What is the role of the *neverland* gene in fungus-feeding insects?

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**Introduction**

In insects, the enzyme Neverland converts cholesterol to 7-dehydrocholesterol by introducing a double bond [1], a reaction necessary for molting hormone production. However, fungus-feeding insects consume large amounts of ergosterol, a molecule related to cholesterol (see Figure 2) which already contains this double bond. I therefore hypothesised that the *neverland* gene may develop alternative functions in insects with exclusively fungus diets, such as the attine ants, through mutations which change the Neverland protein sequence. Natural selection in favour of amino acid substitutions can be detected by calculating the ratio of nucleotide substitutions which alter the protein (K_r) to those which do not (K_s) in cross-species gene comparisons. If Neverland catalyses different reactions in attine and non-attine ants, then we expect to see a high K_r/K_s value when comparing Neverland genes between these groups.

**Methodology**

I used the *neverland* protein sequence from the vinegar fly *Drosophila melanogaster* to identify Neverland genes in seven attine and three non-attine ant species, as well as in eleven additional *Drosophila* and three other fly species. As none of these flies exclusively consume fungi, this group serves as a negative control. These gene searches used BLAST [2], a program which assesses DNA or protein sequence similarity. When the probability of a match occurring by chance was less than 1 x 10^-5, I classified the sequence in question as a potential homologue. When comparing the *neverland* sequences to that of *Drosophila* and other flies (0.173) I calculated a p-value of 18.4%, indicating similar selective pressure in both groups. Both these values are similar to the intra-attine mean, with a p-value of 22.0% for a comparison with the *Drosophila*-other fly data.

I also observed multiple copies of *neverland* much more frequently in attine ants than in the other groups. Of the seven attine species, four had two *neverland* genes and two had three. In contrast, none of the other ants and only two of fifteen fly species seemed to have two *neverland* genes, and these may simply represent the two alleles of a diploid genome. As ant genome sequences derive from haploid males, such errors cannot explain their apparent duplications.

**Results**

The mean K_r/K_s ratios (see Table 1) of *neverland* sequence pairs within attine ants (0.173) and between attine and other ants (0.132) reveal selective pressure to maintain the Neverland protein sequence in attine ants, as ratios less than one indicate an excess of mutations which do not cause amino acid substitution. Contrary to my hypothesis, this pressure appears significantly stronger between attine and other ants than within the former group; the probability that a difference as large or greater than the one between these values will arise by chance (the p-value) is around 0.1%. 5% is the usual threshold for excluding chance variation between groups as an explanation for observed differences. In contrast, when comparing the mean *neverland* K_r/K_s ratio within *Drosophila* (0.186) to that between *Drosophila* and other flies (0.173) I calculated a p-value of 18.4%, indicating similar selective pressure in both groups. These values are similar to the intra-attine mean, with a p-value of 22.0% for a comparison with the *Drosophila*-other fly data.

**Discussion**

The low K_r/K_s ratio of *neverland* genes in attine ants does not support my hypothesis that natural selection will favour functional changes in this gene in fungus-feeding species. However, we do observe a minor increase in the proportion of mutations which alter the Neverland protein sequence within the attine ants. The presence of multiple *neverland* genes in many of these species may explain this finding; mutations altering enzyme activity will have less severe fitness effects if a second copy of the affected gene can compensate. Alternately, as the inter-attine mean K_r/K_s ratio was similar to that of flies, selection against Neverland protein sequence changes may be unusually strong in non-attine ants. This hypothesis could be tested by measuring this ratio in additional insect groups.

There are several reasons for caution in interpreting these results. Mean K_r/K_s ratios were estimated from a small number of species, and my exclusion of sequence pairs in which fewer than 25% of nucleotides were identical (repeated substitution at a large proportion of sites prevents accurate K_r calculation in these cases) further reduced my dataset. In addition, the software used for protein reconstruction was not optimised for ant genes, potentially mean K_r/K_s value was similar to that of flies, selection against Neverland protein sequence changes may be unusually strong in non-attine ants. This hypothesis could be tested by measuring this ratio in additional insect groups.

**Conclusion**

The low proportion of substitutions which alter the Neverland protein sequence in the fungus-feeding attine ants suggests maintenance of its role in 7-dehydrocholesterol production in these species. How this activity benefits these ants is not clear; one potential explanation is that their diet, contrary to previous assumptions, contains substantial quantities of sterols lacking the double bond Neverland introduces in addition to ergosterol.

**References**


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