Evidence for escape from adaptive conflict?

Arising from: D. L. Des Marais & M. D. Rausher *Nature* **454,** 762–765 (2008)

Gene duplication is the primary source of new genes[1], but the molecular evolutionary mechanisms underlying functional divergence of duplicate genes are not well understood[2]. Des Marais and Rausher[3] argued that data from plant dihydroflavonol-4-reductase (DFR) genes support the model that gene duplication allows the escape from adaptive conflict (EAC) among several functions of a single-copy progenitor gene[4]. As the authors indicated, the key predictions of EAC, in comparison to other models[1,5,6], are that (i) adaptive changes occur in both daughter genes after duplication, and (ii) these adaptive changes must improve ancestral functions. Furthermore, EAC indicates that (iii) the improvement of several ancestral functions is constrained before duplication, although this last point was not explicitly stated. Here we show that contrary to the predictions of EAC, only one of the duplicated DFR lineages exhibited adaptive sequence changes. Owing to the lack of information on enzyme concentrations[3] we question the accuracy of enzyme activity comparisons, and it is thus not clear that any ancestral function has been improved in either lineage.

To test the predictions for one daughter lineage, the authors first investigated patterns of DNA sequence evolution[3]. It was shown that after the duplication of the progenitor *DFR* gene, the lineage leading to the common ancestors of *DFR-A* and *DFR-C* exhibited a non-synonymous-to-synonymous rate ratio significantly exceeding 1, indicative of adaptive evolution by positive selection. However, subsequent biochemical assays showed that these enzymes have "essentially no activity" on any ancestral substrate[3] and therefore do not demonstrate improvement of ancestral function, violating the above criterion (ii).

The other daughter gene from the duplication, *DFR-B*, showed no evidence of positive selection from sequence analysis[3], calling into question whether adaptive evolution has occurred. To test potential improvement of ancestral enzyme function after duplication, the authors made recombinant proteins from a heterologous system and measured catalytic activities on five substrates. Although the same volume of cell culture extract was used in all assays, the recombinant proteins were neither quantified nor equalized in concentration. It is known that the protein yield from heterologous systems can vary by several orders of magnitude, depending on a wide variety of factors[7]. In fact, an earlier study in a heterologous system nearly identical to the one used by Des Marais and Rausher reported very different yields for different DFR enzymes[8]. Thus, it is crucial to quantify the concentration of the obtained recombinant protein for any quantitative measure of activity, as has been done in previous studies of duplicate gene evolution[9,10]. Without enzyme concentration information, the apparently higher activity of DFR-B depicted in their Fig. 2 could be due to a larger amount of enzyme rather than a truly higher enzyme activity as the authors interpreted[3].

One may argue that the ratios of activities against different substrates can be compared among enzymes to control for differences in protein concentration. For example, one could infer from their Fig. 2 (ref. 3) that the relative activity on dihydrokaempferol (DHK) to dihydroquercetin (DHQ) is enhanced in DFR-B, compared to that of the progenitor enzyme. However, such relative activity information does not indicate whether the enhancement is due to increased activity on DHK, reduced activity on DHQ, or both. It could also be posited that the activity of DFR-B on DHQ is unlikely to be reduced compared to the progenitor enzyme if DHQ is the main precursor of most anthocyanins produced by post-duplication species. However, because Michaelis–Menten kinetic parameters were uncharacterized for these enzymes, even if the activity of DFR-B is lower than that of the progenitor, it remains possible that it is high enough for normal physiology, particularly if DHQ concentrations are high. Furthermore, one cannot exclude the possibility that the activity on DHQ is retained in DFR-A and/or DFR-C but reduced in DFR-B. Even if different enzymes are compared using these ratios, it is clear that DFR-B is no better with naringenin and eriodyctiol (the less typical but proposed to be improved-upon substrates) compared to DHK, DHQ and dihydromyricetin (DHM) (the typical substrates of DFR) than the progenitor enzyme from *Evolvulus* (Fig. 1). Thus, there does not seem to be any unambiguous evidence for improvement of any ancestral function in DFR-B. Coupled with the failure to detect positive selection in DFR-B, these results go against both criteria (i) and (ii). Because there is no evidence of improvement of ancestral function in any of DFR-A, -B and -C, criterion (iii) obviously cannot be established.

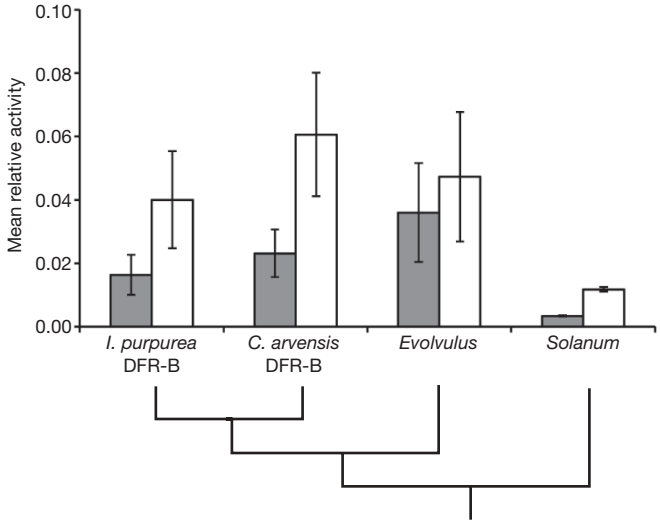

Figure 1 | Results of enzyme activities against different substrates. Shown are enzyme activities against naringenin (grey bars) and eriodyctiol (white bars), relative to those against DHK, DHQ and DHM. A greater number indicates a relative preference for narigenin (or eriodyctiol) compared to DHQ, DHK and DHM. Error bars denote one standard error. For a given enzyme, we calculate its activities against naringenin (or eriodyctiol) relative to DHK, DHQ and DHM, and show the average of the three relative activities and its standard error. The evolutionary relationships among the four enzymes are shown by the phylogeny. The relative activities are calculated from the data presented in Fig. 2 of ref. 3, graciously supplied by the authors.

In conclusion, none of the three key features of the EAC model have been clearly demonstrated in the evolution of DFRs. Although EAC may be a valid model for describing gene family evolution, rigorous tests of the predictions associated with it are needed to describe its general importance.

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Des Marais & Rausher reply

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Barkman and Zhang[1] level two criticisms at our report of escape from adaptive conflict (EAC)[2]. The first criticism is that our evidence of repeated adaptive substitution in the *DFR-A/C* lineage is consistent with either EAC or neofunctionalization in that lineage. As indicated in our original report[2], we do not disagree with this claim, but believe it misses the point that the hallmark of EAC is the adaptive improvement of function in both duplicate lineages. The repeated positive selection in the *DFR-A/C* lineage simply demonstrates improvement of some as yet unspecified function in that lineage.

Their second criticism[1] is that we failed to quantify enzyme concentration in our assays, and because of this our results do not demonstrate improvement of function in the *DFR-B* lineage. However, an examination of the possible effects of uncertainty in enzyme concentration indicates that our inference of improved catalytic function on anthocyanin precursors at the base of the *DFR-B* lineage remains robust. We can represent uncertainty in enzyme concentration by the relationship

$$C_1 = \gamma C_B \quad (1)$$

in which C_1 and C_B are the absolute enzyme concentrations in the assays using the single-copy *DFR* and the *DFR-B* copy, respectively. Furthermore, we represent the specific activity of enzyme type i (single-copy or B-copy) on substrate k by A_i^k, in which specific activity is defined by

$$P_i^k = C_i A_i^k \quad (2)$$

Here P_i^k is the concentration of the anthocyanidin reaction product produced by enzyme i acting on substrate k. Combining equations (1) and (2) yields

$$A_B^k = \gamma A_1^k \left(P_B^k / P_1^k\right) \quad (3)$$

Consider first activity on substrate dihydroquercetin (DHQ). In our assays, we detected no difference in product concentrations between DFR enzymes occurring as single gene copies (*Evolvulus* and *Solanum*) and the B copy in post-duplication species (*Convolvulus* and *Ipomoea*), indicating that $\left(P_B^k / P_1^k\right) = 1$. Thus, in this case, equation (3) reduces to $A_B^{DHQ} = \gamma A_1^{DHQ}$. Because DHQ is the main precursor of most anthocyanins produced by post-duplication species[3], it seems probable that purifying selection would eliminate mutations reducing activity on DHQ in the lineage leading to these species. We therefore consider it very unlikely that $\gamma < 1$. If $\gamma = 1$, then there was no improvement of activity on DHQ in the B-copy lineage, as we initially concluded. The other possibility, that $\gamma > 1$, would indicate improvement in function on DHQ, which we did not originally claim our data supported, but which would suggest improvement in DFR-B function on normal substrates after duplication.

Next, consider activity on substrate dihydrokaempferol (DHK). We demonstrated previously that the post-duplication B-copies produced about five times as much pelargonidin product as the pre-duplication single copies, indicating that $P_B^{DHK} / P_1^{DHK} = 5$, approximately. Substituting into equation (3) yields $A_B^{DHK} = 5\gamma A_1^{DHK}$, indicating an improvement in function on this substrate in the DFR-B lineage unless $\gamma < 1/5$. Again, we believe such a small value of γ to be very unlikely because it would indicate that activity on substrate DHQ had been reduced fivefold in the DFR-B lineage when that function is subject to purifying selection. A similar argument can be made for improvement of function on naringenin and eriodyctiol in the DFR-B lineage.

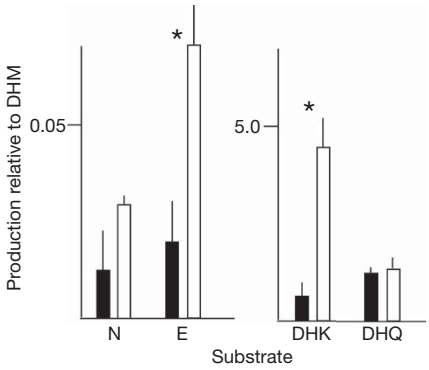

Figure 1 | Product production on different substrates relative to DHM production. Production (μg product per μM substrate) from equation (2). Relativization was performed separately for each species. Values reported are means for pre-duplication species (filled bars) and for post-duplication species (open bars). Error bars denote one standard error. E, eriodyctiol; N, naringenin. $*P = 0.05$, significant difference between pre- and post-duplication species (*t*-test).

Finally, Barkman and Zhang[1] report that their Fig. 1 shows that DFR-B exhibits no improvement on substrates naringenin and eriodyctiol even when these are expressed relative to the production of DHK, DHQ and dihydromyricetin (DHM). Although they do not describe how these values were calculated, we suspect that they may have erroneously relativized naringenin and eriodyctiol to the average of DHK, DHQ and DHM. The appropriate analysis[4] is to relativize all but one substrate to the remaining substrate for each species, then compare the relativized averages. When this is done, using the same data we provided them with, it is clear that the catalytic function of DFR-B on both eriodyctiol and DHK is significantly improved in the post-duplication species (Fig. 1).

In conclusion, the above arguments indicate that our failure to quantify enzyme concentration in our assays probably does not invalidate our original conclusion that subsequent to duplication, the *DFR-B* lineage improved function on several ancestral substrates. This improvement, along with our demonstration of adaptive substitution in the lineage leading to the DFR-A and DFR-C copies, satisfies the criterion for EAC of adaptive improvement of function in both post-duplication lineages.

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