. dardanus*, it is interesting to note that the males of this species are demonstrably promiscuous with respect to female color patterns, consistent with the amusing comment of W. C. Hewitson following the recognition of female-limited polymorphism in *P. dardanus* (then *P. merope*) by Roland Trimen: "it would require a stretch of the imagination, of which I am incapable, to believe that the *P. Merope* [sic] of the mainland, having no specific difference, indulges in a whole harem of females, differing as widely from it as any other species in the genus." (quoted by Trimen 1874: p140; see Cook et al. 1994 for field observations on mate choice by male *P. dardanus*). Whether or not all wild *nandina* hybrids are sired by *P. dardanus* is a matter for speculation at this point, but it should be remembered that many populations of *P. phorcas* also exhibit female-limited polymorphism—although this is not so spectacular as that seen in *P. dardanus* (Vane-Wright & Boppré 1993).

This molecular investigation demonstrates the value of pinned collections as a source of both morphological and molecular data, and the importance of molecular studies for taxonomy. A similar methodological approach has already been used to investigate another demonstrably hybrid "species", *Erebia serotina* Descimon & de Lesse, 1953, as reported by Descimon & Mallet (2009). The value of the technique presented here lies in the fact that it is not dependent on fresh material; we propose the use of both mitochondrial and nuclear markers on museum material as a valuable tool to assess putative hybrids.

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