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Dispersal between Shallow and Abyssal Seas and Evolutionary Loss and Regain of Compound Eyes in Cylindroleberidid Ostracods: Conflicting Conclusions from Different Comparative Methods

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Abstract.—Complex organs such as eyes are commonly lost during evolution, but the timescale on which lost phenotypes could be reactivated is a matter of long-standing debate, with important implications for the molecular mechanisms of trait loss. Two phylogenetic approaches have been used to test whether regain of traits has occurred. One way is by comparison of nested, continuous-time Markov models of trait evolution, approaches that we term tree-based tests. A second way to demonstrate statistical support for trait regain is through use of node-based tests that employ explicit estimation of ancestral node states. Here, we estimate new molecular and morphological phylogenies and use them to examine the possibility of eye regain and dispersal between abyssal and shallow seas during the history of cylindroleberidid ostracods, a family of about 200 species, comprising both eyeless and sighted species. First, we confirmed that eye presence/absence is correlated with habitat depth. Parameter estimates from a phylogenetic model indicate that speciation is more rapid in deep-sea eyeless clades compared with shallow-water sighted clades. In addition, we found that tree-based statistical tests usually indicated reversals, including both transitions from deep to shallow seas and regain of eyes. In contrast, node-based statistical tests usually failed to show significant support for reversals. These results also hold for simulated phylogenies, indicating that they are not unique to the current data set. We recommend that both tree-based and node-based tests should be examined before making conclusions about character reversal and that ideally, alternative character histories should be tested using additional data, besides just the phylogenetic distribution of presence/absence of the characters. [Character evolution; comparative methods; compound eye; Cylindroleberididae; deep sea; dispersal; Dollo's Law; Ostracoda.]

A central topic of macroevolutionary study is whether or not evolution is biased in a particular direction. If so, such trends could indicate an element of predictability in the history of life (reviewed in Gregory 2008). Such alleged trends may apply to continuously varying traits, like mammalian body size (e.g., Alroy 1998) or metazoan complexity (e.g., Marcot and McShea 2007), as well as to transition biases in traits with discrete states, including floral symmetry (Ree and Donoghue 1999), fungal fruiting body (Hibbett and Binder 2002), plant breeding systems (Weiblen et al. 2000; Takebayashi and Morrell 2001), gene expression domains (Oakley et al. 2006), and arthropod limbs (Adamowicz and Purvis 2006). Analyses of trends and biases in character evolution have become increasingly sophisticated and now rely on explicit statistical models of character evolution in a phylogenetic context (e.g., Pagel 1994; Huelsenbeck and Rannala 1997; Oakley 2003a; Ronquist 2004; Maddison et al. 2007). In some case studies, character evolution trends have also been associated with potentially causal ecological correlates (e.g., Roalson 2008). For example, Givnish et al. (2005) found that net venation and fleshy fruits were gained more often than lost in monocots, and these traits showed a significant association with living in shady habitats. Bergmann et al. (2009) found a directional trend toward stockiness in horned lizards, which is correlated with multiple specializations for ant-eating. A full understanding of biases and trends during macroevolution includes the genetic, environmental, and biogeographical factors that drive the mechanisms of character evolution.

One specific topic in the more general debate on trends and biases—the potential irreversibility of trait evolution or Dollo's Law (Dollo 1893; Gould 1970)—originally had a strong ecological perspective. However, recent work on Dollo's Law has focused more on genetic factors and less on environmental or biogeographic factors. Initial genetic considerations of this originally pre-genetic idea argued that back mutation and recombination are common genetic events such that reversion is theoretically possible (Muller 1939; Simpson 1953) and now empirically observed (Teotonio and Rose 2001; Borowsky 2008). Despite known cases of reversion, it is commonly argued—especially in comparative methods literature—that complex trait regain is far less likely than loss, particularly over longer timescales (Maddison 1994; Omland 1997; Trueman et al. 2004), because a single mutation in any one component can knock out a trait, whereas evolving (or regaining) complex integrated traits may require numerous independent mutations (Maddison 1994; Omland 1997; Reznick et al. 2002). However, the relative ease of the mutations leading to loss of complex traits versus gain could be far less important than the relative ease of *fixation* of those mutations. No matter the mutational ease, complex traits might rarely be lost if they remain central to organismal function and fitness, a regime that could remain constant until a major change in environment occurs, perhaps caused by dispersal. Conversely, assuming that loss mutations are reversible, traits could be regained, but only after a return to the environment where the trait is central to function and fitness.

Eye evolution provides a useful example to illustrate this relationship between environment (light level) and phenotype (eye complexity). In marine organisms, the frequency of dispersal of shallow-water sighted species to the lightless deep sea could be a major determinant of rates of eye loss. If presence of eyes is central to fitness of shallow marine organisms, eyes should be retained, no matter the mutational ease of loss. A recent study on asellote isopods (Raupach et al. 2009) supported this "submergence hypothesis," with multiple colonization events to the deep sea (and concomitant loss of eyes), without finding the reverse (we herein term dispersal from abyssal to shallow seas "ascension"). Although shallow versus deep sea is not defined the same way by all authors, the isopod results are consistent with the commonly noted pattern termed "onshore–offshore," where deep-sea diversity results from shallow-water dispersers (Moseley 1880; Jablonski et al. 1983; Jacobs and Lindberg 1998). Conversely, the rates of dispersal of deep-sea eyeless species to illuminated environments could be a major determinant of rates of eye (re)gain during evolution. Although eye regain was not applicable or not addressed, multiple taxa are known to have dispersed from deep to shallow seas, including stylasterid corals (Lindner et al. 2008) and neogastropod mollusks (Jablonski and Bottjer 1991; Jablonski 2005). Here, we examine the history of dispersal between shallow and abyssal seas in association with loss and potential regain of compound eyes in cylindroleberidid ostracods.

Cylindroleberidid ostracods are a particularly valuable group for testing hypotheses about eye evolution and the relationship between rates of character evolution and biogeography/ecology. This family, within the ostracod subclass Myodocopa, is well defined by the presence of paired flat gills at the posterior of the body (Vannier et al. 1996). All 221 described living species of cylindroleberidids are marine, living at depths from 0 to 4500 m (Syme and Poore 2006). Also assigned to the family are multiple exquisitely preserved fossils (Weitschat 1983; Siveter et al. 2003). All known cylindroleberidids, even those living at abyssal depth, have a single dorsal median eye. In addition, most shallow-water cylindroleberidids possess paired, lateral compound eyes (hereafter compound eyes), whereas most bathyal and abyssal species lack them. This mixture of deep-sea species lacking compound eyes (hereafter eyeless, even though all species also possess a median eye) and shallow-water species possessing eyes suggests the occurrence of dispersal between distinct environments as well as transitions in presence or absence of eyes.

Here, we present the first phylogenetic analysis of cylindroleberidid ostracods, including genetic data from 5 deep-sea species, which we use to examine the status of reversibility of dispersal to the deep sea and of the correlated loss of compound eyes. We use the resulting cylindroleberidid phylogenies to first confirm that absence of eyes is correlated with deep-sea habitat (living at >1000 m depth). We then test, using full tree–based and node-based tests for character state reversibility, whether the compound eyes of cylindroleberidid ostracods were ever regained and the correlated question of whether deep-sea lineages have ever invaded shallow water. Using tree-based tests, we find support for reinvasion of shallow seas and regain of compound eyes in cylindroleberidid ostracods. In contrast, node-based, ancestral state reconstruction tests unambiguously support only losses of eyes and the submergence/onshore–offshore hypotheses. We suggest that both tree-based and node-based methods should be examined when testing for bias in rates of evolution between character states because they can yield different conclusions. Even when such results are in conflict, these analyses generate testable evolutionary hypotheses.

Methods

Data

Outgroup selection.—As outgroups for the cylindroleberidids, we included one species from each of 3 other myodocopid families, plus the more distantly related ostracod species *Manawa staceyi*. We did not include the sister taxon to Myodocopida—the mainly planktonic Halocyprida—because those species have high rates of molecular evolution and their genes are difficult to align with those of other taxa (Oakley and Cunningham 2002; Oakley 2005; Tinn and Oakley 2008).

Molecular data.—A challenge to estimating phylogenetic relationships and character histories in many invertebrate groups, including cylindroleberidids, is that exhaustive species-level taxon sampling of molecular data is very difficult. First, perhaps half or more of all living ostracod species are not yet even described (Horne et al. 2002). Unknown species are found even in areas where other marine groups are well known (Lum et al. 2008). Second, as a family, cylindroleberidids have a global distribution, yet many described species are known only from one or a few specimens from remote localities that would often require expensive ocean-going vessels to obtain. Therefore, obtaining specimens preserved for molecular work is by necessity opportunistic. Even when specimens can be preserved for molecular work, ostracods' small size, often patchy distribution (sometimes leading to few individuals collected), and substantial divergence from other groups (Tinn and Oakley 2008), makes obtaining molecular data from multiple genes for many species an additional challenge. In contrast to molecular data, morphological information can be obtained from museum samples and published species descriptions and used to estimate phylogenetic relationships of cylindroleberidids. In fact, in this study, we include morphological analyses for a high proportion of all known species (see 'Morphological Data' section). The result is a patchwork of available data for phylogenetic inference. Because many other interesting taxonomic groups may also be understudied and/or have a paucity of molecular data (given when including fossils), one major goal of this paper is to

utilize approaches for examining the efficacy of inferences about character evolution, even in the face of missing phylogenetic data (see also FitzJohn et al. 2009).

To obtain DNA data, we used ostracod tissue from either the muscular second antenna or a section of the gills or posterior body. We isolated DNA using the DNeasy tissue kit (Qiagen, Inc.). We amplified gene regions using polymerase chain reaction (PCR) using primers for 16S (Hillis and Dixon 1991; Wakayama and Abe 2006), 18S (Yamaguchi and Endo 2003), and newly designed primers F1UN (5′-ACGGGGATATTCTCCCCTTTTCC-3′), F1N (5′-GACTCTGAATAACTTTTAGCTGAT-3′), R9 UN (5′-GGTTCACCTACGGAAACCTTGTT-3′), R9 N (5′-TCTAAATGGTCAAGTTTGGCCA-3′), and 28S (v-x and ee-mm regions) (Jarman et al. 2000). We sequenced purified PCR products using a Beckman CEQ DNA sequencer, using the same forward and reverse primers as used in the PCR (for 18S, we used additional internal primers [Yamaguchi and Endo 2003]), with Beckman's CEQ reagents according to manufacturer's instructions.

Using MUSCLE (Edgar 2004), we next aligned the sequences of each gene region and removed poorly aligned nucleotides with Gblocks (parameters—$b3 = 7$, $b4 = 5$, $b5 = h$, $t = d$) (Castresana 2000). We then used maximum likelihood (ML) and the Akaike information criterion (AIC), implemented in Modeltest (Posada and Crandall 1998), to determine separately for each gene region the statistically best-fit model of molecular evolution. For analysis in RAxML (Stamatakis 2006), we subsequently selected the best partitioning scheme for the molecular data from 4 different partitioning strategies (not partitioned; 2-partitions: mitochondrial/genomic; 3-partitions: 16S/18S/28S; 4-partitions: 16S/18S/28S vx/28S eemm) using the corrected AIC (AIC_c) (McGuire et al. 2007). These AIC scores depend on the likelihood (calculated in RAxML) and the number of parameters present in each model.

Morphological data.—Using published descriptions and specimens, we coded morphological characters for all well-described cylindroleberidid species (164 out of a total of 221 species; Syme and Poore 2006). We excluded species that had an inadequate published description with very few characters described or illustrated, and those known only from juveniles, as their adult morphology is uncertain.

We coded 69 morphological characters according to the hypotheses of independence (separate characters) and homology (character states) (Pimentel and Riggins 1987). All characters were scored as unpolarized and reversible, which is appropriate where the ancestral state has not been defined and where the cladogram will be rooted after the analysis (Nixon and Carpenter 1993). Continuous characters (such as percentages) were divided into 3 discrete states (Wilkinson 1992) and modeled as ordered through the middle state where possible. All other characters were scored as unordered. Many characters refer to the absence or presence of setae and their counts. In most of these characters, the counts displayed a clear mode. We thus coded the mode as one of the states, and the counts either side of the mode as further states (in some cases, there was only a count of one side of the mode). Resulting 3-state setae-based characters were modeled as ordered when possible, under the hypothesis for ordered characters discussed above. We used 4 characters (#12, 19, 20, 22) from Horne et al. (2005) that correspond to our characters 48, 50, 58, 61 except for minor wording modifications in our character and state descriptions. Appendix 1 lists all morphological characters and character states, whereas the taxa studied and their associated morphological data are presented in Appendix 2 and in MorphoBank Project #175. We coded depth state (abyssal/shallow) and compound eye state (absent/present) for character evolution analyses but did not use these in searches for the best tree topology. Analyses of depth and eye size scored as continuous characters would be of interest, but for most ostracods, depth distribution data are too poorly understood and often reported for only a single individual. Dividing into only two binary states is more consistent with the current state of knowledge of the variation in ostracod depth distributions. Similarly, absolute measures of eye size are unavailable from the literature for many ostracod species.

Impact of missing data.—To test the extent to which missing data and the resulting uncertainty of our phylogenetic estimate influenced our conclusions, we first performed phylogenetic and character analyses on 2 different taxon sets. For each taxon set, we performed bootstrapping analysis in RAxML of the primary concatenated molecular and morphological data, yielding in each case 1000 pseudoreplicated data sets, which were used to test the sensitivity of final conclusions to phylogenetic uncertainty. This bootstrapping approach allowed all analyses—phylogenetic reconstruction, relaxed molecular clock analysis, and character evolution analyses—to be conducted in a maximum likelihood statistical framework. The first taxon set included all 164 cylindroleberidid taxa (we term this the "all" taxon set). Analyzing this data set has the advantage that it represents sampling of most of the known extant members of the family, important because taxon sampling influences character evolution inferences (Ree and Donoghue 1999; Salisbury and Kim 2001). A disadvantage is that molecular data are only available for a subset of taxa, and the overall concatenated data set therefore has much missing data. The second taxon set included all concatenated molecular and morphological data for the subset of taxa with molecular data (30 species including outgroups, which we term the "molecular" taxon set) and a minimal amount of missing data.

We recognize that Bayesian approaches offer an attractive means to account for phylogenetic uncertainty (Huelsenbeck et al. 2000). However, for the current data, such an analysis requires mixing of Bayesian and ML inference methods because not all methods we employ are currently implemented in a Bayesian context. For example, mixed morphological and molecular analyses coupled with relaxed molecular clock analyses are not easily implemented in a Bayesian framework with

currently available software. We did examine multiple trees derived from a Monte Carlo Markov chain (MCMC) search with subsequent ML methods (not shown) and found qualitatively similar results to those found with ML bootstrapping. Developing Bayesian implementations for all these analyses is beyond the scope of the current paper, and we therefore only present the ML bootstrapping results.

In addition to missing data from the matrix of species analyzed, incomplete description of cylindroleberidid species could also introduce a bias, especially if abyssal and shallow species are described at a rate disproportionate to the true value. For example, deep-sea species are difficult to sample and may be described more rarely than shallow-water species, which may be considered easier to obtain. FitzJohn et al. (2009) explored the impact of missing and phylogenetically unresolved species in studies of trait-dependent speciation and found that when missing species are randomly distributed, their results reasonably estimated rates of speciation and extinction. To examine the hypothesis that deep-sea or eyeless cylindroleberidids are undersampled, we traced the proportion of known shallow and sighted species over time using the dates of publication for species descriptions and calculated confidence intervals on those proportions using standard statistical formulas.

Phylogenetic Analyses

MrBayes.—Although we did not use a Bayesian approach for all analyses, we estimated phylogenies with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) to allow for additional insights into the topological support of our phylogenies. We partitioned our data sets according to the best-fit strategy (see results), which included 3 molecular partitions and 2 morphology partitions ("restriction" type for morphology setae characters and "standard" for other morphology characters). As the best-fit model of molecular evolution, we used GTR, including rate heterogeneity modeled as a gamma distribution. We ran 50 million generations with 4 chains in each of 3 runs. We examined the standard deviation of split frequencies in MrBayes, which was 0.08 at the end of our run, and we used Are We There Yet (Wilgenbusch et al. 2004) to assess convergence of the MCMC chains.

RAxML.—We implemented ML analyses using RAxML 7.2.6 using the combined rapid bootstrap and search for ML tree (the "–f a" option) (Stamatakis 2006). We partitioned our data sets according to the best-fit strategy (see Results section), which included 3 molecular partitions and 2 morphology (binary and multistate) partitions. As the best-fit model of molecular evolution, we used GTR, including rate heterogeneity modeled as a gamma distribution. Morphological analyses employed using RAxML assumed all unordered characters with equal rates (Mk), because current software does not allow mixing ordered and unordered characters in the same analysis and the majority of our morphological characters are unordered. RAxML morphological models are not conditioned on characters being variable, and this may result in overestimation of branch lengths based on our morphological data (Lewis 2001).

Calibration and divergence times.—To test whether the evolutionary rate of molecular data was clock-like, we used a likelihood ratio test (LRT) to compare the likelihood of a model that enforces a strict molecular clock with a model with rates free to vary on each branch (Felsenstein 1988), implemented in PAUP* (Swofford 2003). For this global clock test, we assumed the best-fit model of molecular evolution and estimated the parameters and ML separately assuming a strict clock and assuming no molecular clock.

The Markov models of trait evolution we employ assume that more character state changes occur along longer branches of phylogenies (Pagel 1999), and branch lengths that estimate relative time may be more logical predictors of character change than those that estimate genetic distance of phylogenetic markers. Because of this assumption and because a molecular clock does not hold for the data (see Results section), we made the cylindroleberidid phylogenies ultrametric, as done in previous comparative analyses (Schluter et al. 1997) by using penalized likelihood to relax the assumption of a molecular clock (Sanderson 2002). Using r8s (Sanderson 2006), we first rooted the tree by pruning the outgroup taxon *M. staceyi* and then calibrated the branch lengths using myodocopid fossils dated at 425 My (Siveter et al. 2003; Siveter et al. 2007; Siveter et al. 2010), placed at the most recent common ancestor of the myodocopids. In order to allow computational feasibility, we calculated single smoothing values for each ML tree ("all" and "molecular") using cross-validation (CV) in r8s (Sanderson 2002; Sanderson 2006) and applied this value to calibrate each tree from bootstrap pseudoreplicated data.

Character Evolution Analyses

Correlation between eyes and depth.—In many marine species, there is a correlation between living at greater depth and the absence of eyes, and the Cylindroleberididae is no exception (Kornicker 1975). To test formally this correlation in a phylogenetic context, we used a data matrix of depth state (abyssal/shallow) and eye state (absent/present); characters 68 and 69 in Appendices 1 and 2. We divided depth into 2 states: up to 1000 m and greater than 1000 m because 1000 m is an estimate for the limit to which daylight extends (Warrant and Locket 2004). In addition, depth ranges are poorly known in most myodocopids, and 2 broad categories better represent this uncertainty compared with using specific, continuously varying values for depth. We used Mesquite (Maddison and Maddison 2006) to implement Pagel's test of correlated evolution (Pagel 1994). The test calculates likelihoods separately for 2 models of evolution: an independent model, where eye state and depth state evolve independently, and a dependent model, where the rate of change in one character depends on the state of the other character. Following the calculation

of likelihoods under both models for each bootstrap replicate for each of the 2 taxon sets, we performed LRTs to determine whether the dependent model was significantly better than the less complex independent model. We used simulation to determine *P* values (Maddison and Maddison 2006) for each ML tree (all and molecular), but simulation was too computationally expensive to perform for all 2000 bootstrap replicates, so for those, we assumed that the values are distributed according to a chi-square distribution for hypothesis testing. This approach also provides parameter estimates for rates of evolution between different combinations of character states.

Tree-based tests of reversible evolution.—In general, two types of comparative statistical test are used to examine the possibility of reversals in trait evolution (Oakley 2003a; Collin and Miglietta 2008). The first are *tree-based tests*—those that compare models of trait evolution along entire phylogenetic trees. These tests usually assume that the trait in question evolves by a continuous-time Markov process, and they compare different nested models using ML. A similar idea has been generalized to count character transitions in a Bayesian framework (Minin and Suchard 2007). Using ML, if a model where a parameter describing rate of trait gain equals zero is rejected in favor of a model including the possibility of gain, then gain of the trait somewhere on the phylogeny is supported. Such an approach was taken by Takebayashi and Morrell (2001), who unexpectedly found results from this methodological approach to favor evolution from asexual to sexual states in plants.

Using 1000 trees obtained by bootstrapping combined morphological and molecular data for each of 2 taxon sets (excluding the outgroup *M. staceyi* to allow rooting) and calibrated using r8s, we compared alternative continuous-time Markov models of trait evolution using ML. We used Mesquite's (Maddison and Maddison 2006) "Chart" function to calculate likelihood values for various models of character evolution across all 2002 trees (1000 bootstrap replicates plus the ML tree for each of 2 taxon sets). In all cases, we added a taxon with "eyes present" and "shallow depth" on a very short branch at the root, which effectively sets the root state. Adding these root tips is conservative with respect to the hypothesis of reversibility because numerous loss events during evolution could incorrectly support "eyes absent" at the root (Oakley 2000; Oakley 2003b; Oakley 2003a; Nosil and Mooers 2005; Goldberg and Igic 2008) and lead to the incorrect inference of multiple eye gains in the family. In addition to being conservative for the regain hypothesis, eye presence and shallow environment at the cylindroleberidid root are favored by external data, including presence of eyes in most other myodocopid ostracods (Oakley and Cunningham 2002; Oakley 2005), including shallow-water Silurian fossils (Siveter et al. 2003; Siveter et al. 2007; Siveter et al. 2010), some of which are stem-group cylindroleberidids or stem-group myodocopids.

We next compared one-state with 2-state (gain and loss) models (Mooers and Schluter 1999) of character evolution and calculated likelihood values for an irreversible model of evolution where rates of eye gain or transition from deep to shallow were set to zero. In order to account for potentially different speciation and extinction rates in taxa with and without eyes and living at different depths, we also used the BiSSE module implemented in Mesquite (Maddison et al. 2007). BiSSE allows estimates of rates of gain and loss of a trait, in concert with estimates of speciation and extinction rates under each state. To test whether a model including these extra parameters is significantly better than one without, we performed LRTs to compare different models of evolution. We constrained 2 speciation parameters to each other and 2 extinction parameters to each other (4-parameter model) and compared that model with an unconstrained model with separate parameters for speciation and extinction in lineages with each character state (6-parameter model). We finally performed LRTs to compare the unconstrained model (with 6 parameters) with a model where rate of eye gain was set close to zero (0.0000001; setting to zero caused program error) as additional tests of irreversibility in the BiSSE framework.

Node-based tests of reversible evolution.—Besides tree-based tests, the possibility of trait regain can also be tested by *node-based tests*—those that rely on statistical models to make explicit estimates of ancestral states at the nodes of phylogenetic trees. If an ancestral state is estimated to lack a trait and descendents of that node possess the trait, there is evidence for gain (or regain). Several ancestral state reconstruction analyses have suggested reversal of lost traits including myodocopid ostracod eyes (Oakley and Cunningham 2002), shell coiling (Collin and Cipriani 2003), insect wings (Whiting et al. 2003), sexual reproduction (Domes et al. 2007), and indirect development (Chippindale et al. 2004; Wiens et al. 2007). Nosil and Mooers (2005) revisited the question of generalists evolving from specialists using node-based ancestral state reconstruction because estimates of rates of gain and loss in tree-based tests are highly dependent on the number of taxa possessing each state. They recommended in general a comparison of tree-based tests and node-based tests to infer directionality in character state changes (see also Ree and Donoghue 1999; Oakley 2003a).

We also estimated ancestral states of all nodes on each bootstrap replicate in Mesquite for both eye presence/absence and habitat depth. We summarized all state changes separately for each of multiple character evolution models in Mesquite using the "Summarize State Changes Over Trees" option. As with tree-based analyses, node-based ancestral state reconstruction methods were performed after adding a zero-length branch at the root with an operational taxonomic unit (OTU) scored as "eyes present" and "shallow depth," respectively.

Simulation.—After finding different conclusions from tree-based and node-based tests, we simulated phylogenies to test whether or not phylogenetic estimation error is a cause of the discrepancy. Using observed data on presence/absence of eyes in species for the

"molecular" taxon set, we simulated topologies and branch lengths in Mesquite (Maddison and Maddison 2006) under a Yule model (pure birth), where each branch has an equal probability of speciation. The simulations used node depths matching those of the experimental clade (425 My). Other models, such as those allowing for extinction (birth–death), could also be used, but the main point here is to have topologies that are known with certainty to use for comparing results of tree-based and node-based tests to test whether or not phylogenetic uncertainty is the cause of the discrepancy. For each of 100 simulated trees that had eyes present at the root, we performed tree-based and node-based tests of character reversal, as described above for empirical data.

RESULTS

Data

We provide a nearly comprehensive phylogenetic analysis for known members of the family Cylindroleberididae by using 69 morphological characters (Appendices 1 and 2) for all 164 well-described extant species (and 4 outgroup taxa) and by adding molecular data for 26 of those species (plus 4 outgroup taxa) from 4 different gene regions (16S, 18S, 28S vx, 28S eemm). Table 1 summarizes the taxa we obtained for molecular analysis from 4 different gene regions and 30 different species. Collection data for specimens are listed in Supplementary Table S1 (available from http://www.sysbio.oxfordjournals.org). Technical difficulties prevented us from obtaining sequence data from every combination of species and gene region, so we treated some genes as missing data for some species. Undescribed new species are identified by museum accession number of the voucher and a genus name where known, for example, "*Synasterope*_J57067." Some specimens could not be confidently assigned to existing genera by morphology; these are indicated by family designation and voucher accession number, for example, "Cylindroleberididae_J57073." We deposited matrices and resulting phylogenetic topologies in TreeBASE, study #11258.

Phylogenetic Analysis

Model selection.—For molecular data, the statistically best-fit model is the GTR + gamma + I, for each gene region. The best partitioning strategy is to treat each

TABLE 1. Taxa with associated molecular data; new sequences in bold

Species	GenBank accession numbers			
	16S	18S	28S vx	28S eemm
Manawa staceyi		AF363295[a]	AF363351[a]	
Euphilomedes sordida	AY624742[b]	AF363299[a]	AF363349[a]	AF363330[a]
Vargula hilgendorfii	AY624737[b]	AF363301[a]	AF363357[a]	AF363332[a]
Rutiderma apex		AF363308[a]	AF363360[a]	AF363336[a]
Tetraleberis sp.		AF363294[a]	AF363359[a]	
Tetraleberis_J57056		**EU587246**	**EU587309**	**EU587278**
Leuroleberis surugaensis	AY624730[b]			
Leuroleberis mackenziei	**EU587264**	**EU591813**	**EU587314**	
Asteropterygion magnum		**EU591818**	**EU587303**	**EU587279**
Asteropella monambon	**EU587263**		**EU587308**	
Asteropella slatteryi_J57060	**EU587260**		**EU587304**	**EU587280**
Asteropella slatteryi_J57061	**EU587261**		**EU587305**	
Asteropella_J57062	**EU587262**	**EU587242**	**EU587306**	**EU587281**
Parasterope styx		**EU587236**	**EU587310**	**EU587282**
Skogsbergiella sp_ZMH_K-42206(DS 3)		**EU587237**	**EU587311**	**EU587283**
Cylindroleberididae_*new sp_ZMH-K-42207(DS 4)*		**EU587237**		**EU587284**
Archasterope bulla		**EU587239**	**EU587312**	**EU587285**
Cylindroleberididae_*new sp_ZMH_K-42209(DS 7)*		**EU587240**	**EU587313**	**EU587286**
Parasterope pollex		AF363309[a]	AF363348[a]	AF363339[a]
Bathyleberis oculata	**EU587251**	**EU591814**	**EU587287**	**EU587265**
Archasterope efficax		**EU591817**	**EU587288**	**EU587266**
Archasterope_J57065		**EU587249**	**EU587289**	**EU587267**
Synasterope_J57066	**EU587252**	**EU591815**	**EU587290**	**EU587268**
Synasterope_J57067		**EU587250**	**EU587291**	**EU587269**
Cylindroleberis marranyin		**EU587243**	**EU587292**	**EU587270**
Cylindroleberis_J57069	**EU587253**	**EU587244**	**EU587293**	**EU587271**
Parasterope gamurru_J53225	**EU587254**	**EU587245**	**EU587294**	**EU587272**
Parasterope gamurru_J53224	**EU587255**	**EU591819**	**EU587295**	**EU587273**
Parasterope_J57072		**EU587247**	**EU587296**	
Cylindroleberididae_J57073			**EU587307**	
Cylindroleberididae_J57074			**EU587297**	**EU587274**
Cylindroleberididae_J57075		**EU587241**	**EU587298**	**EU587275**
Cylindroleberididae_J57076	**EU587257**		**EU587299**	
Postasterope barensi		**EU587248**	**EU587300**	**EU587276**
Postasterope barensi_J57079	**EU587258**		**EU587301**	
Postasterope corrugata	**EU587259**	**EU591816**	**EU587302**	**EU587277**

Notes: [a]Oakley and Cunningham (2002).
[b]Wakayama and Abe (2006).

TABLE 2. Selection of partitioning scheme for molecular data

	Not partitioned	2-partitions	3-partitions	4-partitions
LnL	−9108.58	−8896.19	−8866.38	−8855.74
pi (params)	13	26	39	52
n (nucleotides)	2094	2094	2094	2094
AIC_c	18,243.2	17,844.4	17,810.8[a]	17,815.5

Note: [a]Best partitioning scheme.

gene as a separate partition, but there is no support for dividing the 2 noncontiguous 28S gene regions into separate partitions (Table 2). Following the recommendation of the RAxML manual, we excluded the proportion of invariant sites parameter and used GTR+gamma, estimating parameters separately for each gene.

Topology.—Many nodes on our phylogenies have low bootstrap support and/or low posterior probability values, especially for the "all" topology that lacks molecular data for many species (Figs. 1 and 2). Although no previously published phylogeny exists for this family, our ML trees (based on the "molecular" and "all" taxon sets) are mostly consistent with current taxonomy at higher taxonomic levels (Syme and Poore 2006). First, there is very strong support for monophyly of the family for the molecular taxon set (Fig. 1) and moderate support for monophyly of the family for the all taxon set (Fig. 2). The phylogeny of all taxa indicates that *Bruuniella* is a monophyletic group that is the sister-group to the rest of the cylindroleberidids, but molecular data are currently unavailable for this rare genus that is taxonomically ascribed to its own tribe within the subfamily Cylindroleberidinae (Kornicker and Harrison-Nelson 2005). With moderate to strong support, depending on the taxon set, the subfamilies Cyclasteropinae and Asteropteroninae are each monophyletic within the polyphyletic Cylindroleberidinae. The only exception to this is the species of *Tetraleberis sp J*57056 that groups with the Asteropteroninae rather than the Cyclasteropinae. However, this specimen is a juvenile and, although it is confidently placed in the Cylindroleberididae based on morphology, its identification to genus is tentative. We find strong support for a sister-group relationship between subfamilies Cylindroleberidinae and Cyclasteropinae in the molecular taxon set and moderate support for the same (excluding *Bruuniella*) in the phylogeny with all taxa, as hypothesized previously (Kornicker 1981).

On the phylogeny with all taxa, many genera are not monophyletic groups (e.g., *Asteropella*, *Synasterope*,

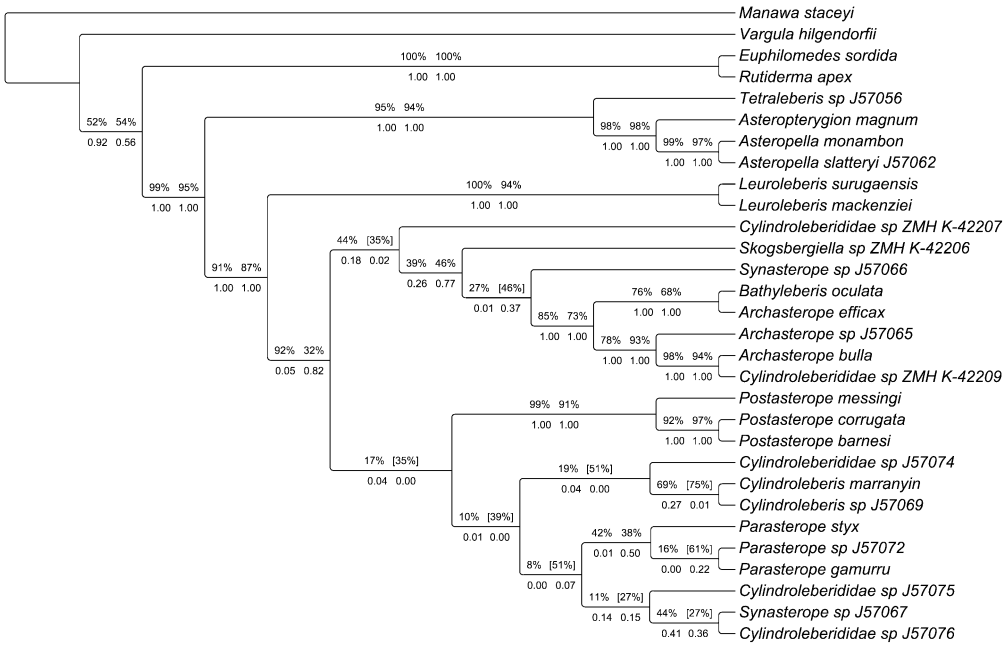

FIGURE 1. Phylogenetic analyses of cylindroleberidid ostracods for data referred to as the "molecular" taxon set in the text. The topology illustrated is the ML tree based on partitioned analysis of combined molecular and morphological data, implemented in RAxML. Numbers above each node are ML bootstrap percentages, based on 1000 pseudoreplicates. The number to the left includes both molecular and morphological data; the number to the right includes only molecular data. Below each node are Bayesian posterior probabilities (proportion of clades encountered in MCMC search). Partitioned Bayesian analyses were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The number to the left includes both molecular and morphological data; the number to the right includes only molecular data. The graphic was produced using phyutility (Smith and Dunn 2008) and TreeGraph (Stover and Muller 2010).

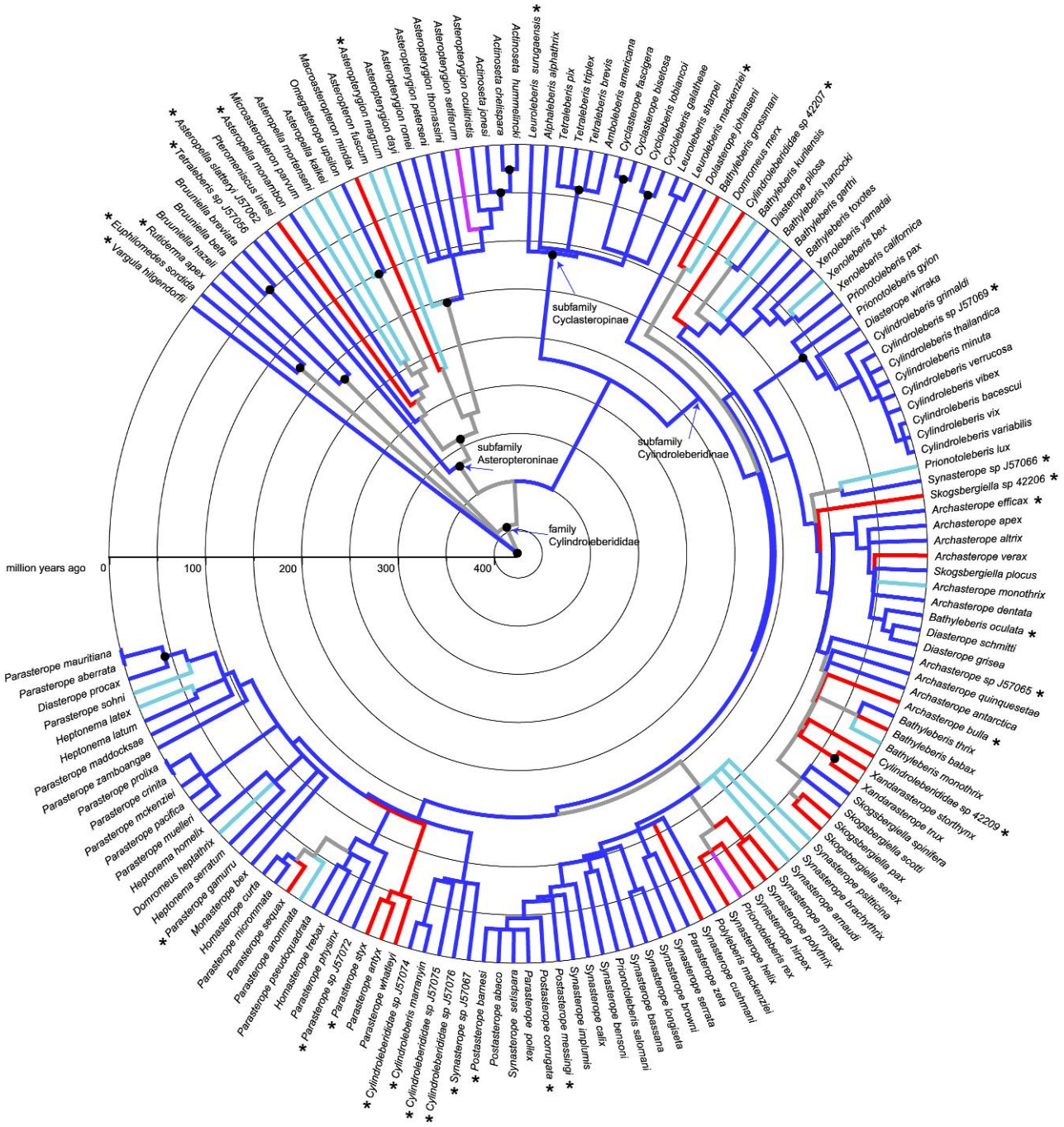

FIGURE 2. Phylogenetic analyses for data referred to as the "all" taxon set in the text. The topology illustrated is the ML tree based on partitioned analysis of combined molecular and morphological data, implemented in RAxML. The branch lengths are made ultrametric using r8s and by fixing the ancestor of myodocopida to 425 My, based on the presence of multiple stem and crown group myodocopids in the Silurian, Herefordshire assemblage. Black circles at nodes represent bootstrap proportions >75%. Branches are colored by ML inferred ancestral states: blue = eye present + shallow depth; red = eye absent + abyssal depth; light blue = eye absent + shallow depth; purple = eye present + abyssal depth; grey = equivocal for one or both character traces.

Parasterope). This is not surprising because many cylindroleberidid genera have not been defined by unique characters, but rather by unique combinations of characters, and the efficacy of these characters for defining natural groups has not been tested previously. Our results indicate that many of these genera may be artificial groups and thus would have limited predictive power or use in classification. A full interpretation of these taxonomic results will be presented elsewhere.

Calibration.—A global molecular clock was rejected in an LRT for the 30 species (including outgroups) with

TABLE 3. Tests of character evolution hypotheses using tree-based approaches

Question	Character[a]	Null	Alt	LRT[b]	df	*P*	Result
Is a 2-rate model best-fit?							
Molecular topology	Depth	Equal rates	Two rates	3.95	1	<0.05	2-rate model favored
	Eyes	″	″	3.95	1	<0.05	″
All topology	Depth	″	″	7.43	1	<0.01	″
	Eyes	″	″	8.78	1	<0.01	″
Reject submergence only/loss only?							
Molecular topology	Depth	D->S = 0	Unconstrained	4.24	1	<0.05	Submergence-only rejected
	Eyes	Eye gain = 0	″	4.40	1	<0.05	Dollo's law rejected
All topology	Depth	D->S = 0	″	14.14	1	<0.01	Submergence-only rejected
	Eyes	Eye gain = 0	″	25.75	1	<0.01	Dollo's law rejected
Is BiSSE model favored?							
Molecular topology	Depth	λ0 = λ1; μ0 = μ1; q01; q10	Unconstrained	1.85	2	0.40	BiSSE model not favored
	Eyes	″	″	1.19	2	0.55	″
All topology	Depth	″	″	4.58	2	0.10	″
	Eyes	″	″	4.29	2	0.12	″
Reject submergence only/loss only with BiSSE?							
Molecular topology	Depth	D->S ∼ = 0	Unconstrained	4.93	1	<0.05	Submergence-only rejected
	Eyes	Eye gain ∼ = 0	″	4.26	1	<0.05	Dollo's law rejected
All topology	Depth	D->S ∼ = 0	″	17.36	1	<0.01	Submergence-only rejected
	Eyes	Eye gain ∼ = 0	″	21.35	1	<0.01	Dollo's law rejected
Are eyes/depth correlated?							
Molecular topology	Depth/eyes	Rates are independent	Rates are dependent	48.72		≪0.01	Eye and depth correlated
All topology				48.72		≪0.01	″

Notes: [a]Scored as binary character with zero-length branch added to the root and scored as present (eyes) or shallow (depth).
[b]Based on median value of separate calculations for 1000 topologies resulting from pseudoreplicated data sets generated by bootstrapping original character data.

molecular data. The log likelihood assuming a molecular clock was −10451.9 compared with the non-clock likelihood of −10352.1, resulting in a likelihood ratio statistic of 199.6 ($P < 0.0001$). CV values estimated in r8s with the "crossv" command from the ML phylogenies were 130,000 for the "all" taxon set and 13 for the "molecular" taxon set and were used for all pseudoreplicated data sets. Because of the rate variation and because we only used a single calibration point at the root, we acknowledge that our divergence time results may have large error ranges, which could be refined in future studies.

Character Evolution

Correlation between eyes and depth.—We found a significant ($p \ll 0.01$) correlation between eye presence/absence and habitat (Kornicker 1975), based on both the "molecular" and the "all" taxon sets (Table 3). Our results support the hypothesis that light environment, including absence of sunlight at depth <1000 m, influences the evolution of lateral eye morphology. Parameter estimates for Pagel's model indicate rates of evolution between different combinations of eye state and habitat depth (Table 4). We find that parameter estimates are not consistent between our 2 taxon sets: the rank correlation between the 8 rate estimates from the 2 data sets is only 0.167 ($P = 0.65$). This inconsistency suggests a sensitivity of these conclusions to taxon sampling. Nevertheless, some results are consistent between data sets. The rate of eye gain in shallow waters is among the highest of all rates. In addition, transitions away from eyeless/deep species show low rates of evolution. Unlike lateral eyes, a median eye is present in all known cylindroleberidid ostracods, indicating that the evolution of different eye types is influenced differently by light environment, which could be the result of different functions and/or developmental constraints. The retention of genes used in median eyes

TABLE 4. ML parameter estimates from Pagel's discrete character correlation model, assuming ML trees

Parameter	Molecular taxon set[a] [Rank] Value	All taxon set[a] [Rank] Value
Eyeless/deep-> eyeless/shallow (q12)	[6] 1.20811	[7] 0.008545
Eyeless/deep-> eyed/deep (q13)	[7] 0.00003	[5] 0.189710
Eyeless/shallow-> eyeless/deep (q21)	[3] 5.21783	[3] 0.700704
Eyeless/shallow-> eyed/shallow (q24)	[2] 58.24567	[1] 1.823280
Eyed/deep-> eyeless/deep (q31)	[1] 164.11474	[8] 0.000003
Eyed/deep-> eyed/shallow (q34)	[5] 2.02314	[2] 1.684425
Eyed/shallow-> eyeless/shallow (q42)	[4] 2.45081	[4] 0.465838
Eyed/shallow-> eyed/deep (q43)	[8] 0.00013	[6] 0.013008

Note: [a]Correlation between parameter values from different taxon sets = 0.167, $P = 0.65$ (Pearson's rank correlation).

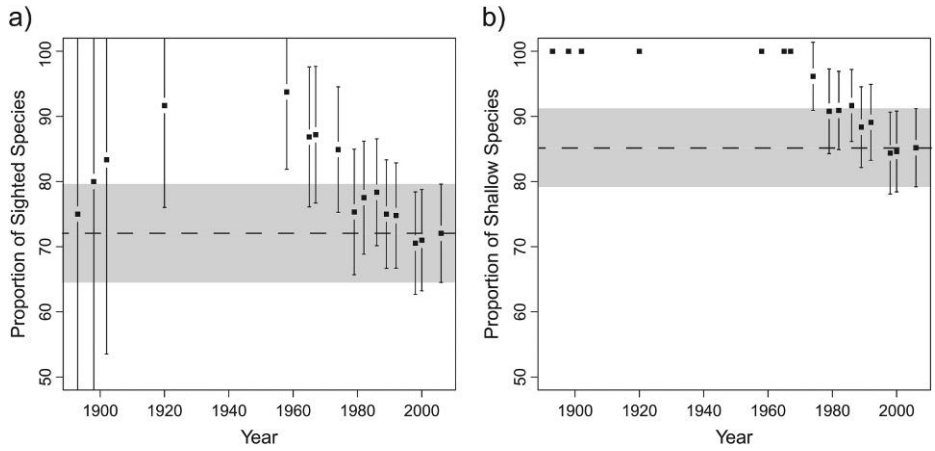

FIGURE 3. The proportion of described cylindroleberidid species with eyes over time (a) and the proportion of described deep-sea species (b) have changed little. We plotted the proportion of species with eyes (a) or collected from >1000 m (b) and confidence intervals by year.

could facilitate regain of compound eyes (Oakley and Cunningham 2002; Oakley 2003b; Oakley et al. 2007), a general process termed switchback evolution (Van Valen 1979).

An important consideration when studying character evolution is whether taxon sampling efforts well represent the true proportions of species with different states. We found that the proportion of described sighted species has changed little over time, and the proportion of deep-sea species has changed little since abyssal species were first described in the 1970s (Fig. 3). The relatively stable proportions of species with different states could be interpreted in multiple ways. First, it could mean that we have a good estimate of the true proportions, an assumption of the methods we employ here. Another interpretation is that there is a bias in describing eyeless:sighted and shallow:deep species, but the bias has always persisted at a similar level.

Node-based tests rarely support reversible evolution.—Although there is some variation across taxon sets and pseudoreplicated phylogenies, overall we find little evidence for character reversals using node-based tests (ancestral state reconstruction methods). Results of the ML phylogenies with branch lengths adjusted by r8s are presented in Figures 2 and 4, for both eyes and depth. Results from the 'Summarize State Changes Across Trees' option from 1000 pseudoreplicated data sets for the molecular taxon set are presented in Figure 5 and for the all taxon set in Figure 6.

The "molecular" taxon set shows very little sensitivity to phylogenetic uncertainty and we find evidence for eye loss and depth-submergence events but little evidence for reversals when using node-based tests. Most pseudoreplicated trees showed 4–5 eye losses and 3–4 shallow-to-deep transitions (submergence events). For both eye and depth analyses, there was more variation in inferred number of eye losses and submergence events when assuming the 2-rate model than the equal-rate model (Fig. 5). In contrast, we found zero eye gains and zero deep-to-shallow transitions in a very high proportion (92–95%) of pseudoreplicated trees, regardless of whether we assumed a equal-rate or 2-rate model of character evolution (Fig. 5).

The "all" taxon set showed some differences in results based on 1- and 2-parameter models and some sensitivity to phylogenetic uncertainty, but like the molecular taxon set, there was more support for loss and submergence than for gain and ascension with the node-based tests. Under a 1-parameter model, pseudoreplicated trees implied about 5–20 eye loss or submergence events, whereas under a 2-parameter model, most pseudoreplicated trees implied 0–10 such events. This result is not due to rate differences between 1- and 2-parameter models because the 2-parameter rates are higher on average (Table 5). Instead, one explanation for fewer events in the 2-parameter model is the increased uncertainty of ancestral reconstructions under a 2-parameter model. To count as a state transition in this analysis, an ancestral node must show significant support for absence and a descendent must show significant support for presence of the trait (with significant support defined as alternative ancestral states differing in log likelihood by 2 or more). Although the number of inferred loss and submergence events heavily depended on model, inferred numbers of eye gains and ascension events differed less between models. Most pseudoreplicates support zero eye gains and zero deep-to-shallow transitions although phylogenetic uncertainty is evident because different bootstrap replicates indicate different numbers of transitions, especially for the 2-rate model (Fig. 6).

Tree-based tests support reversible evolution.—Using tree-based tests, we found (Table 3) models of irreversible evolution often to be rejected for both eyes and depth, regardless of taxon set (at $P = 0.05$, eyes and depth

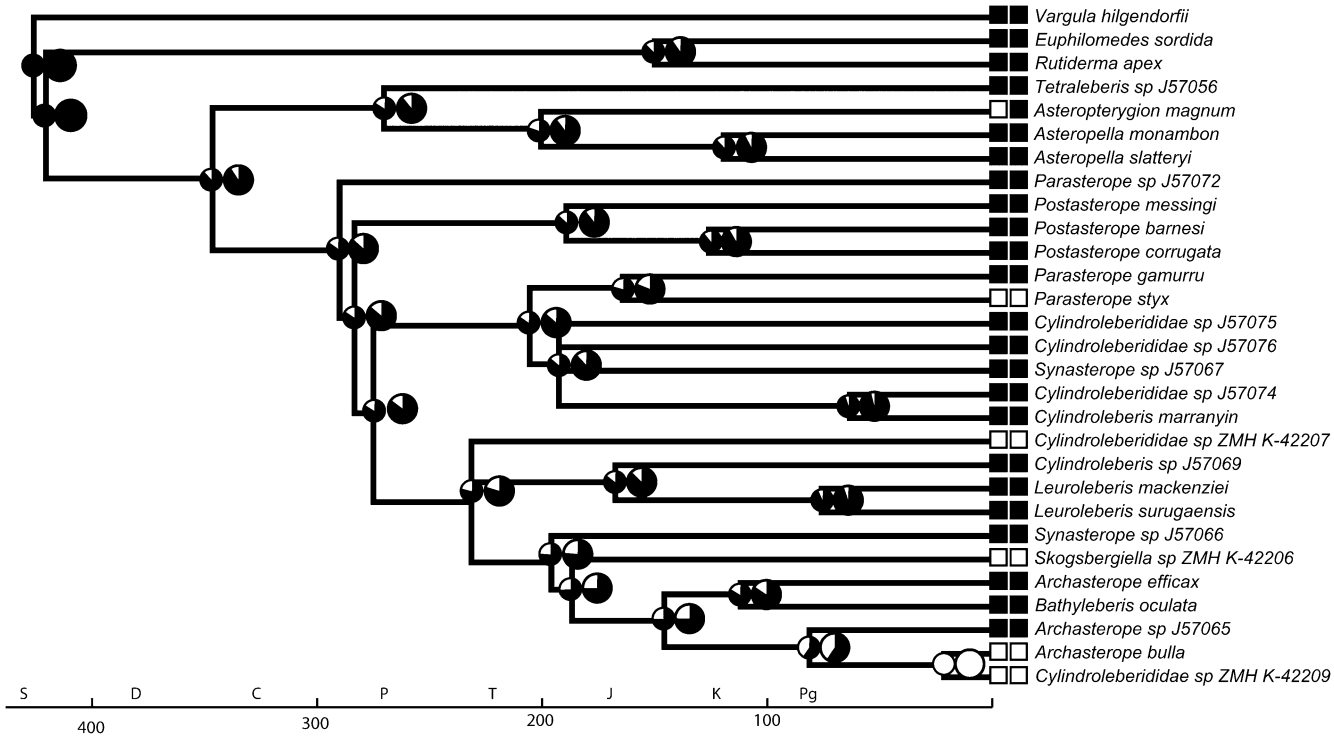

FIGURE 4. ML cylindroleberidid phylogeny based on only molecular data. The branch lengths are made ultrametric using r8s and by fixing the ancestor of myodocopida to 425 My. This tree has some differences with the combined morphological/molecular tree illustrated in Figure 1, including the position of *Leuroleberis*. Ancestral states were estimated using the asymmetric Mk2 model. Smaller pie charts to the left represent proportion of likelihood supporting eye absence (white) versus eye presence (black); slightly larger pies to the right represent proportion of likelihood supporting abyssal (white) and shallow (black) depth. Squares to the right of species represent observed states of eyes (left) and depth (right). Note that most shallow species have eyes, with the exception (in this taxon set) of *Asteropterygion magnum*.

rejected irreversibility in more than 65% of bootstrap replicates for the molecular taxon set and in more than 95% of replicates for the all taxon set) and whether or not we incorporated differential rates of speciation/extinction using the BiSSE model (Maddison et al. 2007). Rejecting irreversibility leads to the perhaps surprising conclusion that lateral eyes have been regained within the cylindroleberidid family and that abyssal lineages have dispersed to shallow waters during the history of the family. Although median values and the majority of all individual pseudoreplicates reject irreversibility, there was some variation among analyses based on different pseudoreplicated topologies, indicating some sensitivity to phylogenetic uncertainty, mainly for the molecular taxon set, presumably because the smaller taxon set has lower statistical power. For the molecular taxon set, 40% (eyes) and 30% (shallow-to-deep migration) of individual pseudoreplicates failed to reject irreversibility ($P > 0.05$). For the "all" taxon set, only a single individual pseudoreplicate failed to reject irreversibility for eyes and 8% failed to reject irreversibility of depth ($P > 0.05$).

Two-rate models were generally favored over equal-rate models regardless of taxon set, but again there was some sensitivity to phylogenetic uncertainty. For the molecular taxon set, 46% and 50% (for eye and depth, respectively) of individual topologies do not support the 2-rate model ($P > 0.05$). There is less variability for the "all" taxon set, where 85% and 74% (eye and depth, respectively) do not support the 2-rate model ($P > 0.05$). The difference in sensitivity is likely related to the number of taxa: greater numbers of taxa more commonly support 2-rate over equal-rate models of character evolution (Mooers and Schluter 1999).

Based on median values for eye and depth characters using the "all" taxon set, we found little support for the 6-parameter BiSSE model (Table 3), which accounts for differential speciation and extinction rates for lineages with different character states (Maddison et al. 2007). Median values fail to reject the null hypothesis and therefore do not support differential speciation and extinction rates. Nevertheless, there is some variability among pseudoreplicated trees (37% and 42% of trees actually do reject the null for eyes and depth, respectively). Similar to the "all" taxon set, analyses using the "molecular" taxon set did not reject the null model using the median likelihood values of pseudoreplicated trees. Here, there is extensive variability due to phylogenetic uncertainty: unlike median values, 50% and 46% of pseudoreplicates reject the null hypothesis for eyes and depth, respectively. Even though the BiSSE

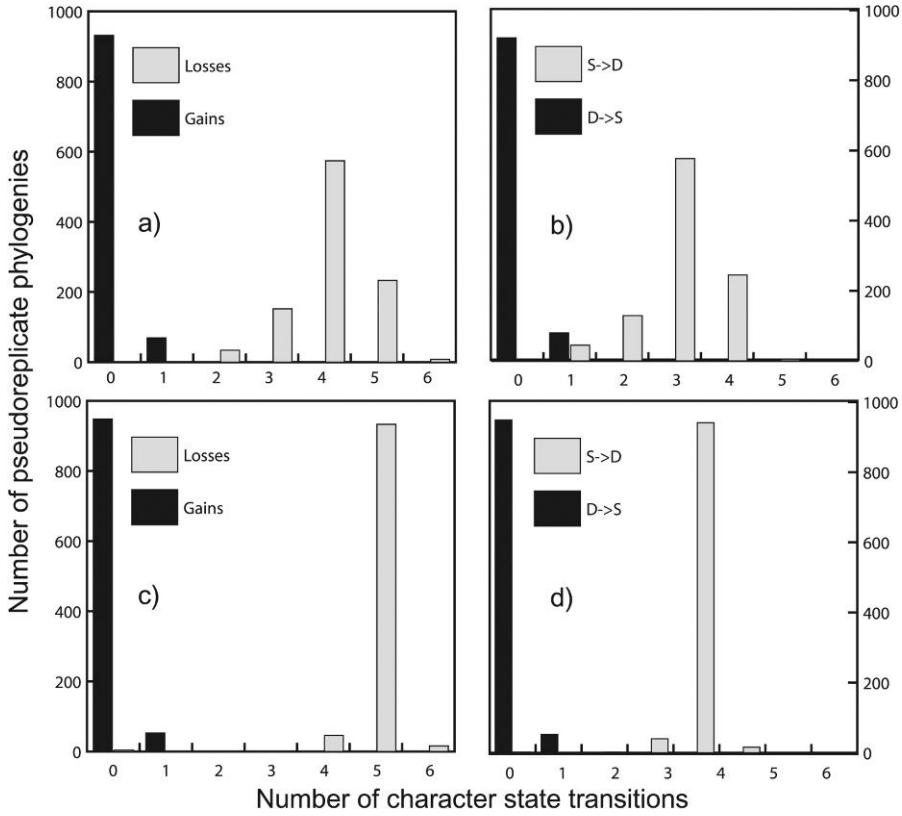

FIGURE 5. Number of character state transitions inferred on bootstrap pseudoreplicates of morphological and molecular data from the "molecular" taxon set phylogeny. Number of gains (black) and losses (grey) of eyes inferred on 1000 trees using a 2-rate model of character evolution (a). Number of deep-to-shallow (black) and shallow-to-deep (grey) dispersal events inferred on 1000 trees using a 2-rate model of character evolution (b). Number of gains (black) and losses (grey) of eyes inferred on 1000 trees using a 1-rate model of character evolution (c). Number of deep-to-shallow (black) and shallow-to-deep (grey) dispersal events inferred on 1000 trees using a 1-rate model of character evolution (d).

model is not favored in most pseudoreplicated trees, we present the parameter estimates of the full BiSSE model in Table 5, which are similar for both taxon sets. These values estimate the rate of speciation to be approximately twice as rapid in the deep sea and eyeless taxa compared with shallow sea and sighted taxa. However, biological conclusions should be tempered by the fact that the full BiSSE model may be overparameterized for the current data sets.

Simulation.—We found discrepancies between tree-based and node-based analyses in simulated trees, consistent with the hypothesis that phylogenetic estimation error is not a cause of the discrepancy between types of analysis. Using observed data on eye presence/absence and a Yule model, 100% of simulated trees rejected irreversibility in tree-based analyses, whereas 95% of simulated trees showed only eye loss in node-based analyses. A representative example of the simulated trees is shown in Figure 7.

DISCUSSION

Whether evolutionary trends or biases are present, and in particular whether traits are reversible or not, is of enduring interest in evolutionary biology (Darwin 1868,1875; Dollo 1893; Cockrell and Ireland 1933; Simpson 1953; Gould 1970; Van Valen 1979; Marshall et al. 1994; Kohlsdorf and Wagner 2006), and for ostracod eyes in particular (Oakley and Cunningham 2002; Dingle 2003; Hunt 2007; Horne 2010). Both reversal and irreversibility are biologically interesting, with different implications for the mechanisms for origin and maintenance of phenotypic traits during macroevolution. Yet distinguishing between alternative patterns of evolutionary change is notoriously difficult and often depends on a priori assumptions about how characters evolve and on the statistical and methodological tools used to assess the patterns (Omland 1997; Ree and Donoghue 1998; Schultz and Churchill 1999; Oakley and Cunningham 2002; Oakley 2003a; Kohlsdorf and Wagner 2006; Goldberg and Igic 2008). While examining the patterns of gain and loss of compound eyes and transitions between shallow- and deep-sea habitat of cylindroleberidid ostracods, we found contradictory results based on tree-based and ancestral node-based inferences, which have different implications and inspire different evolutionary hypotheses. The dependence of conclusions on choice of models and methodology highlights the fact that interpretations about character evolution should be made with caution

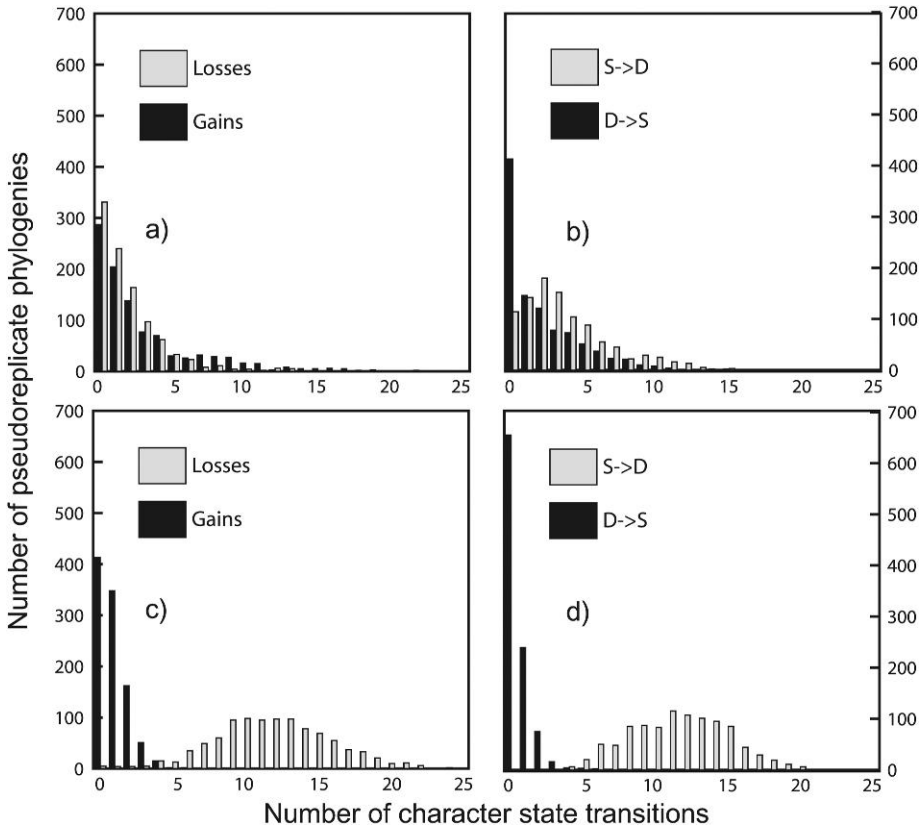

FIGURE 6. Number of character state transitions inferred on bootstrap pseudoreplicates of morphological and molecular data from the "all" taxon set phylogeny. Number of gains (black) and losses (grey) of eyes inferred on 1000 trees using a 2-rate model of character evolution (a). Number of deep-to-shallow (black) and shallow-to-deep (grey) dispersal events inferred on 1000 trees using a 2-rate model of character evolution (b). Number of gains (black) and losses (grey) of eyes inferred on 1000 trees using a 1-rate model of character evolution (c). Number of deep-to-shallow (black) and shallow-to-deep (grey) dispersal events inferred on 1000 trees using a 1-rate model of character evolution (d).

and that final conclusions may often require testing alternatives with other data (Schultz and Churchill 1999; Oakley and Cunningham 2000; Wiens et al. 2007; Goldberg and Igic 2008).

Although the current study included many analyses of varying complexity, the main conclusions can be summarized as follows. First, there is a strong correlation between absence of lateral eyes and living in abyssal depths such that the evolution of these traits seems tightly coupled. For tree-based tests, we find that a 2-rate model is usually favored for eye and depth evolution, meaning that eye regain and deep-to-shallow

TABLE 5. Median values and ratios for parameter estimates for different models, averaged across bootstrap pseudoreplicates

Taxon set	Character	Model	Parameter 1[a]	Parameter 2[a]	(0->1)/(1->0)[b]	Speciation (1)/Speciation (0)[b]
Molecular	Depth	Mk1	Rate = 8.77×10^{-04}	N/A	N/A	N/A
Molecular	Depth	Mk2	Gain = 1.62×10^{-02}	Loss = 2.75×10^{-03}	5.89	N/A
Molecular	Depth	BiSSE	Speciation0 = 0.89	Speciation1 = 0.29	6.80	0.32
Molecular	Eyes	Mk1	Rate = 1.17×10^{-03}	N/A	N/A	N/A
Molecular	Eyes	Mk2	Gain = 1.60×10^{-02}	Loss = 3.50×10^{-03}	4.57	N/A
Molecular	Eyes	BiSSE	Speciation0 = 0.82	Speciation1 = 0.31	4.86	0.36
All	Depth	Mk1	Rate = 1.40×10^{-03}	N/A	N/A	N/A
All	Depth	Mk2	Gain = 1.05×10^{-02}	Loss = 1.72×10^{-03}	6.10	N/A
All	Depth	BiSSE	Speciation0 = 1.07	Speciation1 = 0.50	8.84	0.42
All	Eyes	Mk1	Rate = 3.64×10^{-03}	N/A	N/A	N/A
All	Eyes	Mk2	Gain = 1.15×10^{-02}	Loss = 4.17×10^{-03}	2.76	N/A
All	Eyes	BiSSE	Speciation0 = 0.79	Speciation1 = 0.55	3.19	0.59

Notes: [a]Parameter estimates are low for non-BiSSE models because branch lengths were scaled to absolute time in years. For BiSSE models, all branch lengths were scaled by 0.01 years; otherwise likelihood searches did not converge.

[b]Character states: For depth, 0 = deep, 1 = shallow; for eyes, 0 = absent, 1 = present. As such, speciation0 refers to rate of speciation in deep sea or eyeless species and speciation1 is rate of speciation in shallow or eyed species.

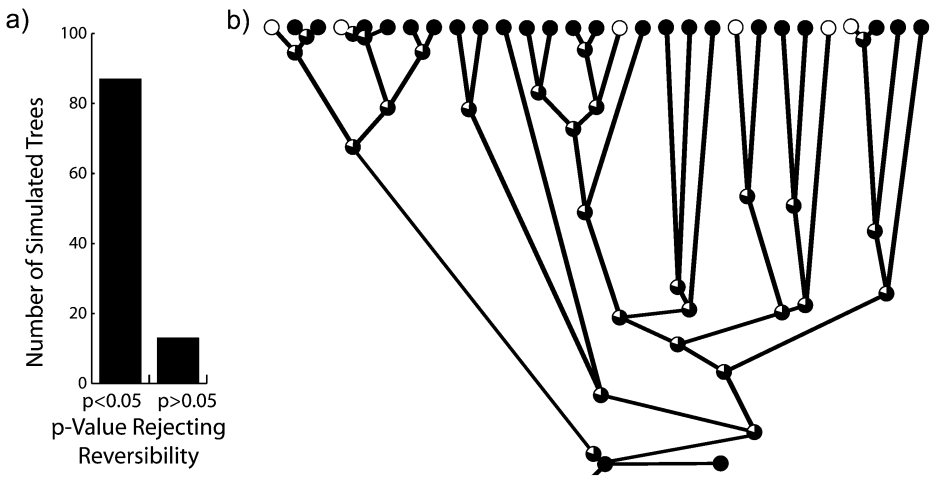

FIGURE 7. Most simulated trees support reversible character evolution with a tree-based test but do not support reversibility with a node-based test. Illustrated are results of 100 Yule model–simulated tree topologies using observed character state distributions of eye presence/absence (i.e., character states were fixed, not simulated). Histogram of *P* values of tree-based tests of character reversibility. For each simulated tree, we compared the likelihood of a 2-rate character model with the likelihood of a Dollo model and inferred a *P* value assuming a chi-squared distribution (a). One representative simulated topology (node height proportional to relative time of speciation in Yule simulation), with ancestral states reconstructed (b). All ancestors show higher likelihood support for presence than absence of the character, in contrast to the implications of the tree-based tests on the same simulated trees.

dispersal are supported. In fact, parameter estimates based on these models indicate that eye gain and ascension from deep to shallow seas are three to eight times faster than eye loss and shallow-to-deep dispersal (Table 5). For node-based tests, we instead find that successive node reconstructions do not unambiguously support eye regain or deep-to-shallow dispersal. This study expands on previous tests for phylogenetic irreversibility and demonstrates via several analyses how different methodological approaches can give different results for the same data set. Thus, researchers need to think carefully about how biology can justify the choice of one model over another and can lead to the refinement of models or to the development of new models/tests.

Why Do Tree-based Analyses Support Reversals and Node-based Analyses Do Not?

We considered multiple hypotheses as to why tree-based analyses support reversible evolution, but node-based tests do not. First, we considered whether our choice of methodology caused the discrepancy between tree-based and node-based approaches. For example, simulations by previous authors indicate that tree-based analyses often incorrectly support reversals if conducted with improper assignment of root-state frequencies or by ignoring the effects of biased speciation and extinction for taxa with different character states (Goldberg and Igic 2008). These difficulties do not explain the discrepancies between tree-based and node-based tests in cylindroleberidids. Although based on external information about fossils and outgroups we added a taxon at the root with character states of eyes present and shallow depth, we found empirically that changing this approach made no qualitative difference with our data. Specifically, we removed the added taxon at the root and assumed equal prior probabilities for the 2 possible states at the root. We found negligible differences (not shown) compared with the results reported. We also used "diversitree" (available from http://www.zoology.ubc.ca/prog/diversitree) to implement different root-state assumptions in tree-based tests for the ML topologies, and again this produced no qualitative differences compared with the results presented. One explanation for the lack of reliance on root state with our data is that a rapid rate of character evolution has effectively erased the history of the root state. Finally, we accounted for different rates of speciation/extinction using the BiSSE model (Maddison et al. 2007). In both cases, regain of compound eyes and dispersal from shallow-to-deep water were still favored in most tree-based analyses.

A second possible explanation for the discord between tree-based and node-based tests is phylogenetic uncertainty. Although phylogenetic uncertainty adds another level of ambiguity to our empirical study, it does not cause differing results between tree-based and node-based analyses. More specifically, using observed data for presence/absence of eyes, most Yule model–simulated trees (known with certainty) showed the same discrepancy between tree-based and node-based analyses, as did many individual trees based on bootstrap pseudoreplicates. Therefore, phylogenetic uncertainty is not the cause.

One reason these methods yield different results is that the node-based test reconstructs many ancestral states with some level of ambiguity. In the case of cylindroleberidids, although all ancestral nodes (except some nodes leading to exclusively eyeless taxa) were reconstructed as more likely to have eyes present and shallow depth, most nodes had at least some uncertainty, some with considerable uncertainty (see also simulation in Fig. 7). Another possible approach to node-based tests could be to use joint, rather than marginal estimates of ancestral states (Pagel 1999). Joint estimates find the entire set of ancestral states that maximizes the likelihood of the states observed at the tips, whereas marginal estimates compare support for one state versus the other at each node in turn. However, uncertainty should be considered even if using joint reconstructions—for example, one might consider the entire suite of joint reconstructions that are not significantly different from the ML estimate. As such, it seems there will be considerable uncertainty in node-based tests (joint or marginal) whenever rates of evolution are high, although the specific nature of that uncertainty may be different for the different methods. In the case of cylindroleberidid eye and depth evolution, there is a strong possibility that one or more of these ambiguously reconstructed ancestors may have actually lacked eyes, and thus when considering the tree as a whole, it is statistically likely that a regain occurred somewhere on the tree. Unfortunately, the ambiguity of node-based tests leads to ambiguity in pinpointing particular evolutionary events for further analysis, such as genetic examinations or estimates of the absolute timing of reversals (Wiens 2011). In the end, the tree-based test, which summarizes this information over the whole tree, appears to be more sensitive in its ability to recover cases of trait reversibility and thus to reject Dollo's Law. This suggests that using node-based tests may be a more conservative way to test Dollo's Law, especially when using equal-rate models of evolution. The incongruence of the results obtained herein provides a framework within which to discuss the biological implications of alternative evolutionary hypotheses.

Tree-based Analyses Favor Deep-to-Shallow Transitions and Eye Regain

If the results based on tree-based tests are correct, then compound eyes were regained at some point and species migrated from abyssal to shallow seas during the evolution of cylindroleberidid ostracods. Although many authors have argued against the regain of complex traits during evolution, we point out two shortcomings of a priori assumptions used to argue against regain of complex phenotypes. First, is the assumption that most components of a trait degrade quickly after loss, thus preventing the possibility of trait regain. Contrary to this assumption, some eyeless crayfishes possess the visual pigment gene opsin (Crandall and Cronin 1997). Eyeless *Astyanax* cavefish possess numerous biochemical components of vision and developmental regulators of eye morphology (Jeffery et al. 2003), which probably made possible their experimental resurrection through hybridization of independently eyeless populations (Teotonio and Rose 2001; Borowsky 2008). In general, the multifunctionality (pleiotropy) of genes may often prevent their loss, even after loss of one or more functions (Marshall et al. 1994). Interestingly, in cylindroleberidids, median eyes are present in all known species and could serve to maintain genes formerly used in lost compound eyes (Oakley 2003b). The presence of genetic components coupled with trait absence in cavefish suggests that developmental regulatory processes—rather than gene loss—are responsible for loss of eyes, as supported by the observation that upregulation of *sonic hedgehog* (*Shh*) is associated with cell proliferation leading to eye loss during *Astyanax* eye development (Jeffery et al. 2003). Presumably, regulatory changes such as increased expression of *Shh* could be reversed during evolution, especially because gene expression level probably evolves very quickly (Oakley 2005).

A second potential shortcoming of the arguments that complex traits should commonly be lost and should only rarely be regained is the focus on the origin of mutations (genetics) and a lack of focus on fixation of mutations (microevolution driven by ecology). For example, regardless of the potential ease of a mutation for eye loss, traits like eyes should not be lost in illuminated environments when they are central to organismal function and fitness. Only after a change in habitat might those mutations be fixed. (A caveat is that ostracods with highly ornamented carapaces may not benefit from eyes.) Similarly, reverse mutations after loss in the deep sea might never be fixed, until the lineage returns to illuminated environments where eyes are useful. (A caveat is that light sources such as bioluminescence may exist in the deep sea.) These considerations illustrate the general importance of ecology and biogeography for trait loss and gain, and eye evolution is a useful model system with which to understand these factors. The frequency of dispersal and maintenance of shallow-water species with eyes to the deep sea should be a major determinant of rates of eye loss. Conversely, dispersal and maintenance of deep-sea eyeless species to illuminated environments could be a major determinant of rates of eye (re)gain during evolution.

The maintenance of genetic components after eye loss eyes could facilitate regain, perhaps through regulatory genetic changes. Common switching between dark and illuminated habitats over evolutionary time could even lead to higher-level selection, as discussed and sometimes demonstrated in other systems (e.g., Rabosky and McCune 2010; Gould 2002). In this case, deep-sea clades maintaining the ability to regain eyes and therefore to recolonize shallow waters could be maintained more often than those lineages that lose the genetic capabilities to develop eyes, a hypothesis that remains untested.

Node-based Analyses Favor Only "Submergence" and Eye Loss

Although our tree-based tests significantly reject irreversible evolution under a variety of assumptions, node-based tests favor zero eye gains with multiple eyes losses and favor zero deep-to-shallow transitions (the submergence hypothesis) with multiple shallow-to-deep dispersal events, especially when assuming equal-rate models of evolution (Fig. 6c,d). The implication of the results based on node-based methods is that cylindroleberidids have commonly dispersed from shallow to deep water and that eyes have been commonly lost, but not the reverse. Similar results have been found in other organisms. Although Dingle (2003) suggested eye regain and ascension in a podocopid ostracod, a subsequent thorough phylogenetic analysis indicates that eyes (in this case, the median eye) have only been lost, not regained (Hunt 2007). A recent analysis of isopods (Raupach et al. 2009) came to the same conclusion: multiple shallow-to-deep transitions and eye loss events occurred, without the reverse (although no formal analysis of character evolution was conducted on the phylogenies). In stylasterid corals, Lindner et al. (2008) did find transitions from deep-to-shallow oceans, but the definition that they used for deep ocean (>50 m) is much different than we use here (>1000 m), and although they found defensive features to have originated in deep water animals, correlation of characters with habitat depth was not addressed. In fact, the different physiological and life history requirements of the abyssal sea could make dispersal from deep-to-shallow waters highly improbable (Childress 1995; Raupach et al. 2009), thereby favoring the conclusions in cylindroleberidids indicated by node-based methods.

This result—that eyes are commonly lost but rarely regained—provides testable predictions for the rapid loss or mutation of genes involved in compound eyes, as demonstrated in other systems. For example, the pigment gene *cinnabar* showed mutations consistent with nonfunctionality in subterranean beetles (Leys et al. 2005). Following evolutionary transitions in flower color, genes have also been lost in angiosperms that are critical for pigment production (Zufall and Rausher 2004; Whittall et al. 2006). Future research in cylindroleberidids could examine whether the genomes of eyeless species possess the genetic components of compound eyes, such as functional opsins or other pigment genes like *cinnabar*. Examination of multiple case studies from individual cylindroleberidid species and other eyeless arthropods (e.g., Villacorta et al. 2008) could uncover patterns in the order and timing of hypothesized gene losses. Although deep-sea cylindroleberidids are very difficult to access for such detailed molecular work, a number of shallow-water (and therefore more accessible) and eyeless species exist. These species (e.g., *Asteropella mortenseni* and *Asteropterygion magnum*) tend to possess highly calcified and/or ornamented carapaces, which would obstruct the path of light to the compound eyes, presumably making them less functional and relatively expendable.

Summary

In our tests of trait irreversibility, we show incongruent results from tree-based and ancestral node-based tests. This discrepancy between approaches is also found while analyzing simulated phylogenies. We suggest that such discrepancies will be common when rates of evolution are high, which will lead not only to ambiguity in ancestral reconstruction at particular nodes in node-based tests but also to a strong conclusion from tree-based tests that a reversal must have happened at some point. Although it may seem unsatisfying, we conclude that it is not possible with currently available data to distinguish between rapid one-way dispersal from shallow to deep (with only eye loss) and two-way dispersal (including eye regain events). These alternatives could be disentangled by more complex models of character evolution (Skinner 2010), through better phylogenetic resolution or through detailed genetic study. Nevertheless, based on the phylogenetic patterns presented here, we suggest that cylindroleberidid ostracods provide rich opportunities for studying the mechanisms of eye loss (and possibly regain) during evolution.

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APPENDIX 1

Summary of Morphological/Geographical Characters Used in the Phylogenetic Analyses. All Characters Are Unordered Except Where Noted Otherwise

1. Carapace with vertical ridge posterior to incisur: (0) present or (1) absent
2. Carapace with posterior nodes: (0) present or (1) absent
3. Carapace with horizontal ridges: (0) present or (1) absent
4. Carapace rostrum anterior margin: (0) smooth or (1) scalloped
5. Carapace with dentition on inner dorsal margin: (0) absent or (1) present (from mid to posterior)
6. Carapace of male with posterior row of hairs: (0) present or (1) absent
7. Carapace posterior infold with ridge between list and valve margin: (0) present or (1) absent
8. Antenna 1: article 2 with lateral setae: (0) present or (1) absent
9. Antenna 1: article 2 with: (0) 1 or (1) 2+ lateral setae
10. Antenna 1: article 2 with dorsal setae: (0) present or (1) absent
11. Antenna 1: article 2 with: (0) 1 or (1) 2+ dorsal setae
12. Antenna 1: article 3 with: (0) 1–5, (1) 6 or (2) 7+ dorsal setae [ordered]
13. Antenna 1: article 5 with: (0) 0 or (1) 1+ dorsal nodes
14. Antenna 1: article 5 s-seta proximal filament: (0) present or (1) absent
15. Antenna 1: article 5 s-seta with: (0) 1 or (1) 2+ proximal filaments
16. Antenna 1 article 5 s-seta terminal filaments: (0) one or more tips bifurcate or (1) terminal filaments all of similar length, serially branching from a common stem
17. Antenna 1: article 5 s-seta with: (0) up to 5, (1) 6 or (2) 7+ terminal filaments [ordered]
18. Antenna 1: article 5 s-seta of male: (0) filamentous or (1) not filamentous, similar to female
19. Antenna 1: article 6 medial seta: (0) present or (1) absent
20. Antenna 1: article 6 medial seta: (0) short, not reaching past 8th article or (1) long, reaching past 8th article
21. Antenna 1: article 7 a-seta shape: (0) claw-like or (1) seta-like
22. Antenna 1: article 8 d-seta: (0) present or (1) absent or minute
23. Antenna 1: article 8 d-seta shape: (0) filamentous (blunt tip) or (1) seta-like (tapered tip)
24. Antenna 1: article 8 d-seta length as a percentage of e-seta: (0) <30% or (1) >50%
25. Antenna 1: article 8 d-seta: (0) bare or (1) with spines
26. Antenna 1: article 8 e-seta: (0) with spines or (1) without spines
27. Antenna 1: male c- and f-setae: (0) similar length to b- and g- setae or (1) very long: 1.5–2× length of Antenna 1
28. Antenna 2: protopod distomedial seta: (0) present or (1) absent
29. Antenna 2: protopod distomedial seta: (0) with spines or (1) without spines
30. Antenna 2: endopod article 1 with setae: (0) present or (1) absent
31. Antenna 2: endopod article 2 with: (0) present or (1) absent
32. Antenna 2: exopod article 2 of male: (0) 1× length of article 3 or (1) 3× length of article 3
33. Mandible: basale dorsal proximal setae: (0) present or (1) absent
34. Mandible: basale dorsal midlength setae: (0) present or (1) absent
35. Mandible: basale dorsal midlength with (0) 1 or (1) 2+ setae
36. Mandible: basale dorsal distal (not terminal) setae: (0) present or (1) absent
37. Mandible: basale dorsal terminal setae pair: shorter seta length as a percentage of longer setae: (0) <40%, (1) 40–75%, or (2) >75% [ordered]
38. Mandible: exopod length as percentage of dorsal margin of endopod article 1: (0)<40%, (1) 40–75%, or (2) >75% [ordered]
39. Mandible: endopod article 1 dorsal terminal spines: (0) present or (1) absent
40. Mandible: endopod articles 2 and 3: (0) fused or (1) separate
41. Mandible: endopod article 2 dorsal setae a, b, c, and d: (0) are clearly distinguished or (1) are not clearly distinguished
42. Mandible: endopod article 2 with lateral e-seta: (0) present or (1) absent
43. Maxilla: ventral setal comb: (0) present or (1) absent
44. Maxilla: endopod article 1 alpha setae: (0) present or (1) absent
45. Maxilla: endopod article 1 with (0) 1 or (1) 2+ alpha setae
46. Maxilla: endopod article 1 beta seta: (0) present or (1) absent
47. Maxilla: endopod terminal article with: (0) 1 or (1) 2+ setae
48. Fifth limb: (0) leg-like or (1) feeding structure
49. Fifth limb: epipod (0) present or (1) absent
50. Sixth limb: endopod shape (0) leg-like or (1) compacted
51. Sixth limb: setae at/above upper endite: (0) present or (1) absent
52. Sixth limb: at/above upper endite with (0) 1 or (1) 2+ setae
53. Sixth limb: setae at/below lower endite: (0) present or (1) absent
54. Sixth limb: at/below lower endite with: (0) 1 or (1) 2+ setae
55. Sixth limb: epipodial setae: (0) present or (1) absent
56. Seventh limb: shape (0) leg-like or (1) worm-like
57. Seventh limb: with (0) 6–11, (1) 12 or (3) >12 setae [ordered]

58. Seventh limb: terminal process: (0) symmetrical or (1) asymmetrical
59. Seventh limb: shape of symmetrical terminal process: (0) flat or (1) with opposing combs
60. Seventh limb: number of combs: (0) single comb or (1) double combs
61. Furca: structure: (0) 3–4 main claws followed by secondary claws and setae or (1) series of claws decreasing evenly in size
62. Furca: with setae between claws: (0) present or (1) absent
63. Gills: (0) present or (1) absent
64. Gills: development: (0) weakly developed, less than 4 pairs of small or narrow lobes or (1) well-developed, more than 5 pairs
65. Posterior of body: shape: (0) without process or only slightly rounded, (1) with medium process, or (2) with long finger-like process [ordered]
66. Median eye: (0) present or (1) absent
67. Bellonci organ: (0) present or (1) absent
68. Depth: (0) deep, >1000 m, (1) shallow, 0–1000 m [not used in phylogenetic analyses]
69. Compound eye: (0) absent, (1) present [not used in phylogenetic analyses]

Appendix 2. Taxa/character matrix for morphological characters used in the phylogenetic analyses

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                                  1         2         3         4         5         6
                        12345678901234567890123456789012345678901234567890123456789012345678901234567890123456789

Manawa_staceyi          11100111?0000?????????????????????0?????????????1????010?????0??????1??1110
F_Nymphatelina          111?????????????????????????????????????????????1????000?????0????1?1??11?1
F_Colymbosathon         111?????????????????????????????????????????????1????100?????1?????20120??1
F_Triadocypris          111?1????010?????????????????????????????????????10??????12?????00????1
Vargula_hilgendorfii    11100111?1?00????10110010100100010012211?1????101?????121??111?00011
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Asteropterygion_romei   10000111?0100010000000010110001001?022111?0010110101010120100101200011
Asteropterygion_setiferum 10100?11?0100010?0000?101?0101?01?022111?0010110101010120100101200001
Asteropterygion_oculitristis 10100110001000100100001010000100000221117?0010110101011120100101200011
Asteropterygion_peterseni 10000?11?0100010?00?00101?0101?01?022111?0010110101010120100?0120011
Asteropterygion_dayi    10000?11?0100000?00?00101?0101?01?022111?0010110101010120100101200011
Asteropterygion_thomassini 10000111?0100010000000010100101?01?022111?0010110101010120100101200011
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Actinoseta_chelispara   10001111?0100010010000?10100000?01?022111?0010110101011120100101200011
Actinoseta_jonesi       11101?11?0100010?00?00001?0100?01?022111?0010110101011120100101200011
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Asteropella_slatteryi_J57060 10000?11?0100????00101???1001??011?1?2111?01?0110101011?00?0101?0011
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Alphaleberis_alphathrix 11100010101200112001100101101017102211?0000110101010120100101000011
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Cyclasterope_bisetosa   11100?10001200112?01?0?101?0100?10101211?0010110101010120111100100011
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Postasterope_abaco             11100001?0010????00101???111???111?11111000000010100001110101101200l1
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Prionotoleberis_pax            11100?10000100011?0101???1?0111?10012111010000010100001110101101?0011
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Skogsbergiella_scotti          11100?00000100011?01000101?0111?10112010000000101000011201011011001l
Skogsbergiella_senex           11100?00000200012?01000101?0111?10111010000000101001?112010110110000
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Skogsbergiella_sp_ZMH_K-42206  11100???????3?????01000101???11?11?1?2010?00000101?????????????01?0000
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Synasterope_serrata            11100?10000001?11?0101???1?0111?10012111010000010100001110101101000 11
Synasterope_setispara          11100?01?00101?11?0101???1?1?11?11?111110100000101000011101011012 0011
Synasterope_J57067             11100?10000101?11?0101???1???11?11?121110100000101000011101011010 0011
Synasterope_J57066             11100?100001?1????0101???1?01???10012111010000010100001110101101000 11
Xandarasterope_storthynx       11100?10000100011?0100010 1?0111?11?121110000000010100001120101001000 00
Xandarasterope_trux            01100110000100011001000101001 11?11?121110000000010100001110101001000 00
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Xenoleberis_yamadai            11100011?00100011001001101 10111?10012011010000010100001110101101000 11
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Cylindroleberididae_42209      11100?10000100011?0100010 1?01???11?121110000000101?????12010??0110000
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