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SCIENTIFIC OPINION

SHOULD HYPOTHESES CONCERNING SPECIES STATUS BE CONSIDERED ALONGSIDE OTHER HYPOTHESES IN GENETIC STUDIES OF SPECIES COMPLEXES? A RESPONSE TO VAN BORTEL AND COOSEMANS 2003

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Anopheles mosquitoes exist typically as complexes of closely related species. This was not recognized in early studies, but over time, complex members have been uncovered by the application of morphological, chromosomal, cross-mating, and molecular analyses. Even today, when studying the genetic diversity of known complex members, it is prudent to bear in mind that there is an alternative hypothesis of the existence of other species in the complex. At present, there are 2 generally accepted members of the *Anopheles minimus* complex in mainland Southeast Asia—*An. minimus* species A and C—but several studies have suggested that additional species might exist there (Yu and Li 1984, Yu 1987, Baimai 1989, Sharpe et al. 2000, Somboon et al. 2001). (It is worth noting that suggesting an additional species might exist is not the same as proposing a new species.) In the latest of these papers (Somboon et al. 2001), our suggestion was based on novel sequence variants of the D3 loop of 28S ribosomal DNA and our interpretation of existing allozyme data in Van Bortel et al. (1999).

In their response to Somboon et al. (2001), Van Bortel and Coosemans (2003) said that "it is premature to decide on whether the observed variation at the D3 region reflects intra- or inter-specific patterns" (p. 262). We fully agree with this. There are at least 2 hypotheses that could explain the observed genetic data in the *An. minimus* complex: the existence of previously undetected cryptic species and intraspecific variation within the known species. Although alluded to in Van Bortel et al. (1999), hybridization was only put forward explicitly as a 3rd possible hypothesis in Van Bortel and Coosemans (2003). In our opinion, there is insufficient evidence to reject any of these hypotheses at the present time. The 1st 2 were considered in Somboon et al. (2001), in which we made the cautious statement that we "tentatively suggest the possibility of up to 4 species in the *An. minimus* complex in Vietnam" (p. 262). Clearly, we were not, and are not, adamant that additional species exist within the *An. minimus* complex—merely that there is the possibility that they do and that this possibility should not be overlooked.

Specific points were raised by Van Bortel and Coosemans (2003) that we would like to respond to. First, there is the issue of heterozygote deficiency at the allozyme loci, *Ldh* and *Gpi,* that persists in the *Odh* form II group after splitting *An. minimus* s.l. into 2 groups on the basis of the *Odh* genotype (Van Bortel et al. 1999). Van Bortel et al. (1999) used *Odh* forms I and II to define samples as *An. minimus* A and C, respectively, following the work of Green et al. (1990) in Thailand, although there are more *Odh* alleles in Vietnam than Thailand. As we noted in Somboon et al. (2001), the heterozygote deficiency within form II (*An. minimus* C) is suggestive to us of unresolved species. Van Bortel et al. (1999) and Van Bortel and Coosemans (2003) state that this deficiency only occurred once when there was a relatively high proportion of hybrids in the sample. In fact, the presence of hybrids would, if anything, lead to an excess of heterozygosity, rather than a deficiency, although at the low levels of hybridization noted (<1%), this is unlikely to be detectable. Van Bortel and Coosemans (2003) suggest that hybridization might explain the heterozygote deficiency, but their explanation seems unnecessarily complex. Furthermore, they offer no previous examples in which heterozygote deficiency is associated with hybridization. The commonest alleles at the *Ldh* and *Gpi* loci are at a high frequency within form II (0.985 and 0.958, respectively, over all the sites and collection times; Van Bortel et al. 1999:table 6), so heterozygote deficiency is presumably because of the presence of a small number of rare-allele homozygotes. If these rare-allele homozygotes do belong to a cryptic species, they might be present at a low level in the human- and cattle-baited collections made. The fact that heterozygote deficiency was only detected at 1 site at 1 time (in the largest sample size) might not therefore be surprising, particularly because species abundance can vary not only with collection method but geographically and seasonally. However, the simple chance occurrence of an unlikely combination of rare alleles cannot be excluded.

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Second, as Van Bortel and Coosemans (2003) point out, we clearly did not have a large enough sample size of *An. minimus* from Vietnam to estimate fixation indices for the D3 sequence data, which is why we did not do this. We did note that given the previous observations of the lack of within-species genetic diversity at this locus over a wide geographical area, the observation of variation was surprising. We also noted that it was unexpected for these novel alleles to occur in the homozygous state but acknowledged that "the sample size is too small to be conclusive" (Somboon et al. 2001, p. 111). A homozygous state was inferred from an unambiguous peak on an electropherogram, whereas the presence of a polymorphic site in an individual can typically be detected by the presence of overlapping peaks of 2 bases (providing alleles are equally represented, as is expected if the template is abundant).

Third, Van Bortel and Coosemans (2003) raised the issue that there might have been errors of mislabeling or contamination in the samples we used. As always, it is impossible to rule out such a possibility, although we believe it to be unlikely. In any case, it would not offer an alternative explanation for variation in the D3 data because the novel sequences cluster within the *An. minimus* clade. We also think that *Taq* error is an extremely unlikely explanation for the novel sequences because it would require that multiple errors had been introduced (including 2 in 1 fragment of <400 base pairs) in early rounds of amplification. Although a technical possibility (Hillis et al. 1996), it is really only a problem when the concentration of template is very low, which is particularly unlikely in a multicopy locus.

Fourth, Van Bortel and Coosemans (2003) comment on the greater variation present in *An. minimus* in Vietnam relative to Thailand and draw the comparison with *Anopheles gambiae* s.s. west and east of the Rift Valley in Kenya. This analogy is particularly interesting because the debate as to whether or not the different chromosomal and molecular forms of *An. gambiae* s.s. in West Africa should be considered separate species continues actively and is the focus of considerable research efforts. A recent review concludes that the S and M molecular forms are at the very earliest stages of speciation (della Torre et al. 2002). Speciation and the acquisition of full reproductive isolation can be viewed as a process (Wu 2001) rather than an event, and species at the earliest stages of this process will be those most difficult to detect. If species exist in the *An. minimus* complex that have not yet been recognized, they are likely to fall into this category.

Neither the hypothesis of intraspecific polymorphism nor that of the presence of additional cryptic species as explanations for the genetic diversity in *An. minimus* in Vietnam can be rejected as yet. In order to reject one of these hypotheses, we agree with Van Bortel and Coosemans (2003) that further research is required and that this will require a population genetic approach with the use of multiple loci.

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