Molecular phylogenetic evidence for the independent evolutionary origin of an arthropod compound eye

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Eyes often take a central role in discussions of evolution, with debate focused on how often such complex organs might have evolved. One such debate is whether arthropod compound eyes are the product of single or multiple origins. Here we use molecular phylogeny to address this long-standing debate and find results favoring the multiple-origins hypothesis. Our analyses of DNA sequences encoding rRNA unequivocally indicate that myodocopids—the only Ostracoda (Crustacea) with compound eyes—are nested phylogenetically within several groups that lack compound eyes. With our well-supported phylogeny, standard maximum likelihood (ML) character reconstruction methods significantly reconstruct ancestral ostracods as lacking compound eyes. We also introduce a likelihood sensitivity analysis, and show that the single-origin hypothesis is not significantly favored unless we assume a highly asymmetric model of evolution (one favoring eye loss more than 30:1 over gain). These results illustrate exactly why arthropod compound eye evolution has remained controversial, because one of two seemingly very unlikely evolutionary histories must be true. Either compound eyes with detailed similarities evolved multiple times in different arthropod groups or compound eyes have been lost in a seemingly inordinate number of arthropod lineages.

The number of times eyes originated during evolution is often debated, including within anthropods (1–3). Many biologists argue that arthropod compound eyes are the product of a single origin because detailed similarities exist among the eyes of diverse groups. For example, genes involved in eye development such as *Pax-6* and *sine oculis* appear functionally conserved across phyla and may also be conserved within Arthropoda (2). In addition, a highly stereotyped number and arrangement of cells develop in a similar manner to form the individual eye facets of different arthropod groups (4–8). Finally, neural circuitry of the optic lobe is highly conserved in many arthropods (9).

Despite the many similarities, some scientists suggest that compound eyes may result from multiple origins. Nilsson (10) argues that the different biophysical properties of some eyes make homology unlikely. Others postulate, based on phylogenetic arguments drawn from taxonomy, that compound eyes may have multiple origins (11, 12). We have taken advantage of the power of molecular systematics and the recent advances in methods for analyzing character evolution to study the question of compound eye evolution in a phylogenetic framework.

Here we use these tools and examine the phylogeny of the Ostracoda (Crustacea) to test the hypothesis that one ostracod group independently evolved compound eyes with respect to all other arthropods (11, 12). The Ostracoda are a diverse and ancient group of bivalved crustaceans with a superb fossil record dating back at least 500 million years (13). Taxonomically, ostracods are often divided into three major groups: Podocopa, Palaeocopa, and Myodocopa (13). The Myodocopa are further divided into the Halocyprida and Myodocopida. Although most Podocopa and Myodocopida have a non-image-forming and anterodorsally located eye called the "median eye," the Myodocopida (myodocopids) are the only ostracods that also have a pair of lateral compound eyes. Our molecular phylogeny clearly indicates that myodocopids are monophyletic and are nested within several groups lacking compound eyes. Based on this phylogeny, methods of character reconstruction significantly favor the independent origin of myodocopid compound eyes, constituting the strongest phylogenetic evidence to date for multiple origins of arthropod eyes. If this is not an independent origin, and compound eyes were actually lost many times, then this is a case where commonly used methods of historical inference are positively and significantly misleading.

Methods

Taxa. Our analysis contains representatives of the major groups of Ostracoda (13), with the possible exception of the Platycopida, which lack both median and compound eyes and are often placed within the Podocopa (14). We sampled all five taxonomic families of Myodocopida, the only ostracods with compound eyes, to test for monophyly.

As outgroups we chose two maxillopods, crustaceans thought to be close relatives of ostracods (e.g., ref. 5). Like most maxillopods, both chosen outgroups have a median eye (5). However, only one of these outgroups also has compound eyes (*Argulus* sp.: Branchyura), the other does not (*Tigriopus*: Copepoda). This outgroup choice is conservative with respect to the independent compound eyes hypothesis, because most maxillopods lack compound eyes (5) and the inclusion of additional outgroups lacking compound eyes could strengthen but is unlikely to weaken our conclusion of independent origins. Collection details for all taxa are available from T.H.O.

Sequences and Phylogenetic Analysis. We used standard primers and methods to obtain a complete sequence for DNA encoding 18S rRNA (rDNA) and a partial 28S rDNA sequence including the ddff, eemm, and vx regions (15, 16). We aligned sequences with CLUSTALX (17) and removed ambiguously aligned regions, although the same maximum likelihood (ML) tree topology was obtained when including all data (results not shown). We determined the best-fit model of molecular evolution to be Tamura–Nei (18) + gamma + invariant sites by using MODELTEST (19). We then fixed parameters to their ML estimates (transversions = 1; A–G = 3.5081; C–T = 4.1785; gamma shape = 0.6894; proportion of invariant sites = 0.3228) for a ML heuristic search and for 500 ML bootstrap pseudoreplicates in PAUP* (20). We estimated relative branch lengths by using the ML tree and assuming a molecular clock.

Ancestral State Reconstruction. Taxa were scored for presence/absence of compound eyes and separately for presence/absence

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Abbreviations: rDNA, rRNA-encoding DNA; ML, maximum likelihood.

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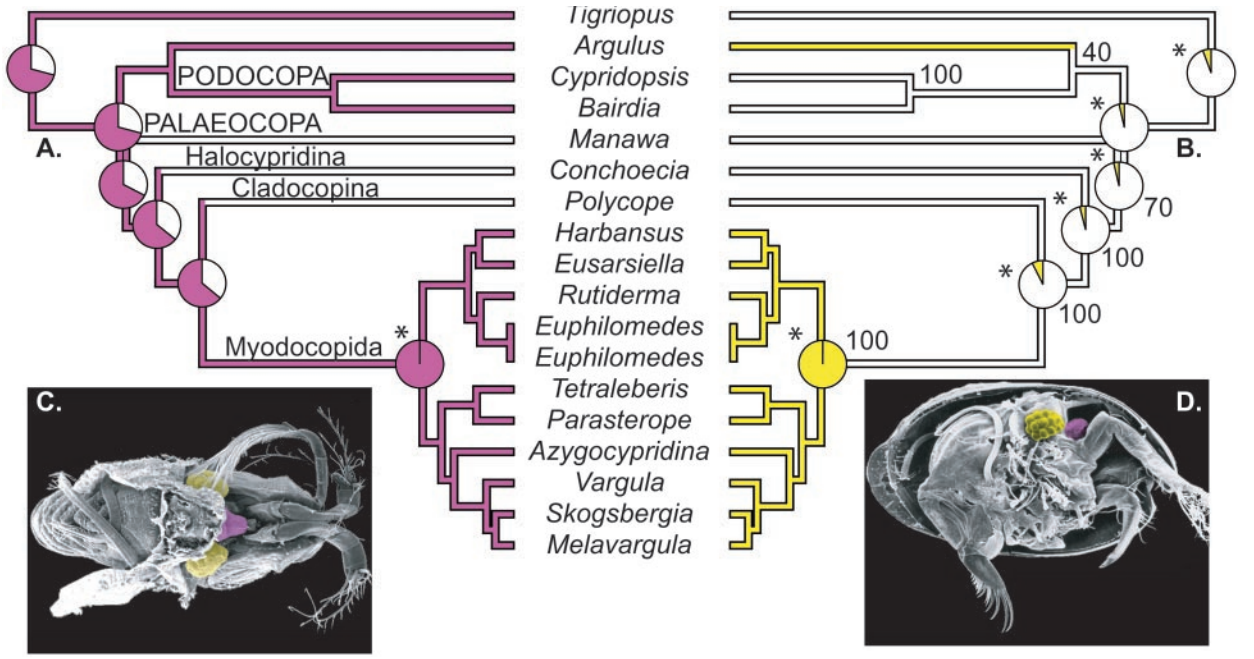

Fig. 1. (*A* and *B*) Ostracod rDNA phylogeny using best-fit ML and ML character mapping of two eye types. Numbers at right represent bootstrap support for the phylogeny (nodes within Myodocopida were also well supported by bootstrapping, but values are not shown here). Character mapping results support homology of the median eye and the independent origin of myodocopid compound eyes. Pie charts in *A* represent relative ML support at ancestral nodes for presence (purple) and absence of median eyes (white). Pie charts in *B* represent relative ML support at ancestral nodes for presence (yellow) and absence (white) of compound eyes, asterisks indicate a significant result (ln likelihood difference > 2) using the same rDNA phylogeny and equal rates models of character evolution. (*C* and *D*) Dorsal (*C*) and lateral (*D*) views of ostracods with compound eyes, shaded yellow, and median eye, shaded purple.

of median eyes. *Azygocypridina lowryi* was scored as having compound eyes, although its lateral eyes are hirsute flaps (11). Parker (11) hypothesized that *Azygocypridina* are basal myodocopids and that hirsute flaps may represent transitional compound eyes.

We used ML to estimate ancestral states (21–23) of compound and median eyes scored as discrete characters either by using DISCRETE 4.0 (21, 23) or by calculation with MATHEMATICA (Wolfram). We used the "global" method assuming a Markov model (22, 23) with equal transition rates between gain (α) and loss (β) of eyes. ML parameter estimates were $\alpha = \beta = 2.77$ for compound eyes and $\alpha = \beta = 4.58$ for median eyes. We did not use two-rate models in either case (which also showed significant support for an independent origin of compound eyes and identical results as an equal rates model for median eyes) because they did not fit the data significantly better than one-rate models. The "local" ML method (23) was more conservative, but did not qualitatively change results, nor did using alternative phylogenies with similar ML support, including one with ostracods monophyletic (results not shown). We used branch lengths estimated under a molecular clock assumption, although our sequence data reject a molecular clock. In such cases, Schluter *et al.* (22) set all branch lengths equal. When a tree with equal branch lengths was assumed, ancestral nodes still showed significant support for absence of compound eyes.

Likelihood Sensitivity Analysis and Confidence Interval (CI) Test. We developed an analysis to determine how skewed the evolutionary model must be before our data are consistent with the single-origin hypothesis of arthropod compound eyes. We examined evolutionary models ranging from symmetric (rate of loss = rate of gain) to highly asymmetric (35 $\times$ rate of loss = rate of gain) and compared the likelihood of two different hypotheses under different models. First, the "homology" hypothesis is the set of ancestral states where myodocopids and their basal ancestors were fixed to have had compound eyes. Second, the "independent origin" hypothesis refers to the set of ancestral states where all ancestors basal to myodocopids were fixed to lack compound eyes. Parameters were constrained to the ML values calculated by summing over all possible ancestral-state reconstructions that were consistent with each hypothesis for each of the various asymmetric models (i.e., as global analyses, where parameters were not re-estimated after fixing ancestral states to homologous or to independent origin constraints). A potential criticism of this test is that fixing nodes basal to the Myodocopida as eyeless ignores the uncertainty associated with those nodes. Although this criticism is true, our test is nevertheless conservative as follows. If compound eyes were absent in any one of the five nodes basal to myodocopids, then that alone would be evidence for an independent origin of compound eyes somewhere in the phylogeny. In fact, we used the most extreme case, where all five nodes were forced to lack eyes, so our test should only underestimate the asymmetry required to favor the alternative hypothesis of compound eye homology.

We also reconstructed ancestors by using a value for the loss parameter that was just outside the 95% confidence interval estimated from the data. We used MATHEMATICA with a two-parameter model (parameters are rate of gain and loss) to calculate ML parameter estimates and the likelihood surface of the function. We next found the 95% critical value, assuming a χ^2 distribution with 1 degree of freedom and plotted it on the likelihood surface (24). We then used the largest value on the y axis of the 95% confidence area as the value for the loss parameter for reconstructing ancestors. This calculation tests whether an independent origin of compound eyes is favored even when the loss parameter is allowed to take a value significantly greater than that estimated from the data.

Results

We report the best-fit ML phylogeny of partial 28S and complete 18S rDNA data (Fig. 1). We found strong support for monophyly

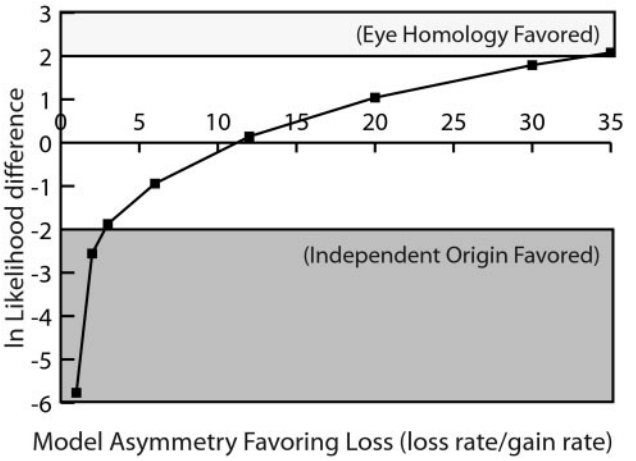

Fig. 2. A sensitivity analysis comparing likelihood models with differing amounts of asymmetry in rate of compound eye evolution. The *y* axis represents the difference between the ln likelihood of the compound eye homology hypothesis (five nodes basal to myodocopids fixed with compound eyes present) and the ln likelihood of an independent origin hypothesis (same five nodes fixed to absence). The *x* axis values refer to the amount of asymmetry favoring loss of compound eyes, reasonable models because loss of eyes is probably more likely that gain. For example, 1 on the *x* axis is an equal rate model. A 30 on the *x* axis is a model where loss = 30 × gain ($\alpha = 30\ \beta$).

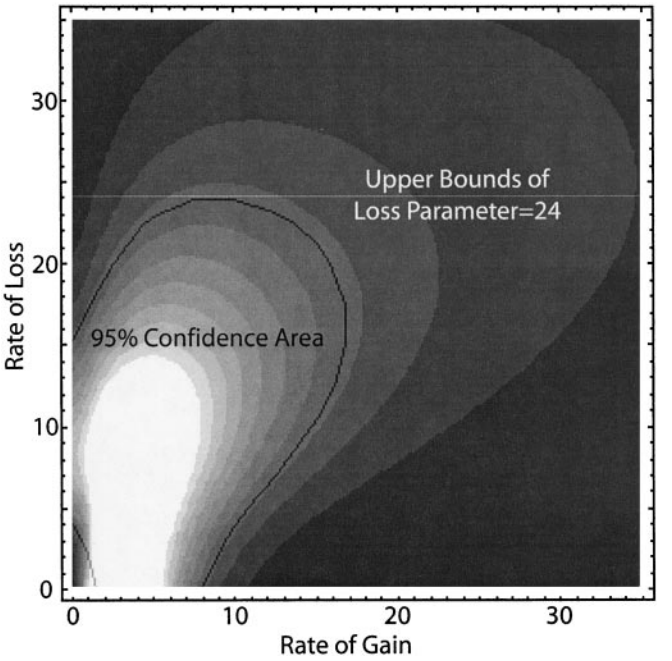

Fig. 3. Likelihood surface for a two-parameter model of ostracod compound eye evolution, assuming the tree in Fig. 1. Lighter areas are parameter combinations with higher likelihood. The critical value was calculated assuming a χ^2 distribution with 1 degree of freedom (24). As illustrated, a value of 24 for the loss parameter fits the data significantly worse than the ML estimate. Even with this significantly high parameter value favoring loss of compound eyes, reconstruction of all nodes basal to myodocopids favor eye absence over presence. These reconstructions are not illustrated. Individually these reconstructions are not statistically significant; all have similar proportions of likelihood favoring compound eye absence (about 0.53).

of the Myodocopida (100% bootstrap, Fig. 1). Furthermore, a monophyletic Myodocopida is nested within Podocopa, Palaeocopa, and a paraphyletic Halocyprida, four ostracod groups that lack compound eyes (Fig. 1). ML methods of ancestral-state reconstruction significantly support the independent origin of myodocopid compound eyes (Fig. 1*B*). The independent-origin hypothesis holds even when we assume models of evolution that favor eye loss over gain (Fig. 2). The assumed model of compound eye evolution must be skewed over 10:1 in favor of eye loss before our data favor the single-origin hypothesis and must be skewed over 30:1 before that result is statistically significant (Fig. 2).

We found that the 95% CI for the loss parameter has a large range (Fig. 3), presumably because this phylogeny has relatively few taxa and estimated rates of eye evolution are therefore based on little data. Larger and broader taxon sampling could provide tighter estimates of rate parameters. Despite the imprecision of rate estimates, the absence of compound eyes is still favored (although not significantly at any one node) at each of five nodes basal to myodocopids, even when using 24 for the loss parameter, a value significantly greater than the rate estimated from the data (which is 0).

In contrast to compound eyes, some ambiguity exists in the reconstruction of median eyes. Homology of the median eyes of ostracods and outgroups is supported by ML reconstructions that used relative branch lengths estimated from rDNA (Fig. 1*A*). This result is different from reconstruction based on parsimony or ML reconstruction with equal branch lengths, both of which favor a loss of median eyes within ostracods coupled with a regain in the myodocopids. The difference in parsimony and ML reconstruction for median eyes clearly illustrates a difference in the assumptions of these methods. Unlike parsimony, ML considers branch lengths as a proxy for time when reconstructing ancestral states. On our phylogeny, long branches separate the three ostracods that lack median eyes from their relatives. ML considers changes (loss of median eyes) along these long branches more likely than changes along the short internal branches, resulting in the difference between parsimony and ML reconstruction. This interpretation is further supported by the fact that ML reconstruction using equal branch lengths concurs with parsimony reconstruction of median eyes.

Our sequence data are equivocal with respect to ostracod monophyly; the ML tree does not support monophyly, but bootstrapping did not show strong support for any alternative tree. The best tree consistent with ostracod monophyly also significantly supports the independent origin hypothesis of compound eyes. Other relationships indicated by our data are consistent with previous views of ostracod phylogeny based on morphology, with the possible exceptions of paraphyletic Halocyprids and Philomedidae (Myodocopida) (25). Our phylogenetic analysis does not support *A. lowryi* as the basal myodocopid, indicating that hirsute flaps are probably reduced compound eyes rather than an intermediate step between absence and presence (11).

Discussion

Our very well supported molecular phylogeny unequivocally indicates that the only ostracods with compound eyes are phylogenetically nested within several groups that lack these eyes altogether. These results indicate one of two possibilities, either arthropod compound eyes have originated more than once or compound eyes were actually lost in several ostracod lineages. ML methods of character reconstruction strongly favor the independent origin hypothesis.

If compound eyes did not evolve independently in myodocopids, then these commonly used methods of historical inference are significantly and positively misleading. Here we found significant support for absence of compound eyes at five nodes ancestral to myodocopids by using ML reconstruction, a method that is often conservative (e.g., ref. 22). A case in point

for ambiguity is ostracod median eyes, where ML ancestral state reconstruction was equivocal at three nodes (Fig. 1*A*) and differed depending on assumptions of branch lengths. But compound eye reconstruction was not ambiguous. Highly significant results were obtained when assuming either equal rates of eye gain/loss or rates estimated by maximum likelihood. Assumptions about branch lengths also did not change the conclusion of an independent origin of compound eyes.

Despite these apparently unambiguous results for compound eyes, an important consideration is that eyes—like other complex characters—are probably more easily lost than gained, which can cause character reconstruction methods to be misleading (26–28). We explored this possibility in two different ways. First, we showed that compound eye absence is favored at all five nodes basal to myodocopids, even when using a value for the rate of compound eye loss that is significantly higher than the ML estimate from the available data (Fig. 3).

Second, we devised a likelihood sensitivity analysis to estimate the extent that loss would have to be favored over gain before the independent origin hypothesis was no longer supported. Herein lies a central problem in character reconstruction analysis; there is no way to know what value of a sensitivity analysis is large enough to be considered significant (28, 29). For example, even though 30 is a seemingly very large number, perhaps eyes really are lost over 30 times as often as gained during evolution. From a perspective of Bayesian statistics, this problem can be stated as a difficulty in choosing an appropriate prior distribution for a parameter that estimates directional bias (22, 30). These prior probabilities will almost certainly vary among different characters and analyses at different taxonomic levels. Therefore, additional data that corroborate unambiguous character reconstruction results or clarify ambiguous results are particularly valuable (28, 31).

For the cases in question, further evidence could be obtained by examining molecular development, eye morphology, or neural circuitry. Of these, only eye morphology has been examined previously in ostracods, and these data are consistent with median eye homology and an independent origin of compound eyes. The ostracod median eye is typical of maxillopods in having a tripartite structure and diagnostic tapetal cells (32). With the exception of Mystacocarida and Tantulocarida, which completely lack eyes, other maxillopods have this diagnostic median eye, consistent with a conclusion of median eye homology in maxillopods and ostracods. Unlike median eyes, ostracod compound eyes are unique among arthropods, as we might expect if those eyes had an independent evolutionary origin. Ostracod compound eye facets (ommatidia) each have six photoreceptive cells (''retinular'' or ''R'' cells) and two lens cells (''crystalline cone cells'') (33, 34). In contrast, the ommatidia of many diverse groups of arthropods, including the maxillopod *Argulus* (an outgroup in our phylogeny), have eight R-cells and four cone-cells (4, 6). This common arrangement of cells has been one of the main arguments for the single-origin hypothesis of arthropod compound eyes (4, 6, 35). The name ''tetraconata'' has even been suggested for a Crustacean/Hexapod clade because of the abundance of species with four cone cells per facet (8).

We consider the deviation of ostracods from the common 8/4 cell pattern important evidence that is consistent with the independent origin hypothesis, yet we recognize that other explanations are possible. One possibility is that there was a change in cell numbers during the evolution of homologous compound eyes, a plausible hypothesis because mutations in single genes like *sevenless* reduce R-cell number from eight to seven in *Drosophila* ommatidia (36, 37). In addition, ostracods are not the only arthropods that vary from the 8/4 pattern (4). Despite possible alternative explanations, phylogeny and facet morphology are both consistent with the independent origin of compound eyes in ostracods.

The possible independent origin of an arthropod compound eye is particularly interesting in light of recent controversy surrounding claims for the homology of all metazoan eyes based on highly conserved developmental genes like *Pax-6* (38). This controversy originally focused on definitions of homology (39, 40), but more recently, two opposing evolutionary models have emerged separate from disagreements about homology definitions. One model is that prototype photoreceptive structures and associated developmental genes evolved once and have been elaborated along independent lines (2). A second model is that photoreceptive structures evolved multiple times and each time co-opted homologous genes for use in those structures (41). The key difference between the models is whether a photoreceptive organ was present in all common ancestors or not. The data presented here suggest that compound eyes were not present in ostracod ancestors. If independently derived eyes of ostracods use the same developmental genes as other eyes, then the co-option model above might be favored, suggesting that a structurally nonhomologous eye co-opted homologous eye development genes during evolution. Investigating the genes involved in ostracod eye development will provide additional insight into these matters.

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