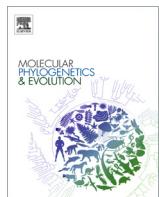




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Cleaners among wrasses: Phylogenetics and evolutionary patterns of cleaning behavior within Labridae[☆]



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ABSTRACT

Cleaner fishes remove and consume ectoparasites and are often categorized by whether they perform this behavior: (1) predominately as juveniles, (2) facultatively throughout ontogeny, or (3) obligately. Through a literature search, we confirmed that with at least 58 species exhibiting cleaning behavior, the Labridae (wrasses, parrotfishes, and allies) contain the highest diversity of cleaner fishes. In fact, there are 3–4 times as many cleaners within labrids as there are in any other marine group. The distribution and underlying causes of this exceptional diversity have not been determined. Here, we assess the topological and temporal patterns of labrid cleaner evolution. We used maximum likelihood and Bayesian approaches to infer the phylogenetic relationships and divergence times between 320 labrid species (50.7% of nominal species). We then employed stochastic character mapping to infer how and when cleaning behavior evolved. We estimate that cleaning has independently evolved 26–30 times in the Labridae, and all such events likely occurred no earlier than in the late Miocene. Given the current sampling and pattern of transitions, we hypothesize that the majority of facultative or obligate cleaning may have evolved through heterochrony.

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1. Introduction

The Labridae (wrasses, parrotfishes, and hogfishes) is a speciose group of marine perciform fishes that occupies diverse ocean habitats worldwide. Labrids are well known for being some of the most common and functionally important inhabitants of coral reef ecosystems, revealing tremendous diversity in morphology and trophic strategies (Wainwright et al., 2004; Bellwood et al., 2006; Price et al., 2011). Labrids feature, among myriad trophic strategies, extreme specializations such as corallivory, planktivory, and molluscivory.

One of the most fascinating specializations within the Labridae is cleaning behavior. Cleaner fishes are taxa that remove and consume ectoparasites off other organisms. The evolution of cleaning behavior presents one of the few examples of mutualisms among vertebrates (Bronstein, 1994; Poulin and Grutter, 1996). While cleaners typically clean other fishes, they have also been observed to inspect a variety of marine vertebrates and invertebrates (see Grutter, 2010 for a review of cleaner fish behaviors). The presence of cleaners in a habitat can have tremendous

ecological consequences. For instance, experimental removal of the bluestreak cleaner wrasse (*Labroides dimidiatus*) has been shown to affect the behavior, recruitment dynamics, and sizes of client fishes (Waldie et al., 2011).

Cleaning is not exclusive to labrids; in fact, at least 18 marine families of fishes include at least one member that cleans. Coté (2000) provides an extensive list of cleaner fishes. According to Coté (2000), 50 species of labrids are documented as cleaners. This is three times as many species as in the next highest group the Gobiidae, within which 14 species of cleaners are recognized. This suggests cleaner fish species richness is not directly proportional to clade diversity, especially when considering the Gobiidae has close to 2000 extant members. Furthermore, of the various groups of marine fishes in which cleaning is found, the overwhelming majority contain five or fewer species that clean (Coté, 2000). These metrics underscore the exceptional diversity of labrid cleaners, marking labrids as a model clade within which to explore the evolution of cleaning.

Cleaner fishes can be categorized by whether they perform the behavior (1) predominately as juveniles, (2) facultatively throughout ontogeny, or (3) obligately (Coté, 2000). Obligate cleaners are more conspicuous and most of what is known about cleaning behavior has been determined through observing species in the obligate cleaner genus *Labroides*. For example, *L. dimidiatus*

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commonly maintains “cleaning stations”, small areas that attract visiting “client” organisms (Youngbluth, 1968). In *L. dimidiatus*, cleaning interactions often begin with the cleaner fish approaching a potential client and presenting itself by swimming in a vertical oscillatory pattern (Randall, 1958; Gorlick et al., 1978). A receptive client will then pose to solicit cleaning by holding still in the water column, spreading its pectoral and pelvic fins, opening its jaws, and flaring its opercula laterally (Losey, 1972; Coté et al., 1998). The cleaner will dart around the client's body as it picks off ectoparasites, most commonly gnathiid isopod larvae (Grutter, 1996) that may be embedded in the fins, gills, buccal cavity, and pharyngeal chamber of the client (Grutter, 1996, 2010; Coté, 2000).

The complexity of behaviors that labrid cleaners exhibit varies widely across the diversity of taxa that clean. Contrary to what is recorded for the obligate cleaner genus *Labroides*, most labrid cleaners do not perform oscillatory swimming, and many do not hold cleaning stations. What does unify cleaners in the Labridae, however, is (1) the ability to detect, remove, and consume ectoparasites off other taxa, and (2) acknowledgment by client species, who allow cleaners to approach and inspect them.

There is growing evidence that cleaners share a variety of morphological characteristics related to feeding. When removing and consuming prey off a substrate, the cleaners *Thalassoma lutescens*, *Larabicus quadrilineatus* and *Labroides dimidiatus* employ low-displacement, fast jaw movements that allow for rapid gape cycles on individually-targeted items (Baliga and Mehta, 2015). Furthermore, the cranial skeletons of cleaner fishes in *Thalassoma* show reduced vertical gape sizes, smaller bite forces, and jaws with reduced mobility when compared to those of non-cleaner congeners (Baliga and Mehta, 2014). Thus, while it may be reasonable to predict that this feeding specialization, which may involve a variety of behavioral and morphological adaptations, would show relatively few independent origins, the staggering species richness of cleaners in the Labridae compared to that of other marine families is perplexing.

How and when cleaning behavior arose in the Labridae has not been established and requires further study with phylogenetic information. While several recent efforts have put forth several sound phylogenetic hypotheses for labrids (Kazancioğlu et al., 2009; Cowman and Bellwood, 2011), these phylogenies do not extensively cover all genera in which cleaning is known to occur, notably in the genus *Bodianus* and close allies. Fortunately, thanks to more recent sampling efforts (e.g. Hubert et al., 2012), we find opportunity here to add further insight to the history and topological organization of the Labridae by incorporating additional species into our analyses. Furthermore, while Coté's review (2000) was exhaustive, additional taxa in the Labridae have recently been identified as cleaners (e.g. *Austrolabrus maculatus* by Shepherd et al., 2005). Thus, in order to formally examine cleaning diversity in labrids, a more current literature search is warranted. Armed with an extensive phylogeny of 320 species and a more comprehensive list of cleaners, we map the evolutionary history of cleaning in the Labridae.

Our investigation thus involved (1) inference of phylogenetic relationships between 320 labrids, (2) an extensive literature search to identify cleaner fishes within the group, and (3) stochastic character mapping to identify evolutionary transitions to cleaning.

2. Materials and methods

2.1. Phylogenetic inference and divergence time estimation

We reconstructed phylogenetic relationships using a molecular dataset that comprised four mitochondrial (12S, 16S, COI, and

CytB) and three nuclear gene regions (RAG2, TMO4c4, and S7), with 5462 total base pairs. We obtained all sequences for 320 labrids and a 24-taxon outgroup from GenBank (see Tables A.1–A.4 for accession numbers and information on genetic sampling).

Our taxon sampling included 261 of 519 wrasses, 52 of 100 scarids, and seven of 12 odacids (see Table A.5 and Fig. A.1 for more details on sampling methods). While some sources (Nelson, 1994; Froese and Pauly, 2015) classify the Scaridae and Odacidae as distinct families, separate from the Labridae, others have found these groups' phylogenetic origins to be nested within the Labridae (Clements et al., 2004; Westneat and Alfaro, 2005).

We thus consider the Labridae to include 631 total species (Froese and Pauly, 2015), and therefore our genetic dataset contained 50.7% of nominal species (90% of nominal genera; Table A.5, Fig. A.1). Following previous studies, we used 24 outgroup taxa that comprised members of the Pomacentridae, Cichlidae, Embiotocidae and other perciforms (Kazancioğlu et al., 2009; Cowman and Bellwood, 2011).

We aligned each gene sequence separately using the built-in algorithm in Geneious 4.8.5. Each alignment yielded high similarity to those found in previous studies (Kazancioğlu et al., 2009; Alfaro et al., 2009). We then trimmed flanking regions that contained sequences from less than 60% of taxa. To identify the best-fitting model of nucleotide substitution for each gene, we used jModelTest 2.0 (Darriba et al., 2012). In each case, we found the best-fit (assessed via AIC and BIC scores) to be a GTR + I + Γ model, or a close variant thereof (Table A.6).

Using SequenceMatrix 1.7.8 (Vaidya et al., 2011), we concatenated nucleotide marker datasets into a supermatrix. We partitioned this supermatrix by individual molecular markers and performed a maximum-likelihood (ML) analysis in RAxML (Stamatakis, 2006). We ran a bootstrap analysis under a GTR + Γ model with 1000 pseudoreplicates and used the phylogenetic tree with the best likelihood score to guide further analyses (Supplementary File Tree B.1).

We then used BEAST 2.2.1 (Bouckaert et al., 2014) to simultaneously estimate topology, branch lengths, and divergence times in a Bayesian framework. Using a relaxed log normal clock model approach, we partitioned the supermatrix by sequence, and fit a separate model for each partition based on our results from jModelTest.

To estimate divergence times, we placed informative parametric priors on nodes of the tree to reflect the somewhat sparsely but available paleontological history of the group (Table 1). We identified descendant members of each node based on the topology of the ML tree. The crown group calibration was based on the K/T boundary extinction, as no full fossil specimens of labrids have been found before this event, and our prior was informed by the 5–95% HPD for crown labrids found by Near et al. (2013). While the fossil history of the Labridae is somewhat sparse, previous studies have described six fossil taxa belonging to the group (see Table 1). For these, we used the estimated age of the fossil as a hard bound on the minimum age of the node, and priors were log-normally distributed. This information assimilated into our analysis of fossil data and historical biogeographical events that have been used by previous studies (Kazancioğlu et al., 2009; Alfaro et al., 2009; Cowman and Bellwood, 2011; Near et al., 2013). One key difference is our extension of the prior related to the closure of the Isthmus of Panama (IoP), which informs the divergence time between *Halichoeres pictus* and *H. dispilus*. Traditionally, the closure of the IoP is estimated to have occurred ~3.1–3.5 MYA (Coates and Obando, 1996), but a recent study uncovered evidence that suggests this closure may have occurred 13–15 MYA (Montes et al., 2015). We took a conservative approach to parameterizing our priors for this event by using a normally distributed prior with 3.1–15.9 MYA as the 5–95% intervals.

Table 1

Fossil and biogeographic information used for divergence time estimation in BEAST.

Group	Fossil or event	Age (MY)	Distribution	Prior (5–95%)	Source
Root (crown Labridae)	K/T boundary	66.2 ^a	Normal	62.2–70.3	Near et al. (2013)
Hypsigenyines	<i>Phyllopharyngodon longipinnis</i>	50 ^b	Lognormal	51.5–63.1	Bellwood (1990)
Labridae (-Hypsigenyines)	<i>Eocoris bloti</i>	50 ^b	Lognormal	51.5–63.1	Bannikov and Sorbini (1990)
	<i>Bellwoodilabrus landinii</i>	50 ^b		51.5–63.1	Bannikov and Carnevale (2010)
<i>Pseudodax moluccanus/Achoerodus viridis</i>	<i>Trigondon jugleri</i>	14 ^b	Lognormal	15.1–44.0	Schultz and Bellwood (2004)
<i>Calotomus/Sparisoma</i>	<i>Calotomus preisli</i>	14 ^b	Lognormal	15.1–44.0	Bellwood and Schultz (1991)
<i>Halichoeres dispilus/H. pictus</i>	Isthmus of Panama	9.5 ^c	Normal	3.1–15.9	Coates and Obando (1996) Barber and Bellwood (2005) Montes et al. (2015)
<i>Bolbometopon muricatum/Cetoscarus bicolor</i>	<i>Bolbometopon</i> sp.	5 ^b	Lognormal	6.1–11.1	Bellwood and Schultz (1991)

We placed parametric prior distributions on the MRCA of lineages as specified above.

^a The K/T boundary is often used as a guide for the estimation of the age of the crown group; no fossil labrids have been found before this event.^b Minimum age for the fossil.^c Estimation for this biogeographic event incorporates information from traditional sources that estimate the closure of the Isthmus of Panama to have occurred 3.1–3.5 MYA as well as a recent study that presents evidence for the closure instead occurring 13–15 MYA.

To ensure that each BEAST MCMC sampling converged on the target distribution, we conducted five separate runs, each from a different random starting tree. We ran each MCMC sampler for 200 million generations, sampling every 20,000 generations. We also ran a similar analysis in which the supermatrix was not partitioned, but found that the MCMC runs had great difficulty attaining stationarity, even after 75+ million generations. We assessed convergence via Tracer 1.6 (Rambaut et al., 2014) by plotting likelihood vs. generation and estimating the effective sample size (ESS) of each parameter.

Once we discarded the burn-in from each run (the first 15–20%), we combined runs via LogCombiner 2.2.1 (Bouckaert et al., 2014). The combined set included 41,323 trees, which we used to assemble the maximum clade credibility (MCC) tree in TreeAnnotator 2.2.1 (Bouckaert et al., 2014). Within each BEAST run, the ESS of all parameters were generally >200, with the lowest ESS still >100. After we discarded the burn-in and combined the results of all five runs, the ESS of all parameters were >600, but the vast majority of parameters had ESS >2000.

2.2. Identifying cleaner fishes

We conducted an exhaustive literature search to gather information on cleaning behavior within the Labridae. Explicit information for each cleaner species is available in Table A.7, and our categorizations of cleaning follow a modification of Côté (2000). We categorized each species in our genetic dataset to one of four states: (1) non-cleaner, (2) juvenile cleaner, (3) facultative cleaner, or (4) obligate cleaner. Juvenile cleaners are those that clean predominately as juveniles or sub-adults. Facultative cleaner species clean throughout ontogeny, although they do not rely on cleaning behavior as their sole means of food acquisition. Obligate cleaners are notable for depending on cleaning to obtain nearly all sources of food. We used these assigned categories in our SIMMAP analyses (below). These states were designed to be discrete and non-overlapping. We encountered some uncertainty in determining states for only one species (see Section 3 and Table A.7). Species for whom cleaning behavior had not been recorded in the literature were simply assigned to the non-cleaner category.

2.3. Inferring the history of cleaning

To simulate the evolutionary history of cleaning behavior on our phylogenetic trees, we used stochastic character mapping (Nielsen, 2002; Huelsenbeck et al., 2003; Bollback, 2006). This method enabled us to (1) sample simulated histories of cleaning

evolution, and (2) estimate the temporal patterns of transitions from non-cleaning to cleaning. In our analyses, we pruned all trees to include just the 320 species of labrids; outgroup taxa were removed.

In analyzing transitions between states, we performed stochastic character mapping via SIMMAP 1.5 (Bollback, 2006) on a sample of 10,000 trees from the posterior distribution of trees provided by BEAST. To select parameters of the prior distributions for our mapping analyses, we first performed an MCMC analysis using built-in functions in SIMMAP. We sampled values for the parameters of the overall rate prior (i.e. the Γ prior) using our MCC tree. Using the prsummary() function (distributed with SIMMAP) in the R 3.1.3 environment (R Core Team, 2014), we used samples from the posterior distribution of these parameters to find the best-fitting parameters for the prior distribution. We then employed these “best fit” priors in samplings of 10 stochastic character maps for each tree in the 10,000 posterior distribution trees. This allowed us to incorporate uncertainty about the topology into our analyses (Huelsenbeck and Rannala, 2003), while sampling character histories in proportion to their posterior probabilities, given the tip states. We imported these maps into the R environment, and used the describe.simmap() function in the phytools package (version 0.4.57; Revell, 2012) to summarize our findings for each 10-map set. We then collected data across the (10,000 total) map summaries to quantify the number and types of state changes, and the relative timing spent in each state.

Because state changes (count data) were nearly all Poisson-distributed, we used a Poisson test to test the hypothesis that each set of transitions was greater than zero, and used a Šidák correction to account for multiple testing (Šidák, 1967). To assess whether certain transitions occurred more frequently than others, we tested the hypothesis that mean numerical counts of different transition types differed significantly via ANOVA. We excluded groups whose mean counts did not significantly differ from zero (via the aforementioned Poisson test) from the ANOVA. Since group variances were unequal, we used Tamhane's T2 to make comparisons between all pairs of groups.

To identify the timing of transitions from non-cleaning to cleaning, we employed two separate analyses. In the first analysis, we used the character histories from the above SIMMAP sampling and extracted the most probable state for each node in each tree. We then matched these node states to their ages (in millions of years from the root), and computed summary statistics. In particular, we recorded the 5th, 50th (median), and 95th percentiles of ages for each category, and used the width of the 5th–95th percentile range to make comparisons among groups. This analysis

allowed us to compare ages of node states across a span of varying topologies, and using the 5th–95th percentile range in ages reduced bias from extreme outliers. However, assigning each node to its most probable state and then using the set of nodes as the only basis for comparison has two limitations: (1) the general loss of resolution when each node is assigned to its most probable state, and (2) the lack of incorporating changes that may occur along branches. These limitations are especially key in situations where transitions consistently occur (i.e. across many mappings) along relatively long branches; using information only from nodes may thus bias estimations of where state transitions occur toward recency.

Thus, in a second analysis, we used SIMMAP to sample 1000 stochastic character maps on only the MCC tree. We then imported the maps into the R environment, and used the `mergeMappedStates()` function in `phytools` to merge together the histories of all three cleaner states. The resulting set of maps thus contained character histories for a binary set of states: “non-cleaning” and “combined cleaning” in which the three cleaning categories were collapsed. We then integrated information across this set of stochastic maps into a Bayesian posterior probability (BPP) for each part of each branch in the tree via the `densityMap()` function in `phytools`. We adopted this binary approach on the MCC tree due to the intractability of simultaneously incorporating both topological uncertainty and multi-category complexity into assigning BPPs of cleaning along mapped edges. To estimate the timing of transitions from non-cleaning to cleaning, we identified the earliest point along each branch at which the BPP of being in a cleaning state reached 0.5, and matched such points to their corresponding times. We chose to quantify times corresponding to a BPP of 0.5 because subsequent (i.e. more recent) points along a branch are more likely to be in a cleaning state than not. We only identified points on branches along which there was an overall increase in the posterior probability of cleaning in order to avoid quantifying information on secondary losses of the behavior. A similar analysis in which we initially coded cleaning as a binary trait before running SIMMAP (i.e. bypassing the need to employ the `mergeMappedStates()` function) yielded nearly identical results.

3. Results

3.1. Phylogenetic inference

Our Bayesian analysis yielded a well-resolved phylogeny that was largely congruent with those found in previous studies (Fig. 1, [Supplemental File Tree B.2](#)). We found the origin of crown labrids to be approximately 62.08 MYA (95% HPD: 57.90–66.67 MYA), which is close to the 59.92 MYA (95% HPD: 54.4–66.7 MYA) estimate that Cowman and Bellwood (2011) found, albeit slightly earlier. The ages of major groups in our MCC tree (i.e. those shown in Fig. 1) are highly congruent with those found in previous studies (Alfaro et al., 2009; Kazancioğlu et al., 2009; Cowman and Bellwood, 2011). Additionally, all BEAST runs converged on a MRCA time for *Halichoeres dispilus* and *H. pictus* of 5.71 MYA. This time is closer to traditional estimates for the closure of the IoP, and is too recent to fit the estimates of 13–15 MYA put forth by Montes et al. (2015).

Within the hypsigenyines, the only major disagreement between our MCC and ML trees was the placement of the MRCA of *Achoerodus viridis* and *Pseudodax moluccanus*. In the MCC tree, we found the MRCA to be immediately sister to the group containing *Bodianus* and close allies with a posterior probability of 0.84 (Fig. 1, [Supplemental File Tree B.2](#)). In contrast, our ML tree placed this MRCA as sister to the odacines, *Choerodon* et al., and *Bodianus* et al. with bootstrap support of 99 ([Supplemental File Tree B.1](#)).

The placement of this MRCA on our ML tree is more similar to the topology of the MCC tree produced by Cowman and Bellwood (2011).

Another source of disagreement between our MCC and ML trees was within the organization of the pseudocheilines. Cowman and Bellwood (2011) identify *Paracheilinus* as a monophyletic genus (though only two species were included), sister to the monophyletic *Pteragogus*. Our analyses incorporated information for six additional *Paracheilinus* species. Whereas our ML tree yielded a topology largely congruent with that of Cowman and Bellwood, our MCC tree suggests *Paracheilinus* is part of a paraphyletic group that includes *Malapterus reticulatus* and placement is closer to the novaculines.

The positions of various groups within the julidines is also unclear. *Macropharyngodon*, *Anampses*, *Pseudojuloides*, *Hemigymnus*, the labrichthyne, and *Sagittalarva* each occupy different positions when comparing our MCC and ML trees. Adding to these complications is the extreme polyphyly that *Halichoeres* and, to a lesser extent, *Coris* exhibit.

Though the organization of major groups (typically genera) in relation to each other is still problematic, shallower nodes generally had higher support. Topology within each genus was largely congruent between our MCC and ML trees.

3.2. Literature search

Our literature search identified 58 species of labrids that are known to engage in cleaning behavior ([Table A.7](#)). The vast majority, 43 species (74.1%), were reported to clean predominately as juveniles. Less common are species that engage in cleaning facultatively throughout ontogeny (11 species; 19.0%). The rarest strategy is obligate cleaning (8.6%), which is exclusively found in all five *Labroides* species.

Only in one case (*Labropsis polynesica*) did we encounter uncertainty in assigning cleaning status. There is little evidence in the literature of this species engaging in cleaning behavior (Randall, 1981). We therefore conservatively coded *L. polynesica* as a non-cleaner in all SIMMAP runs. We note, however, that since many other *Labropsis* species engage in cleaning as juveniles (e.g. *L. australis*), it is possible that *L. polynesica* shares this characteristic.

Our final tally of 58 cleaners (which excludes *L. polynesica*) thus includes 7 species that have been described to clean since Coté's (2000) review: *Austrolabrus maculatus* (Shepherd et al., 2005), *Bodianus anthiooides* (Schiaparelli and Alvaro, 2009), *Centrolabrus caeruleus* (Azevedo et al., 1999), *Halichoeres nigrescens* (Sadovy and Cornish, 2000), *Halichoeres penrosei* (Coni et al., 2007), *Halichoeres radiatus* (Grossman et al., 2006), *Labrus bergylta* (Steigen et al., 2014), and *Pseudocheilinus hexataenia* (Sano et al., 1984). Of these 58 cleaners, 50 appeared in our genetic dataset, and thus a substantial majority of labrid cleaners (86% of known species; 100% of genera) was represented in our phylogenetic analyses.

3.3. Transitions between states

In stochastic character mappings performed on 10,000 posterior distribution trees (summarized in Fig. 2), we found that cleaning evolved from a non-cleaning state on average 28.10 times (SD: 2.43) and was secondarily lost 6.50 times (SD: 2.29). Our analysis also shed light on 8.05 transitions (SD: 2.34) between different cleaning states, the majority showing the pattern of juvenile cleaner transitioning to facultative cleaner. In Fig. 3, a single (and representative) stochastic character map is superimposed on the MCC tree.

Within each transition type, the mean and median did not differ appreciably. Furthermore, the median and mode were identical in

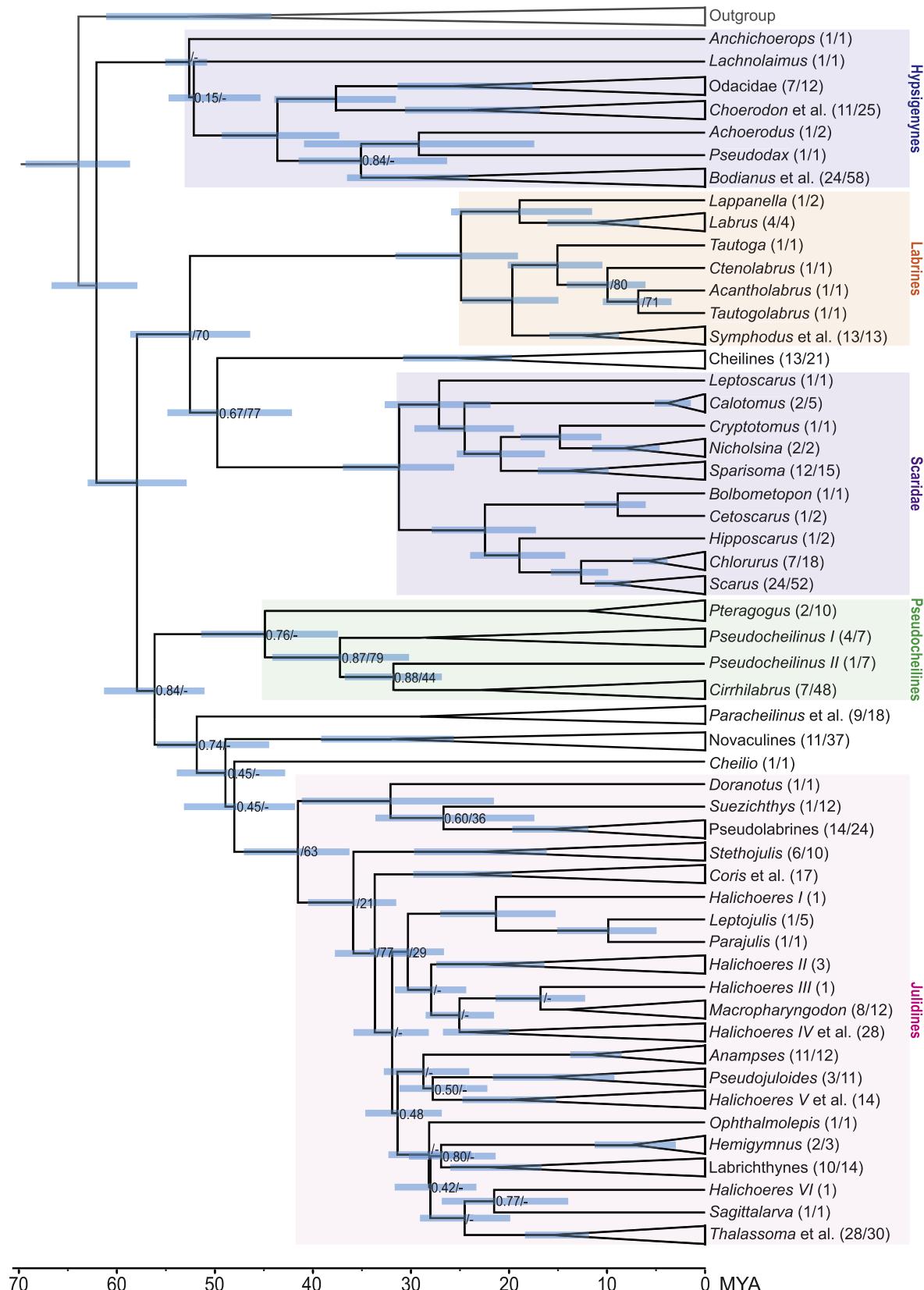


Fig. 1. Maximum clade credibility tree from Bayesian MCMC analyses. Tree is simplified to show only the relationships between major groups within the Labridae. Blue bars show 95% HPD intervals for node ages. Nodes are labeled with support values in the following order: Bayesian posterior probabilities (BPP)/bootstrap support (BS). Dashes (–) indicate no nodal support in the ML tree. Unlabeled nodes have Bayesian posterior probabilities (BPP) ≥ 0.90 and bootstrap support (BS) ≥ 90 . Triangles at the tips indicate that a clade is collapsed and simplified. Tip labels denote genus or group names with the proportion of species sampled in parentheses. The genera *Halichoeres* and *Coris* are polyphyletic, and proportions are not shown for these groups due to the difficulty of assigning taxon placements for non-sampled species. Vertical text and box shading delineate major groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

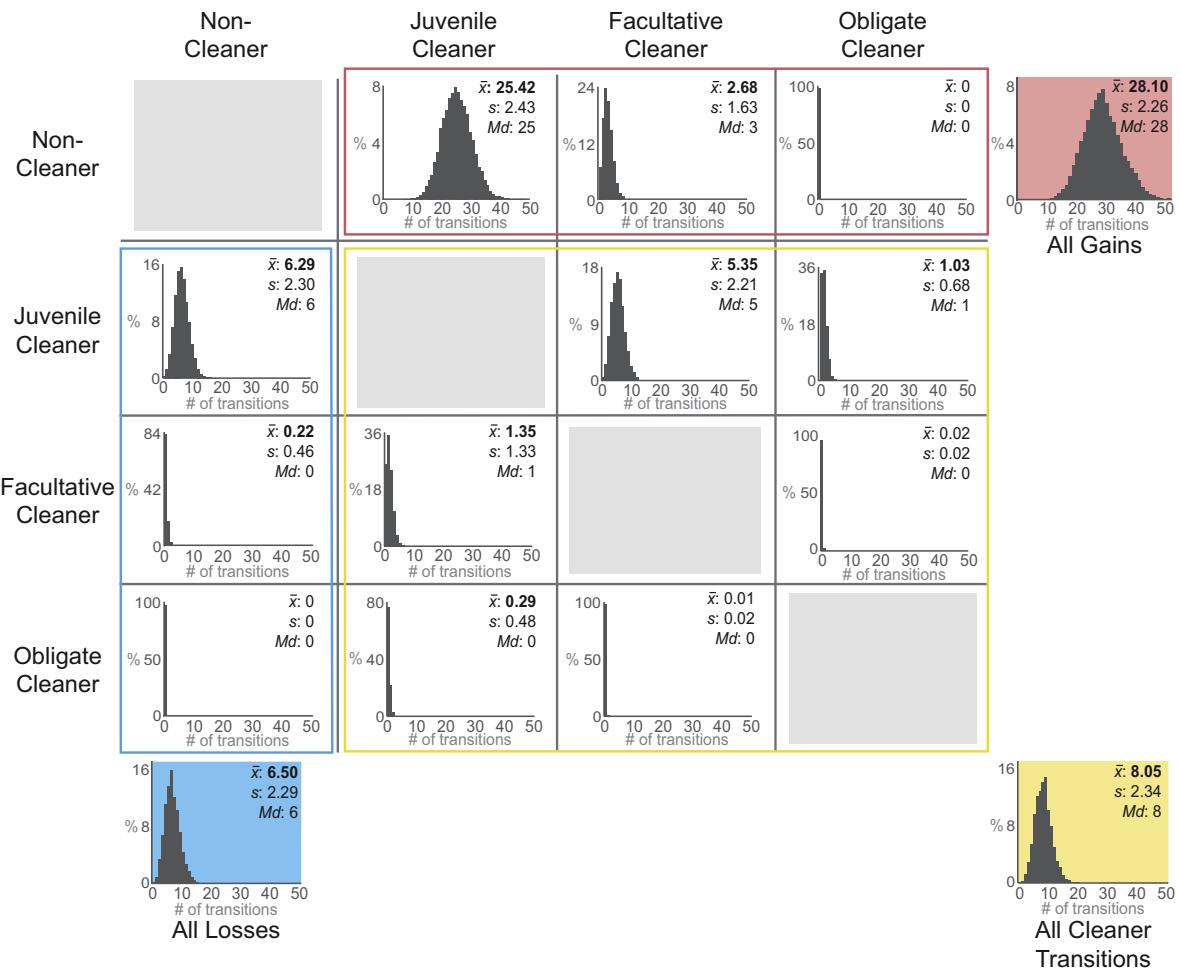


Fig. 2. State changes in stochastic character mappings on 10,000 Bayesian posterior distribution trees. Summary statistics and histograms of state changes in stochastic character mappings performed in SIMMAP on 10,000 trees from the posterior distribution obtained via BEAST. Row names indicate the “from” state, and column headings list the “to” state. Within each box, sample means (\bar{x}) are listed first, sample standard deviations (s) second, and sample medians (Md) third. Modes are not listed, as they are equivalent to medians. State changes that amounted to gains in cleaning behavior (i.e. all non-cleaner to cleaner transitions) are summarized in All Gains, while losses (i.e. from cleaner to non-cleaner) are summarized in All Losses. All Cleaner Transitions integrates information across all transitions between different cleaner states. Bold sample means are significantly greater than zero, as determined by Šidák-corrected Poisson tests.

each case. All mean counts for transitions were significantly greater than zero, except for the following: (1) non-cleaner to obligate cleaner, (2) obligate cleaner to non-cleaner, (3) facultative cleaner to obligate cleaner, and (4) obligate cleaner to facultative cleaner. Notably, these transitions were extremely invariant across mappings on trees; they had medians equal to zero, means near zero and standard deviations equal to or below 0.02 (Fig. 2). We then excluded these four transition types in an ANOVA on mean transition counts, which showed significant differences between groups (df: 7, F-ratio: 401,686.709; p-value < 0.001). A Tamhane's T2 test revealed significant differences between each pair of transitions (all p-values < 0.001). Thus, all transitions represented in Fig. 4 are not only significantly different from zero, but also are significantly different from each other.

3.4. Timing of transitions to cleaning

After matching node states to their ages in mappings on 10,000 trees, we pooled together node ages, which were measured in millions of years from the root (Fig. 5). Given that across our 10,000 trees, the root age of the Labridae ranged from 54.91 to 71.54 MYA, each 5–95th percentile range is firmly within the most recent third of the phylogenies. The 5th–95th percentile age range

for each set of nodes was: juvenile cleaners: 45.13–67.38 MY; facultative cleaners: 52.02–64.61 MY; obligate cleaners: 49.85–62.61 MY. Fig. 5 also shows the node ages for non-cleaners, for which the 5–95th percentile range was 24.83–68.63 MY.

Table 2 showcases the results of our within-branches estimation through 1000 mappings on the MCC tree alone, and Fig. 6 shows graphical representations of the estimates in several clades. We found that the earliest evolution of cleaning likely occurred close to or more recently than 18.36 MYA, with connecting nodes aged 21.26 and 17.89 MYA. The second-oldest age estimate, leading to cleaning in *Pseudodax moluccanus*, is found in a completely separate part of the labrid tree: the hypsigenyines (Figs. 1 and 3). This evolution provides an example of how changes in states can occur within long branches, as the node-to-tip distance is 29.19 MY. Our estimation places the evolution of cleaning within this branch around or after 15.20 MYA. Among all of our estimations of cleaning evolution, the median was 6.11 MYA (Fig. 6D), the mean was 6.80 MYA, and the standard deviation was 4.91 MY.

4. Discussion

The present study aimed to determine when and how cleaning behavior evolved in the Labridae. Through a genetic dataset

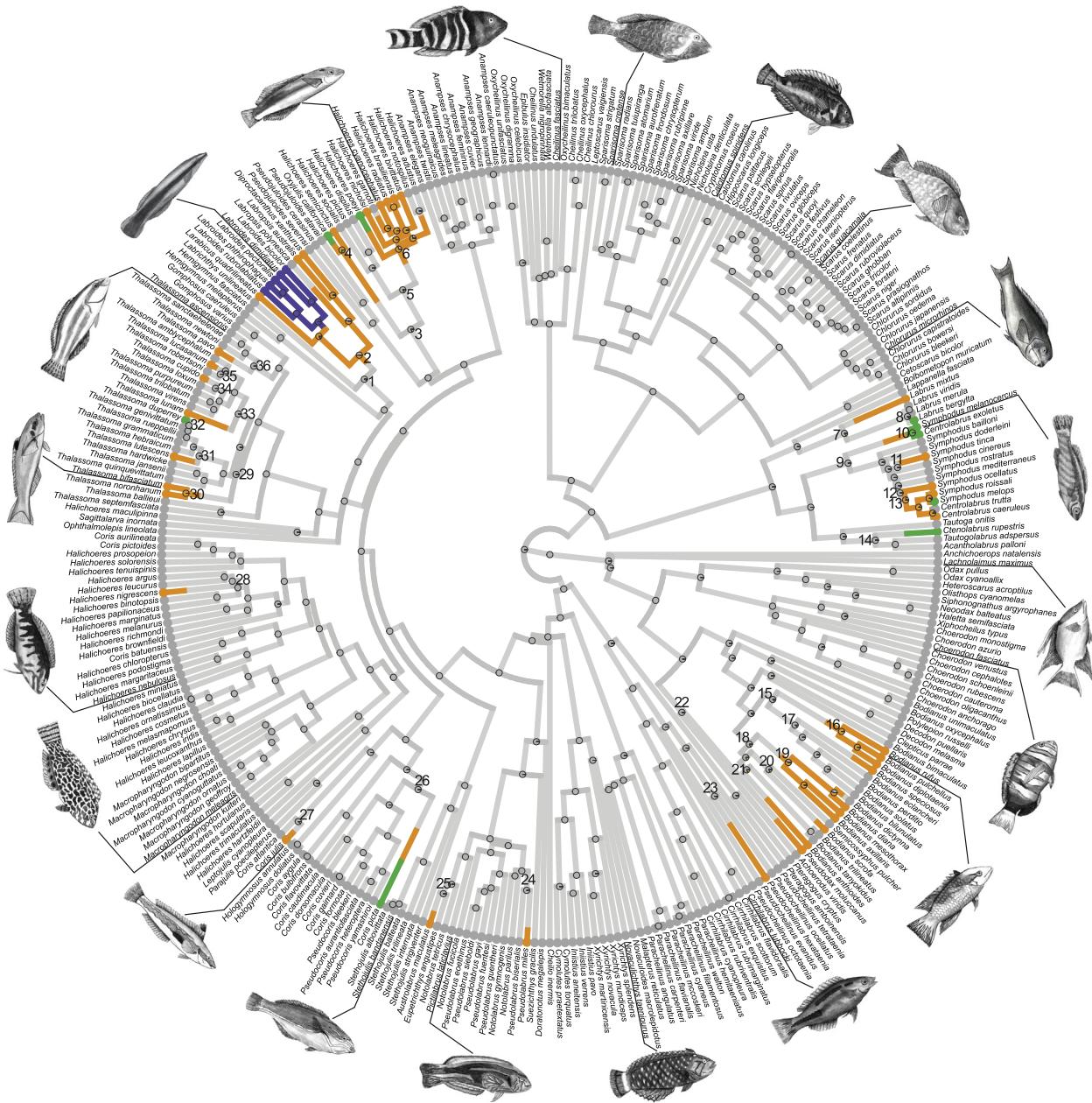


Fig. 3. Maximum clade credibility tree with simulated history of cleaning. A single stochastic character map out of 1000 is laid over the topology of the MCC tree. Gray colors correspond to non-cleaners; orange to juvenile cleaners; green to facultative cleaners; and purple to obligate cleaners. Circles at terminal tips indicate cleaning status for extant taxa, while branch colors depict simulated history. On each node, a pie chart shows the relative Bayesian posterior probability of each character state. Key nodes are numbered (refer to in Table 2 for additional details). Illustrations of fishes encircling the phylogeny are not systematically representative of a particular age class or sex.

comprising sequences from previously published molecular studies, we first reconstructed the most complete phylogeny of the family to date. After identifying the cleaning status of each taxon in our phylogeny, we inferred the history of cleaning behavior using stochastic mapping methods.

4.1. Phylogenetic analyses

Our study provides an extension of previous efforts to resolve relationships within the Labridae (i.e. Cowman and Bellwood, 2011) by including genetic data for 44 additional species. With these additional taxa, we attained 55.41% coverage of genes for the ingroup, while sampling slightly more than 50% of all nominal species. These metrics compare favorably to those of Cowman and Bellwood (2011), which achieved 52.4% coverage of genes for 46%

of nominal species. Our increases in coverage of sequences and taxa reap the benefits of recent sampling efforts (e.g. Hodge et al., 2012; Hubert et al., 2012; Steinke et al., 2009).

Our Bayesian analysis also converged on a MRCA time for *Halichoeres dispilus* and *H. pictus* of approximately 5.7 MYA, reasonably close to findings from previous studies (Kazancioğlu et al., 2009; Cowman and Bellwood, 2011). This estimate lends credence to traditional estimates of the closure of the IoP around 3.1–3.5 MYA, and is in line with similar findings in geminate species pairs of echinoids, crustaceans, molluscs, and other fishes (Lessios, 2008).

While the topology of our MCC and ML trees lacked congruence in specific areas, the majority of disagreements were among the relative positions of major groups (i.e. the organization of deep nodes). Within most genera, the topologies of the MCC and ML trees were far more similar and well resolved; posterior

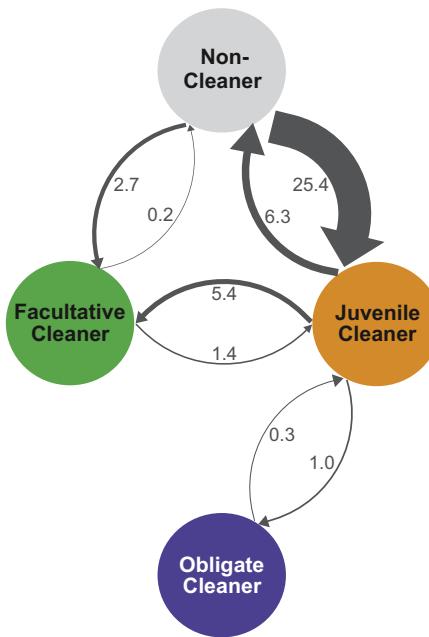


Fig. 4. Mean number of state changes in stochastic character mappings on 10,000 Bayesian posterior distribution trees. A visual representation of the relative frequencies of state changes summarizing simulated character histories on 10,000 trees. The widths of the arrows to/from each state are approximately proportional to the mean count of transitions between connecting states. Transition counts that were not significantly different from zero are not depicted.

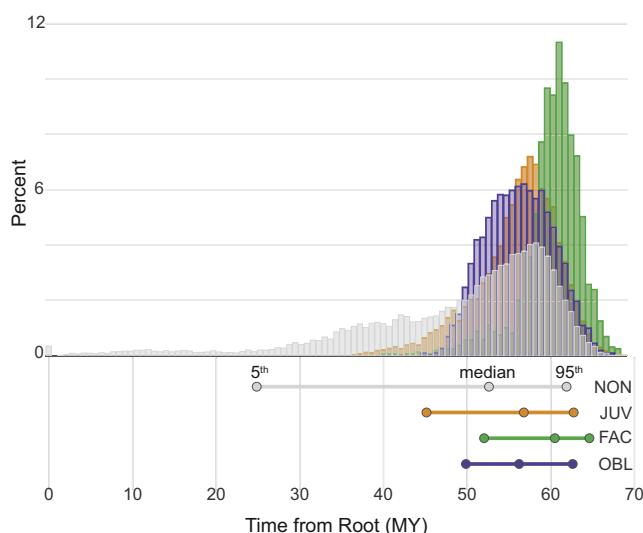


Fig. 5. Distributions of node ages in stochastic character mappings on 10,000 Bayesian posterior distribution trees. After stochastic character mapping was applied to each of 10,000 trees from the Bayesian posterior distribution of trees, each node within each tree was assigned to its most probable state. The age of each node (measured in millions of years from the root) was extracted from its tree. Node ages are pooled and represented in a histogram. Horizontal bars below the histogram show values for the 5th, 50th (median), and 95th percentile of ages within each category. Abbreviations: NON – non-cleaner; JUV – juvenile cleaner; FAC – facultative cleaner; OBL – obligate cleaner.

probabilities and bootstrap support values were generally higher than 0.90 and 90%, respectively. The lack of consensus on the organization of major groups is a problem that other studies have identified as well, especially concerning relationships among genera in the julidines (Kazancioğlu et al., 2009; Cowman and Bellwood, 2011).

Furthermore, the lack of monophyly in some genera could be a result of sampling. For example, in *Paracheilinus*, no nuclear markers were included in the analysis of this group, while only one mitochondrial region (COI) was present. While mitochondrial markers typically provide good resolution for estimating the topology of terminal branches, due to their fast mutation rates, mtDNA are not ideal for estimating basal topology. No doubt, through increased species and gene region sampling, future studies will be better equipped to resolve these organizational problems. Regardless, since a number of our stochastic character mappings involved integrating results from maps on 10,000 trees, we folded topological uncertainty into our analyses of cleaning evolution.

4.2. The organization and inferred history of cleaning

Through stochastic character mapping on 10,000 posterior distribution trees, we infer that cleaning evolved in the Labridae 26–30 times. Most of these events occur within the julidines, but many notable examples occur within the hypsigenyines and labrines as well. Cleaning is conspicuously absent in the speciose scarids and some species-rich genera such as *Cirrhilabrus*, *Choerodon*, *Iniistius*, and *Paracheilinus*. With 50 of 58 known cleaners in the Labridae appearing in the present work, we feel confident that we inferred most of the major evolutions of cleaning herein.

The remaining eight species that were not present in our genetic dataset are: *Coris sandageri*, *Halichoeres poeyi*, *Halichoeres penrosei*, *Halichoeres zeylonicus*, *Labropsis micronesica*, *Labropsis xanthonota*, *Pseudolabrus luculentus*, and *Suezichthys aylingi*. Of these species, almost all have congeners in our phylogeny that clean. It is thus conceivable that some or many of these missing species are sisters to cleaner congeners in the present study and thus could have evolved cleaning through events that are already included in our analyses. Only in the case of *S. aylingi* is the only congener present in our phylogeny (*S. gracilis*) a non-cleaner. Cleaning in *S. aylingi* could be the result of an additional point of cleaning evolution, but would depend on the position of *S. aylingi* in the labrid tree, especially if *Suezichthys* proves to be non-monophyletic. Ultimately, until all of these missing taxa can be incorporated into future phylogenetic analyses, the number of additional evolutions of cleaning beyond the 26–30 described herein remain unknown. Cleaning in the Labridae may have involved up to eight additional independent evolutions, but such assessments remain speculative.

In the present study, of all possible transitions from the non-cleaner to a cleaner state, the most common by far involved transitions to the juvenile cleaner state, occurring 23–28 times on mappings performed on 10,000 trees. The frequency of this repeated transition across various parts of the tree accounts for most of the extant diversity of labrid cleaner fishes (Fig. 3). Secondary losses were relatively rare (typically 4–8 times per tree).

Selection towards juvenile cleaning from a non-cleaner origin requires changes to the juvenile life history stage, but its effects on adult morphology and behavior are presently ambiguous. It is possible that these changes to the juvenile stage occur via similar (and perhaps simple) genetic changes, while selection towards facultative or obligate cleaning might require changes that are more extensive. This may account for the higher frequency to juvenile cleaner relative to transitions to facultative or obligate cleaning from a non-cleaner origin. The lack of secondary losses emanating from the facultative or obligate states adds credence to this hypothesis.

Whether a similar suite of morphological traits is found in juvenile individuals within this cleaning state has not been extensively tested, although there is some evidence of this in the *Thalassoma* clade (Baliga and Mehta, 2014). Baliga and Mehta (2014) show that juvenile cleaners in this group consistently exhibit weak bite forces

Table 2

Estimated ages of transitions to cleaning along branches.

Group	Taxon _i	Age _i	Taxon _j	Age _j	Estimate
Labrichthynes	Node 1	21.26	Node 2	17.89	18.36
<i>Pseudodax</i>	Node 21	29.19	<i>Pseudodax moluccanus</i>	0	15.20
<i>Bodianus</i> et al.	Node 15	19.57	Node 16	7.53	14.42
<i>Halichoeres</i> V et al.	Node 3	19.75	Node 4	3.12	13.25
<i>Bodianus</i> et al.	Node 18	18.07	Node 19	11.49	13.14
<i>Halichoeres</i> V et al.	Node 5	13.66	Node 6	7.59	10.04
<i>Labrus</i>	Node 7	11.13	<i>Labrus mixtus</i>	0	9.78
<i>Bodianus</i> et al.	Node 21	15.45	<i>Bodianus anthiooides</i>	0	9.74
<i>Coris</i> et al.	Node 26	19.02	<i>Coris picta</i>	0	9.51
<i>Bodianus</i> et al.	Node 20	13.02	<i>Bodianus scrofa</i>	0	8.30
<i>Pseudocheilinus</i>	Node 23	15.32	<i>Pseudocheilinus hexataenia</i>	0	8.26
<i>Bodianus</i> et al.	Node 17	14.31	<i>Bodianus speciosus</i>	0	8.03
<i>Syphodus</i> et al.	Node 9	12.19	Node 10	0.91	6.87
<i>Thalassoma</i> et al.	Node 29	10.29	Node 30	3.07	6.64
<i>Halichoeres</i> IV et al.	Node 28	11.14	<i>Halichoeres nigrescens</i>	0	5.58
<i>Ctenolabrus</i>	Node 14	9.96	<i>Ctenolabrus rupestris</i>	0	5.21
<i>Syphodus</i> et al.	Node 12	5.21	Node 13	4.52	4.80
<i>Syphodus</i> et al.	Node 24	8.28	<i>Pseudolabrus miles</i>	0	4.23
<i>Thalassoma</i> et al.	Node 33	7.42	<i>Thalassoma lunare</i>	0	3.73
<i>Thalassoma</i> et al.	Node 36	5.97	<i>Thalassoma pavo</i>	0	3.02
<i>Austrolabrus</i>	Node 24	5.26	<i>Austrolabrus maculatus</i>	0	2.69
<i>Syphodus</i> et al.	Node 11	4.75	<i>Syphodus tinca</i>	0	2.39
<i>Thalassoma</i> et al.	Node 31	3.82	<i>Thalassoma lutescens</i>	0	1.94
<i>Coris</i> et al.	Node 27	3.43	<i>Coris julis</i>	0	1.90
<i>Thalassoma</i> et al.	Node 34	2.36	<i>Thalassoma cupido</i>	0	1.20
<i>Thalassoma</i> et al.	Node 35	2.02	<i>Thalassoma lucasanum</i>	0	1.01
<i>Labrus</i>	Node 8	0.79	<i>Labrus bergylta</i>	0	0.73
<i>Thalassoma</i> et al.	Node 32	0.79	<i>Thalassoma duperrey</i>	0	0.40

Group names correspond to those in Fig. 1. Node names listed in either Taxon column correspond to labeling in Fig. 3. All ages are in millions of years before the present. Estimated ages indicate the earliest time along branches between each pair of taxa at which the Bayesian posterior probability (BPP) reaches 0.50. Such ages were only estimated along branches in which there was an overall increase in the BPP of cleaning, in order to avoid estimating timings of secondary losses.

and possess jaws with low mobility when compared to non-cleaner congeners. Upon reaching adulthood, differences in these functional traits begin to vary. In the present study, we infer cleaning in *Thalassoma* to originate from separate events, indicating that juvenile cleaners in this group are morphologically convergent in the juvenile phase.

Transitions between different cleaning states are relatively infrequent, but appear to be important in attaining facultative cleaner or obligate cleaner states. Notably, our analyses revealed that direct jumps from non-cleaner states to obligate cleaner states (and vice versa) were virtually nonexistent. Essentially all transitions to obligate cleaning originated from a juvenile cleaning state. We estimate that the evolution of obligate cleaning was most likely a single event, perhaps as depicted in Fig. 3. Additionally, we found that transitions to facultative cleaner states were nearly twice as common from the juvenile cleaner state as from the non-cleaner state. Essentially, juvenile cleaning in the Labridae presents a fascinating character state that seems to bridge all others (Fig. 4).

Of course, the notion that obligate cleaning very likely evolved from a juvenile cleaning state is perhaps unsurprising given that the monophyletic genus *Labroides* exclusively contains all five species of obligate cleaners. The immediate sister to this group (the monotypic *Larabicus*) as well as other closely related genera (*Labropsis* and *Diploctacanthus*) all contain juvenile cleaners.

That the majority of transitions to facultative cleaning and all transitions to obligate cleaning are preceded by a juvenile cleaner state lends us to hypothesize that facultative and obligate cleaning evolved via a heterochronic process. Potentially, cleaning in the adult stage is merely an extension of a juvenile feeding preference. Juvenile morphological traits may be carried over to the adult stage, giving adult obligate and facultative cleaners neotenous characteristics, at least for traits related to foraging, prey-capture, or cleaner-client recognition. Selective pressures to retain morphological features that are conducive to cleaning may be rare, however, which may explain the relative infrequency of these

hypothesized cases of arrested development. These hypotheses could be tested via a comparative study that examines the ontogeny of clades of facultative or obligate cleaners, giving insight to morphological trajectories therein.

4.3. Estimating temporal patterns of cleaning evolution

Through our analyses, we find evidence that cleaning behavior evolved relatively recently. From our within-branch estimation method, we found the earliest transitions to cleaning occurred within the last 20 million years, while the majority of cleaning evolution occurred within the last 10 million years (Fig. 6D). These results generally hold up when accounting for varying topological estimates of the tree: at the very least, the earliest cleaning states occurred no deeper than a third of the length of the tree (Fig. 5). Based on our (62.08 MYA) and others' estimates (ranging from 55 to 68 MYA; Alfaro et al., 2009; Kazancioğlu et al., 2009; Cowman and Bellwood, 2011) of the age of crown labrids, the patterns we observe point to the onset of cleaning evolutions in the mid- to late-Miocene, and continuing through the Pliocene and Pleistocene.

The Miocene also marks an especially important era in the diversification of reef fishes. Several groups of reef-associated fishes, including tetraodontiforms (Alfaro et al., 2007), chatetodontids, pomacentrids, apogonids, and labrids (Cowman and Bellwood, 2011) appear to have undergone rapid diversification during the mid- to late-Oligocene and early Miocene. In fact several major labrid lineages, including the julidines, scarines, and some hypsigeniines (*Bodianus* et al.), show significantly higher rates of cladogenesis during these epochs. In particular, Alfaro et al. (2009) provide evidence that around 24 MYA, the julidine rate shifted to nearly double that of the background labrid diversification rate, which led to more than 40% of non-scarine labrid diversity. Furthermore, coral reefs themselves show patterns of diversifying and dominating shallow-water marine systems in the late Eocene through early

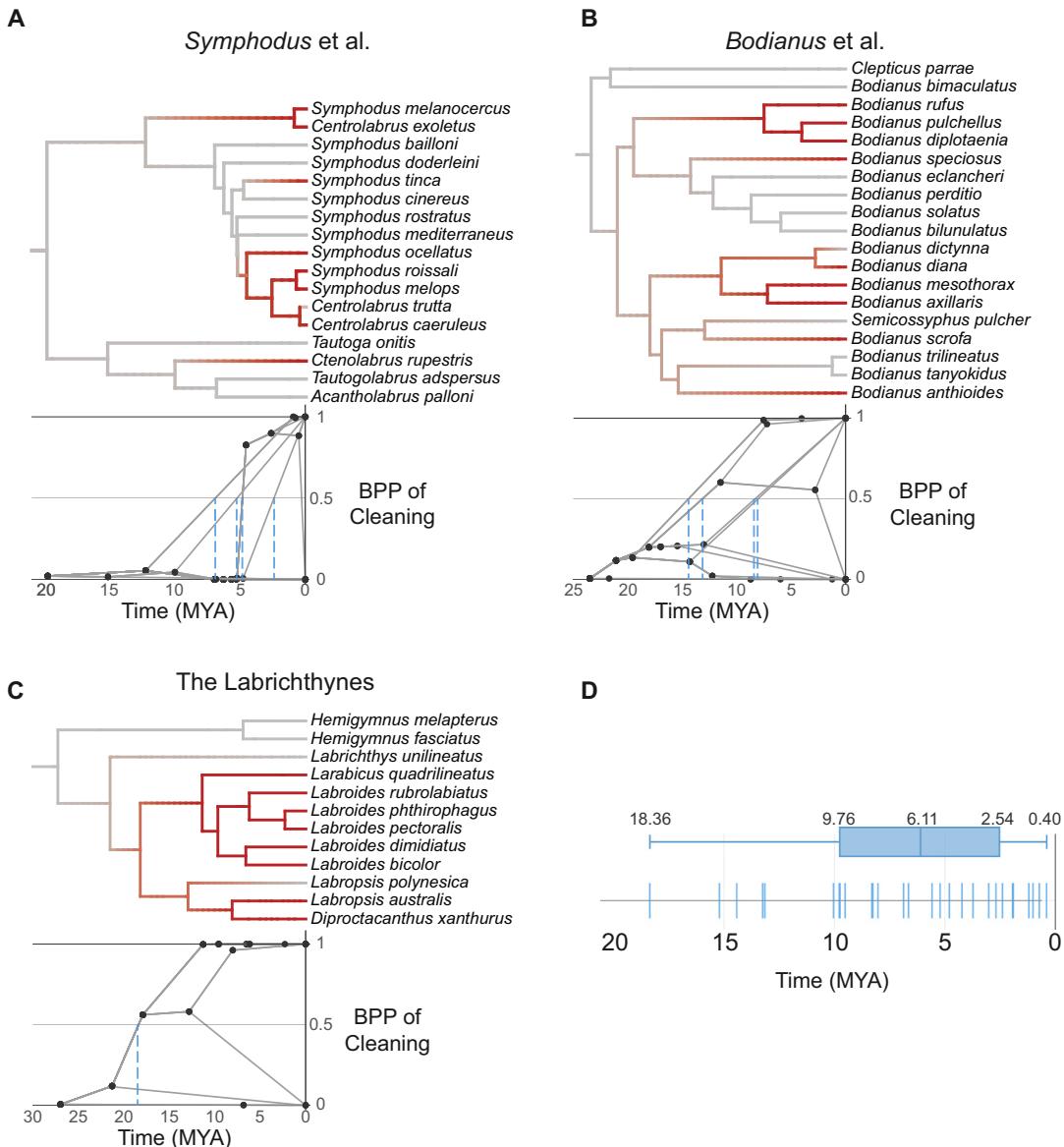


Fig. 6. Estimating temporal patterns of cleaning evolution within branches. (A–C) Graphical representations of the Bayesian posterior probabilities (BPP) of cleaning in three example clades: (A) *Syphodus* et al. (facultative & juvenile cleaners), (B) *Bodianus* et al. (juvenile cleaners) and (C) the labrichthyines (juvenile & obligate cleaners). On the topology of each clade, darker red colors indicate higher BPPs of being in a cleaning state for a node or a portion of a branch. On the accompanying plots, BPPs and ages for nodes and tips are shown using dark gray circles, and BPPs along branches are presented in light gray. Each blue dashed line indicates the time at which a branch attains a BPP of 0.50. Such times were only estimated along branches in which there was an overall increase in the BPP of cleaning, in order to avoid estimating timings of secondary losses. (D): Timing of these events across all clades in the 320-species MCC tree, with a boxplot and summary statistics. See Table 2 for further details.

Miocene (Wood, 1999). Workers have argued that the presence of reefs promoted diversification in fishes by providing habitats of high productivity (Fraser and Currie, 1996), high spatial complexity (Lingo and Szedlmayer, 2006), and high ecological complexity. Price et al. (2011) show that reef-associated labrids exhibit markedly faster rates of trophic morphological diversification and occupy a larger area of trophic morphospace than non-reef species. The tremendous increases in the diversity of reef fishes in the Miocene may have provided cleaner fishes the conditions to expand their potential clientele, thereby increasing the viability of this feeding strategy. Given the patterns we observe in the present study, it appears that cleaning behavior presents a possible example of ecological novelty supported and sustained by labrid diversification on coral reef systems.

On the other hand, extant labrid cleaners are not constrained to occupying coral reef ecosystems. Some taxa, including *Oxyjulis*

californica, and several *Syphodus* cleaner species, occur in temperate, seagrass- or kelp-dominated habitats. Ultimately, the factors that promoted or constrained cleaning evolution in the recent past remain unclear, and may not be homogenous across reef and non-reef habitats. Conceivably, an explosion in either ectoparasite taxonomic diversity and/or population sizes during the late Oligocene or early Miocene could have provided requisite opportunity for sustained directional selection on traits related to cleaning in labrids. Additionally, shifts in climate occurring during these epochs may have contributed to rapid and pronounced restructuring of marine ecological organization, creating space for novel dietary strategies. Whether any or all of these factors contributed to the recurring evolution of cleaning behavior throughout the Labridae can be addressed through future studies that incorporate information on paleoclimatic events or invertebrate diversity.

4.4. Additional remarks

One confound in our analyses is the simple assumption that a lack of observation of cleaning in our “non-cleaner” state is a true representation of the behavioral repertoire of non-cleaner species. In the 15 years since Coté’s review of cleaning, eight additional labrid species have been identified to perform the behavior and none of these eight species was newly-described. This indicates that future observations of cleaning are possible among putatively non-cleaner taxa. While the feeding and social behaviors of many labrids have been extensively documented, this group contains more than 600 taxa, many for which only a paucity of information exists. As scientists continue to document field observations of labrid ecology, additional taxa (including species herein classified as non-cleaners) may be identified to clean.

We issue an additional caveat about our within-branch time estimates of cleaning evolution. One disadvantage of our approach is that as the BPP of being in a cleaning state approaches 0.5, uncertainty reaches a maximum, since uncertainty is proportional to $p^*(1 - p)$. However, we argue that in integrating over our 1000 character maps on the MCC tree, evolutionary transitions between discrete states often occurred within short branch lengths. Of the 20 node-to-tip transitions to cleaning, 12 occurred over branch lengths shorter than 10 million years (Fig. 6 and Table 2). Thus, the relative duration of remaining in a state of high uncertainty in many cases was short. Here, our approach gave us a way to incorporate changes along a branch into our time estimates (thus reducing the recency bias) and provided a comparative metric that could be applied across all transitions to cleaning.

5. Conclusions

Through topological and temporal analyses of labrid evolution, we infer that cleaning evolved 26–30 times in various lineages, leading to an astounding extant diversity of cleaner fishes in this group. Our estimates suggest that these evolutionary transitions began to occur in the mid- to late-Miocene, with the majority occurring within the last 10 MY. Furthermore, we find that direct transitions from non-cleaning to either facultative or obligate cleaning are relatively rare. Transitions to these states are much more common from a juvenile cleaning state, which lends us to hypothesize that some evolutions of facultative or obligate cleaning may involve heterochrony.

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Appendix A. Supplementary material

A. Supplementary Materials – Supplementary Tables A1–A7 and Fig. A1 containing additional data or findings. B. Supplementary Tree Files: B.1 – Maximum Likelihood (ML) tree, obtained via RAxML. B.2 – Maximum Clade Credibility (MCC) tree, obtained via BEAST. Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymprev.2015.09.006>.

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