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Regressive evolution of an eye pigment gene in independently evolved eyeless subterranean diving beetles

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Regressive evolution, the reduction or total loss of non-functional characters, is a fairly common evolutionary phenomenon in subterranean taxa. However, the genetic basis of regressive evolution is not well understood. Here we investigate the molecular evolution of the eye pigment gene *cinnabar* in several independently evolved lineages of subterranean water beetles using maximum likelihood analyses. We found that in eyeless lineages *cinnabar* has an increased rate of sequence evolution, as well as mutations leading to frame shifts and stop codons, indicative of pseudogenes. These results are consistent with the hypothesis that regressive evolution of eyes proceeds by random mutations, in the absence of selection, that ultimately lead to the loss of gene function in protein-coding genes specific to the eye pathway.

Keywords: regressive evolution; eye gene; *cinnabar*; subterranean water beetle; Dytiscidae

1. INTRODUCTION

Regressive evolution has been an area of considerable interest to evolutionary biologists (Darwin 1859; Kosswig 1960; Crandall & Hillis 1997; Culver & Wilkens 2000; Yamamoto *et al.* 2000). One of the key questions is how the loss of function of a character affects the genes that are involved in its developmental pathway and whether loss of a character is a constructive process driven by selection or results from a loss of selection and accumulation of random mutations (Culver & Wilkens 2000). The latter hypothesis predicts that protein-coding genes that are no longer under functional constraints will evolve like pseudogenes: non-synonymous (amino-acid changing) substitutions will evolve at a similar rate to synonymous (silent) substitutions and the coding region may accumulate mutations leading to stop codons or frameshifts. There has been a lack of empirical support for the hypothesis that regressive evolution of eyes is associated with loss of protein-coding gene function at the molecular level, with the most convincing case being a loss of function of the interphotoreceptor retinoid binding protein gene in a marsupial mole (Springer *et al.* 1997). In contrast, a study of *αA-crystallin* in the blind mole-rat (Smulders *et al.* 2002) and *rhodopsin* in cave-dwelling crayfish (Crandall & Hillis 1997) did not find evidence for loss of function, although in the latter study a method was used that may not have had sufficient power to rule out a loss of selective constraint (Leebens-Mack & dePamphilis 2002).

A unique system to test the latter hypothesis is provided by the subterranean diving beetles (Coleoptera: Dytiscidae) that occur in the numerous isolated aquifers of the arid interior of Australia. Over 34 isolated aquifers have been found that are each inhabited by up to five endemic eyeless species (e.g. Watts & Humphreys 2004). Phylogenetic analyses have shown that the majority of species (greater than 80) independently evolved from a small number of widespread, eyed surface species approximately 5 million years ago (Leys *et al.* 2003). These characteristics of the beetle fauna make it an ideal group to study the evolution of genes potentially showing a loss of selective constraints, because their relatively old age elevates the chance of detecting sufficient numbers of mutations, and the numerous independent eyeless lineages are likely to increase the power of the tests.

Here we investigate the eye pigment gene *cinnabar*. The *cinnabar* gene, which codes for kynurenine 3-monooxygenase, has been well studied in several insects, including *Drosophila melanogaster* and the beetle *Tribolium castaneum* (Lorenzen *et al.* 2002). Genomic and functional analyses in these species suggest it is a single copy gene. Mutations in *cinnabar* are known to alter the pigmentation of insect eyes, but do not appear to affect the fitness of individuals, suggesting that the involvement of the gene in other developmental pathways is unlikely (Lorenzen *et al.* 2002). Therefore, the gene provides an ideal candidate to assess whether regressive evolution of the eye is associated with loss of protein-coding gene function in the eye development pathway.

2. MATERIAL AND METHODS

(a) *Taxon sampling*

We attempted to incorporate as many of the subterranean (eyeless) and surface (eyed) lineages of the diving beetle species as possible. Included here are 11 subterranean species, of which nine are completely eyeless and two have strongly reduced eyes with tiny spots of eye pigment, and nine normal-eyed surface species. A table with taxon data, localities and GenBank accession numbers associated with this paper is available in the electronic supplementary material.

(b) *Molecular methods*

DNA extraction and sequencing were performed as described in Cooper *et al.* (2002).

PCR amplification was carried out using a two-step nested PCR procedure as specified by Lorenzen *et al.* (2002), using the following primers: 5′-AAYTAYYTNCAYATHTGGCC-3′(CN5-forward) and 5′-RTARTTRTACATNGC-3′(CN6-reverse), 5′-ACNTTYATGATGATHGC-3′(CN7-forward) and 5′-CNNG CRTTCATNCCYTGNCC-3′(CN8-reverse). A second nested reverse primer was developed specifically for the diving beetles: 5′-TCCAYRTAATTRTACATIGCCARITC-3′(CN4-reverse). All amplifications were run under the same conditions: one cycle 94 °C 9 min, and 40 cycles 94 °C 45 s, 48 °C 45 s, 72 °C 60 s.

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Table 1. Likelihood ratio tests (LRT).
(*Averages (very short branches excluded).)

model	free rates	fixed rates	likelihood	ω_e	ω_b	test	d.f.	LRT	p
(A) $\omega_e=\omega_b$	1	0	−2342.412	0.0602	0.0602				
(B) $\omega_e\neq\omega_b$	2	0	−2325.488	0.0351	0.1614	A versus B	1	33.847	<0.001
(C) $\omega_e\neq\omega_b=1$	1	1	−2366.656	0.0330	1.0000	C versus B	1	82.336	<0.001
(D) $\omega_e\neq\omega_b$ free	10	0	−2307.576	0.0343	0.1747*	B versus D	8	35.824	<0.001
(E) ω_e, ω_b free	38	0	−2294.460	0.0559*	0.1736*	D versus E?	28	26.232	0.5603

Amplifications included 1× reaction buffer (Perkin Elmer, Boston, MA), 0.1 mM of each dNTP, 2.5 pmol of each primer, 0.5 unit of Amplitaq Gold (Perkin Elmer) and 1.5 mM of $MgCl_2$ in 25 µl reaction volumes.

(c) *Data analysis*

Aligned sequence data were analysed with CODEML in PAML (v. 3.14; Yang 1997), and hypotheses of sequence evolution, applying different models for the ratio of non-synonymous to synonymous substitutions ($dN/dS=\omega$) along branches of a phylogenetic tree, were tested using likelihood ratio tests as explained in detail in the electronic supplementary material.

To study whether eyeless and eyed lineages accumulated substitutions at different sites of the cinnabar gene we mapped non-synonymous changes of each lineage separately on a *cinnabar* map marked with conserved sites, based on an alignment of *cinnabar* from *Tribolium*, *Drosophila*, *Bombyx* and *Homo* (Lorenzen *et al*. 2002). Similarly, we investigated whether there were differences in substitution pattern between eyeless and eyed lineages with respect to categories of amino acid substitution, considering hydrophobicity, charge and polarity changes of amino acids, using a χ^2-test. For a neutral pattern of substitutions we expect them to occur randomly across all sites, potentially leading to an elevated number of substitutions that alter the functional properties of amino acids.

3. RESULTS

An aligned sequence dataset of 402 bp (134 amino acids) was produced for 20 species of dytiscid water beetles. BLAST searches using GenBank showed close matches with *cinnabar* sequences of *T. castaneum*, confirming that the diving beetle sequences were derived from the *cinnabar* gene. It appeared difficult to amplify *cinnabar* DNA for many of the eyeless species. Only nine (out of 27) eyeless species produced PCR products suitable for sequencing, as opposed to 11 (out of 13) eyed species ($\chi^2=9.237$, $p=0.0024$). This difference may have resulted from higher substitution rates at conserved primer sites in the eyeless lineages (see below). Despite the use of highly degenerate primers, no cases of multiple amplification products were observed, suggesting that *cinnabar* is most likely a single-copy gene in the diving beetles. Moreover, the phylogenetic gene trees of *cinnabar* and mitochondrial data are highly similar (see electronic supplementary material) providing additional evidence that sequences were derived from orthologous genes. The dataset provides nine independent lineages where dytiscid species have evolved the loss of eyes. The following analyses assess whether there is evidence for a loss of function, relaxed selection or neutral evolution of *cinnabar* in each of these lineages.

Frameshifts caused by nucleotide insertions and deletions were found in two (out of nine) eyeless subterranean species. Two deletions at nucleotide positions 88–90 (3 bp) and 341–344 (4 bp) were detected in *Limbodessus karalundiensis*; however, this did not result in stop codons. A single nucleotide insertion (after position 149) and a single nucleotide deletion (at position 233) were found in *Nirridessus macrotarsis*, resulting in five stop codons, and, therefore, clearly resulting in a non-functional product. No stop-codons were observed in any of the other sequences of subterranean beetles. When contrasted with surface lineages, two (out of six) eyeless lineages from independent evolutionary contrasts showed elevated numbers of amino acid substitutions (see electronic supplementary material).

In order to test whether *cinnabar* in the eyeless lineages evolves at different evolutionary rates compared with the eyed lineages, a series of likelihood ratio tests were performed (table 1). Lineages of eyeless beetles appear to have a significantly different ω ratio (model A compared with B) compared with surface lineages, with the ω ratio being elevated in the eyeless lineages by a factor of four. This result suggests there has been a relaxation of selection on the *cinnabar* amino acid sequence in the eyeless lineages compared with eyed lineages. However, this relaxation of selection did not approach neutral levels of evolution, because ω_b of the eyeless lineages was significantly different from 1 (model C and B compared). A model where each eyeless lineage had a different ω_b ratio was also found to provide a significantly better fit (model B and D compared). The estimated ω_b for the eyeless lineages varied from 0.4626 (*L. karalundiensis*) to 0.0939 (*Bidessodes gutteridgei*). We could not reject the hypothesis that eyed lineages are all evolving with different ω_e ratios (model D and E compared).

The non-synonymous substitutions in the branches for the eyed and the eyeless lineages separately were plotted on a map of the cinnabar gene that included the conserved sites (figure 1). The conserved sites were deduced by comparison of the *cinnabar* sequences of four divergent lineages. Significantly more non-synonymous changes in the eyeless lineages occurred at conserved sites: 10 out of 27 in eyeless lineages compared with 4 out of 77 in surface lineages ($\chi^2=17.399$, $p<0.0001$). A test of whether amino acid substitutions in eyeless lineages were more likely to change the functional (charge/hydrophibicity/polarity) properties of the amino acid than in eyed lineages was not found to be significant ($\chi^2_1=0.38$, $p=0.54$).

4. DISCUSSION

We found clear evidence that the *cinnabar* gene of the eyeless diving beetle lineages evolves like a pseudogene;

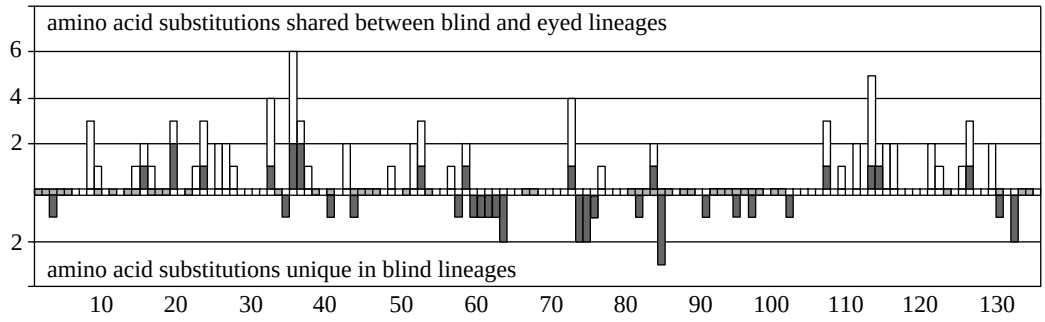

Figure 1. Map of a part of the cinnabar gene. Conserved sites grey, dark bars are amino acid substitutions in eyeless lineages, white in surface lineages.

cinnabar of two eyeless species contained frameshift mutations, one leading to stop codons that would render the gene non-functional. Overall, the ratio of non-synonymous/synonymous substitutions (ω) in all eyeless lineages was increased and non-synonymous changes occur significantly more frequently at conserved sites. Although we detected a significant loss of selective constraint in *cinnabar* of eyeless lineages, we did not find that the ω ratio approached neutral rates of evolution.

Previously, the lack of evidence of neutral rates of evolution in genes that were expected to be non-functional has been explained by alternative, unknown gene functions, e.g. *rhodopsin* in blind cave crayfish (Crandall & Hillis 1997) and *αA-crystallin* in the blind mole rat (Smulders *et al.* 2002). It is likely that *αA-crystallin* has not mutated because it plays an important role in head formation (Culver & Wilkens 2000), but such an explanation for the lack of neutral evolution of *rhodopsin* and *cinnabar* is unlikely. A plausible explanation, not previously taken into account, is that an eyeless lineage, after its divergence from an eyed ancestor, may remain above ground for a significant period of time prior to the transition underground. Before the transition, eye genes are still under purifying selection, but only after the transition will genes that are no longer under purifying selection start to evolve non-synonymous mutations at an accelerated rate. Therefore, unless the transition happened soon after this divergence, it will be unlikely that (composite) estimates for a loss of selective constraint would approach complete neutrality (ω close to 1). We showed by means of likelihood ratio tests that the eyeless beetles each had different elevated ω_b ratios. Combined with our finding that estimates of the timing of transitions in the eyeless lineages are variable, often about half of the lineage time, approximately 5 million years ago (Leys *et al.* 2003), further strengthens the validity of this explanation.

In conclusion, we report here a clear and compelling case of a single copy gene involved in eye development that shows a loss of gene function in a number of eyeless species. The nature of the mutations of *cinnabar* in these species and the fact that they inhabit isolated groundwater aquifers, with no future possibility of geneflow, would imply that the eyeless lineages have permanently lost the function of the cinnabar gene. Similar patterns of regressive evolution of additional structural genes, specific to the eye pathway, would lead to the irreversible evolutionary loss of eyes in these diving beetles. Overall, our study is consistent with the hypothesis that permanent loss of eyes in subterranean animals results from the slow accumulation of mutations, in the absence of purifying selection, that inactivate genes specific to the eye development pathway.

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Cooper, S. J. B., Hinze, S., Leys, R., Watts, C. H. S. & Humphreys, W. F. 2002 Islands under the desert: molecular systematics and evolutionary origins of stygobiont water beetles (Coleoptera: Dytiscidae) from central Western Australia. *Invert. Syst.* **16**, 589–598. (doi:10.1071/IT01039.)

Crandall, K. A. & Hillis, D. M. 1997 Rhodopsin in the dark. *Nature* **387**, 667–668. (doi:10.1038/42628.)

Culver, D. C. & Wilkens, H. 2000 Critical review of the relevant theories of the evolution of subterranean animals. In *Ecosystems of the world* (ed. H. Wilkens, D. C. Culver & W. F. Humphreys) *Subterranean ecosystems*, vol. 30, pp. 381–398. Amsterdam: Elsevier.

Darwin, C. R. 1859 *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London: John Murray.

Kosswig, C. 1960 Darwin und die degenerative evolution. *Abhandl. Verh. Naturw. Ver. Hamburg NF* **4**, 21–42.

Leebens-Mack, J. & dePamphilis, C. 2002 Power analysis of tests for loss of selective constraint in cave crayfish and nonphotosynthetic plant lineages. *Mol. Biol. Evol.* **19**, 1292–1302.

Leys, R., Watts, C. H. S., Cooper, S. J. B. & Humphreys, W. F. 2003 Evolution of subterranean diving beetles (Coleoptera: Dytiscidae: Hydroporini, Bidessini) in the arid zone of Australia. *Evolution* **57**, 2819–2834.

Lorenzen, M. D., Brown, S. J., Denell, R. E. & Beeman, R. E. 2002 Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan oxygenase and kynurenine 3-monooxygenase. *Genetics* **160**, 225–234.

Smulders, R. H. P. H., van Dijk, M. A. M., Hoevenaars, S., Lindner, R. A., Carver, J. A. & de Jong, W. W. 2002 The eye lens protein αA-crystallin of the blind mole rat *Spalax ehrenbergi*: effects of altered functional constraints. *Exp. Eye Res.* **74**, 285–291. (doi:10.1006/exer.2001.1124.)

Springer, M. S., Burk, A., Kavanagh, J. R., Wadell, V. G. & Stanthope, M. J. 1997 The interphotoreceptor retoid binding protein gene in therian mammals: implications

for higher level relationships and evidence for loss of function in the marsupial mole. *Proc. Natl Acad. Sci. USA* **94**, 13 754–13 759. (doi:10.1073/pnas.94.25.13754.)

Watts, C. H. S. & Humphreys, W. F. 2004 Thirteen new Dytiscidae (Coleoptera) of the genera *Boongurrus* Larson, *Tjirtudessus* Watts and Humphreys and *Nirripirti* Watts and Humphreys, from underground waters in Australia. *Trans. R. Soc. South Aust.* **128**, 99–129.

Yamamoto, Y., Stock, D. W. & Jeffery, W. R. 2000 Hedgehog signalling controls eye degeneration in blind cavefish. *Science* **289**, 631–633. (doi:10.1126/science.289.5479.631.)

Yang, Z. 1997 PAML: a program package for phylogenetic analysis by maximum likelihood. *CABIOS* **13**, 555–556. (http://ababcus.gene.ucl.ac.uk/software/paml.html.)

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