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The radiation of the clownfishes has two geographical replicates

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ABSTRACT

Aim The study of adaptive radiations provides an evolutionary perspective on the interactions between organisms and their environment, and is necessary to understand global biodiversity. Adaptive radiations can sometimes be replicated over several disjunct geographical entities, but most examples are found on island or in lakes. Here, we investigated the biogeographical history of the clownfishes, a clade of coral reef fish with ranges that now span most of the Indo-Pacific Ocean, in order to explore the geographical structure of an unusual adaptive radiation.

Location Indian Ocean, Indo-Australian Archipelago (IAA) and Central Pacific Ocean.

Methods We generated DNA sequence data comprising seven nuclear markers for 27 of the 30 clownfish species. We then inferred a Bayesian phylogeny and reconstructed the biogeographical history of the group using three different methods. Finally, we applied a biogeographical model of diversification to assess whether diversification patterns differ between the Indian and Pacific Oceans.

Results The phylogenetic tree is highly supported and allows reconstruction of the biogeographical history of the clade. While most species arose in the IAA, one clade colonized the eastern shores of Africa and diversified there. We found that the diversification rate of clownfishes does not differ between the main radiation and the African clade.

Main conclusions The clownfishes first appeared and diversified in the IAA. Following a colonization event, a geographically independent radiation occurred in the Indian Ocean off East Africa. This rare example of replicated adaptive radiation in the marine realm provides intriguing possibilities for further research on ecological speciation in the sea.

Keywords

Anemonefish, Central Pacific Ocean, diversification, ecological speciation, GeoSSE, Indian Ocean, Indo-Australian Archipelago, mutualism, range expansion.

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INTRODUCTION

Understanding the processes that give rise to new species is key to explaining the geographical distribution of biodiversity. Similar to patterns of terrestrial biodiversity, marine species richness exhibits an uneven geographical distribution. For example, coral reefs cover a small fraction of the world's oceans (Spalding & Grenfell, 1997), but host astonishing

biodiversity (Roberts *et al.*, 2002). Among marine biodiversity hotspots, the Indo-Australian Archipelago (IAA), or 'Coral Triangle' (Hoeksema, 2007), harbours the highest richness of marine species in the world (Roberts *et al.*, 2002). In the IAA, species richness gradients are both latitudinal and longitudinal, following a bull's eye pattern (Potts, 1985; Hoeksema, 2007). Since Wells (1954) first quantified this pattern, many hypotheses have been proposed to explain

the evolution of marine biodiversity in the IAA (Bellwood *et al.*, 2012). Nonetheless, the processes responsible for IAA biodiversity are complex and not yet fully understood.

In marine ecosystems, barriers reducing gene flow can be categorized as 'hard' or 'soft'. Hard barriers physically separate species and make crossing extremely unlikely. An example of a hard barrier is the appearance of land bridges, such as the closing of the Isthmus of Panama, which separated the faunas of the Pacific and Atlantic Oceans around 3.1 Ma (Obando-Rodríguez & Bemis, 1996; Floeter *et al.*, 2007). In contrast, soft barriers, created by physical or chemical properties of the water (e.g. current, salinity) or by large expanses of deep oceanic waters, are permeable to gene flow (Luiz *et al.*, 2012). For example, surgeonfishes (*Naso* spp.) show high genetic connectivity across the Indian and Pacific Oceans (Horne *et al.*, 2008). In such a case, the presence of a high rate of genetic exchange effectively reduces the opportunity for speciation by vicariance, as incipient species are never completely geographically isolated. Despite the permeability of soft barriers, an increasing number of studies describe cases in which marine speciation occurs in the potential presence of gene flow (e.g. Jones *et al.*, 2003), with ecological partitioning being the key process in the appearance of new species (Schluter, 2000). For instance, the spatial genetic structure within the coral reef fish species *Hypoplectrus nigricans* is greater than that measured between *Hypoplectrus puella* and *Hypoplectrus nigricans* over all sample locations (Puebla *et al.*, 2008), consistent with a spatially explicit model of ecological adaptive radiation (Gavrilets & Vose, 2005). Moreover, local processes (e.g. evolution of habitat preferences and colour-based mate choice) that lead to ecological speciation are important in the diversification of some marine organisms (Puebla, 2009), with examples including fish, corals, sponges and algae (reviewed in Bowen *et al.*, 2013).

Adaptive radiation through ecological speciation occurs when numerous species evolve from a single ancestral species and adapt to various ecological niches (Schluter, 2000; Gavrilets & Losos, 2009). Many terrestrial and lacustrine adaptive radiations are replicated in areas separated by physical barriers. For example, *Anolis* lizards show an impressive convergence of ecotypes among the main islands of the Caribbean archipelago (Losos, 2009). Similarly, cichlid fish of the East African lakes show striking parallelisms across their multiple radiations (Wagner *et al.*, 2012). In contrast, examples of species radiation showing parallel outcomes on both sides of a barrier, whether hard or soft, are rare in the marine realm. One such example comes from Bay goby species that developed convergent ecomorphological adaptations when isolated on each side of the Pacific Ocean (Ellingson *et al.*, 2014). It is not yet clear whether this paucity of cases comes from a lack of comparative studies encompassing areas separated by barriers to dispersal or whether it reflects a biological reality.

Here, we use the clownfishes (or anemonefishes; Pomacentridae) to study the effect of geography on species that may have exhibited a high incidence of ecological speciation in

the marine environment. This well-known clade of 30 species (Fautin & Allen, 1997) has diversified through an adaptive radiation process driven by the mutualistic interactions these fishes maintain with sea anemones (Litsios *et al.*, 2012). Indeed, 10 species of sea anemone host clownfishes in the wild. Host specificity varies among clownfish species, from specialists with one host species to generalists having potentially all 10 anemone species as suitable hosts (Fautin & Allen, 1997). Moreover, while ubiquitous in the tropical Indo-Pacific oceans, anemone species differ in their preferred habitats. The segregation of incipient clownfish species in different ecological niches allows speciation to occur despite potential gene flow. While mutualistic interactions with sea anemones have shaped the evolution of clownfishes, little is known of the contribution of biogeographical patterns to the diversification of this group (but see Timm *et al.*, 2008).

In this study we first used seven nuclear markers to infer the dated phylogenetic tree of the clownfishes. We then took advantage of a comprehensive dataset of species distributions to reconstruct the biogeographical history of the clade. Finally, we applied a Bayesian method to infer the effect of the geographical range of species on the diversification patterns of two putatively replicate, geographical radiations of the clownfishes.

MATERIALS AND METHODS

Taxon sampling

We gathered samples from 41 individuals (see Appendix S1 in Supporting Information), representing 27 species of clownfish. We used samples from fin clippings collected in the field (Bali, Indonesia; and Madagascar), loans from other research institutions and specimens from the aquarium trade. All samples were conserved in 90% ethanol. When possible, we sampled individuals across the entire species' ranges and collected all known colour morphs for each species. We used the DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) to extract DNA from tissue samples. We amplified DNA via polymerase chain reaction (PCR) with a 25 µL reaction mixture containing *c.* 50 ng of DNA template, 2.5 µL GoTaq buffer, 3 µL dNTPs (2.5 µM), 1 MgCl₂ (25 µM), 1 µL of both forward and reverse primer (Appendix S1) and 0.3 µL of Taq polymerase (GoTaq DNA Polymerase, Promega, Madison, WI, USA). We purified the amplification products with the QIAquick PCR Purification Kit (Qiagen). We sequenced the purified products for forward and reverse strands with the Big Dye 3.1 Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, and separated the products on an ABI Prism 3100 genetic analyser (Applied Biosystems). We complemented the dataset by adding sequences, available from GenBank, of five species of Pomacentridae to use as outgroup taxa. All newly generated sequences have been deposited in the EMBL database (Appendix S1).

Phylogenetic inference

We visually checked the DNA sequence chromatograms and built a consensus for each forward and reverse sequence using the software GENEIOUS 6 (Biomatters, Ltd, Auckland, New Zealand). We trimmed bases that had more than 5% error probability and aligned the sequences using MAFFT 6.864b with default settings (Katoh *et al.*, 2002). We visually verified all alignments individually before building the final super-matrix. We constructed the phylogenetic trees with MRBAYES 3.2.2 (Ronquist *et al.*, 2012a) and followed the procedure in Ronquist *et al.* (2012b) to infer the dated phylogenetic tree of the clownfishes. All MRBAYES analyses were composed of two parallel runs, each 10 million generations long with four parallel Markov chain Monte Carlo (MCMC) chains, with parameters sampled every 1000 generations. One advantage of a full Bayesian approach is that a priori testing of the best substitution model for each gene partition (e.g. Darriba *et al.*, 2012) is not needed. Indeed, we sampled parameters of the nucleotide substitution process across the GTR model space during the Bayesian MCMC runs (Huelssenbeck *et al.*, 2004). We checked for optimal parameter convergence using the 'sump' command and always removing the first 25% of generations as burn-in.

The phylogenetic analysis pipeline followed Ronquist *et al.* (2012b). We first ran a standard analysis with no assumption regarding a molecular clock and a non-calibrated strict-clock analysis. This allowed estimation of tree height and among-branch rate variation, which we subsequently used to select priors for the following analyses. We compared the performance of the strict clock and three relaxed clock models with Bayes factors as implemented in MRBAYES (Appendix S1). One of the relaxed clock models was similar to the uncorrelated gamma model implemented in BEAST 1.7.5 (Drummond *et al.*, 2006). The strict-clock best explained our data and was used in the final calibrated analysis. No fossil can accurately be placed close to the clownfish group (see Fr  d  rich *et al.*, 2013 for a complete list of available pomacentrid fossils). We used instead the confidence interval of the crown node of the clownfish clade inferred in Fr  d  rich *et al.* (2013) to calibrate our analysis, and applied to this node a uniform prior bounded between 11 and 21 million years. The prior of the strict-clock model was set to a normal distribution (mean = 9×10^{-4} , SD = 5.9×10^{-5}) and the tree age prior was an exponential distribution with shape parameter set to 9×10^{-4} . Finally, we built a majority-rule consensus tree with MRBAYES that included all compatible species groups and this tree was used in the following analyses.

Biogeographical reconstruction

Clownfishes are distributed throughout the greater Indo-West Pacific region, with the exception of several areas located outside of this area (e.g. Hawaiian Islands, Pitcairn and Easter Island; Allen, 1972). We retrieved clownfishes occurrences from the Ocean Biogeographic Information

System web site (<http://www.iobis.org/>), for a total of 1609 records (per species mean = 60, min = 1, max = 265), and used them to assign area membership to each species (Appendices S1 & S2). We defined six distribution areas for our analyses based on a recent quantitative delineation of biogeographical regions for reef fishes (Kulbicki *et al.*, 2013). We manually verified the putative areas with expert based distribution maps because some rare species were only sparsely sampled (Fautin & Allen, 1997).

We used the R package BIOGEOBEARS (Matzke, 2013) to infer the evolution of geographical ranges. This package implements the most common biogeographical history reconstruction methods in a likelihood framework. Moreover, it also proposes a model of founder-event speciation ('+J') and allows the fit of models to be compared using a model choice procedure (Matzke, 2013). In this framework, we reconstructed the biogeographical history of the clownfishes using the dispersal-extinction-cladogenesis model (DEC; Ree, 2005), dispersal-variance analysis (DIVA; Ronquist, 1997) and the BayArea model (Landis *et al.*, 2013). The three models were also tested under the possibility of founder events (+J). We did not constrain the directionality or timing of dispersal in our analyses by, for example, ordering areas, and we set the maximum number of ancestral areas to four, which is the current maximum number of areas inhabited by a single clownfish species. We assessed the fit of each model using the Akaike information criterion (AIC) and AIC weights (ω_i). For completeness, we also used the computational capabilities of the standalone version of BayArea (Landis *et al.*, 2013), but this time with 24 areas that represented all marine provinces in which clownfishes occur (*sensu* Spalding *et al.*, 2007; Appendix S1). The analysis using 24 areas was not feasible with the other software mentioned above.

Geographical range and diversification

We applied a likelihood-based model to estimate region-dependent rates of speciation, extinction and range evolution (GeoSSE; Goldberg *et al.*, 2011). The approach applies the DEC model but treats speciation and global extinction as stochastic processes. We used the implementation available in the R package DIVERSITREE (FitzJohn, 2012). In its current form, GeoSSE can accommodate only two areas. We thus modified the regions that were used in the BIOGEOBEARS analysis to fit this constraint by defining two broader areas: the Western Indian region (areas A and B; Fig. 1) and the Central Indo-Pacific and Central Pacific regions (areas C to F; Fig. 1; Kulbicki *et al.*, 2013). This choice was made following the results obtained from BIOGEOBEARS. Species were grouped as occurring in either of the zones or as being widespread if they occurred in both areas (Appendix S1). We used the Bayesian implementation of the method and ran the analysis for 10,000 generations. This approach estimates model parameters such as speciation, extinction and range evolution and allows their direct comparison. Note that in

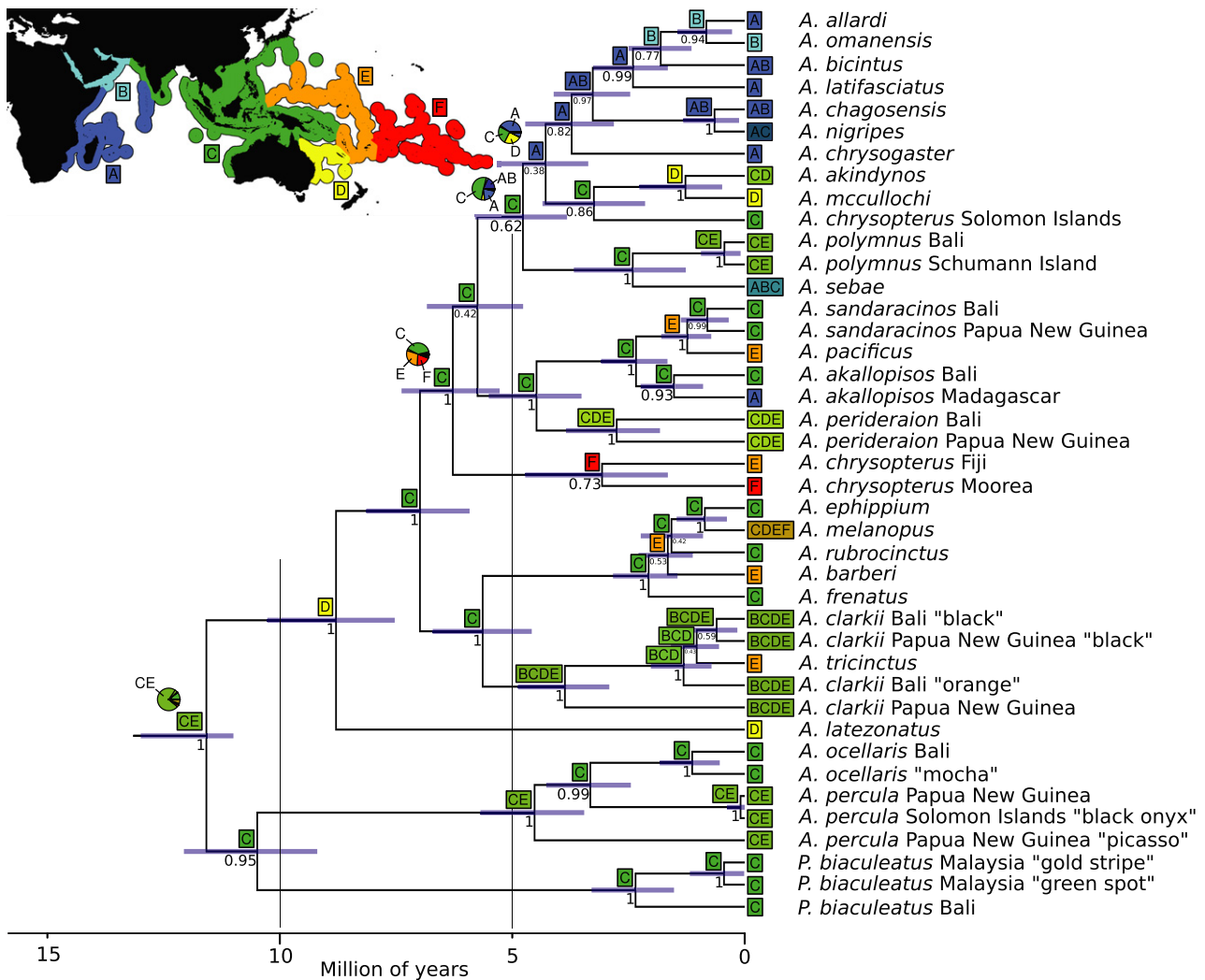


Figure 1 Majority-rule consensus tree of the clownfishes (genera *Amphiprion* and *Premnas*) inferred in MRBAYES. Numbers below nodes indicate Bayesian posterior probabilities. The size of the numbers varies in order to be reader friendly. Localization or phenotype is given for species in which several individuals were sampled (complete information is given in Appendix S1). Credible intervals for the age estimates are shown on nodes as blue bars. Letters from A to F represent the provinces (see inlayed map) used for the biogeographical reconstructions. A, Western Indian province; B, North-western Indian province; C, Central Indo-Pacific province; D, South-western Pacific province; E, Central Pacific province; F, Polynesian province. Ancestral areas inferred using the BayArea+J model are shown above nodes and current distributions are shown left of the species names. Coloured pie charts show the probability of each area on specific nodes. Outgroup species were only used to root the tree and are not shown.

the GeosSE model, only speciation rate is estimated for the widespread category while rates of extinction and range expansion are inferred for the two areas. We rescaled the phylogeny to a height of 1 prior to the analysis to facilitate parameters convergence. We interpreted posterior samples of a given parameter (e.g. speciation rate) to differ between groups when their 95% credible intervals did not overlap.

RESULTS

Phylogeny of the clownfishes

Our phylogeny is nearly complete and spans 27 out of the 30 described species of clownfish. The only missing species

are *Amphiprion fuscocaudatus* (endemic to the Seychelles and extremely rare since the 1998 coral bleaching event; J. Bijoux, Institut de Recherche pour le Développement, Seychelles, pers. comm.) and both *Amphiprion leucokranos* and *Amphiprion thiellei*, which we chose not to include in our analyses because they are likely to have been described based on hybrid specimens (Fautin & Allen, 1997). The topology we obtained is highly supported (Fig. 1). All unsupported nodes are between closely related species and, thus, have little impact upon the organization of the tree. Classically, the Amphiprioninae subfamily has been divided into six morphology-based complexes (Appendix S2; Allen, 1972, 1980, 1991). We found incongruences in all complexes except the *Premnas biaculeatus* and the *Amphiprion percula*/*Amphiprion*

ocellaris complex. Moreover, we recovered a topology based on nuclear markers that differs from previously published phylogenetic trees, which were obtained using a combination of mitochondrial and nuclear sequences (Litsios *et al.*, 2012; Frédérick *et al.*, 2013). Notably, our new analysis places *Amphiprion clarkii* as sister to the clade of *Amphiprion frenatus* and both clades are placed at the base of the *Amphiprion* crown group. We further sequence for the first time *Amphiprion tricinctus*, which is paraphyletic with *A. clarkii*. Also, the distances separating different *A. clarkii* specimens are relatively high when compared with other species groups, and cluster by coloration rather than by geographical origins. The position of the *Amphiprion chrysopterus* individual collected in the Solomon Islands is surprising as it clusters with *Amphiprion akindynos* and *Amphiprion mccullochi*, and is phylogenetically distant from the two other individuals of its species.

Biogeographical inference

The best model identified in the BioGeoBEARS analyses is the one implemented in BayArea. The AIC difference is even greater when considering the extra parameter of founder-event speciation ($\omega_i = 0.78$; Table 1). The origin of the clownfishes radiation is inferred with high confidence in the Central-Indo Pacific and Central Pacific provinces (C & D in Fig. 1). Only *Amphiprion melanopus* and *A. chrysopterus*, which have recently diverged, have managed to colonize the most eastern areas (area E in Fig. 1). We also found that one clade has colonized the Indian Ocean and subsequently diversified there (areas A and B, Fig. 1). Three additional species have expanded their ranges in this ocean (*Amphiprion sebae*, *Amphiprion akallopisos* and *A. clarkii*). The reconstructions using 24 areas in BayArea yield qualitatively similar results and show that the colonization of the Western Indian region proceeded via the Western and South Indian shelf, then the south of the Western Indian province to finally reach the Red Sea and Persian Gulf (Appendix S3).

Table 1 Comparison of the fit of the BayArea, dispersal–extinction–cladogenesis (DEC) and dispersal–vicariance analysis (DIVA) biogeographical reconstruction models, all with the possibility of founder-event speciation ('+J'). We used these models to infer the ancestral geographical ranges of clownfishes on our phylogeny. Results are ordered by model fit. The log-likelihood (lnL) of each model is given as well as the Akaike information criterion (AIC) values. The Δ AIC shows the difference in AIC value compared with the best model, and the Akaike weight (ω_i) gives the relative likelihood of the model.

Model	lnL	AIC	Δ AIC	ω_i
BayArea+J	−113.9	233.7	0.0	0.78
BayArea	−116.1	236.2	2.5	0.22
DEC+J	−128.5	262.9	29.2	0.00
DEC	−129.5	262.9	29.2	0.00
DIVA+J	−133.8	271.6	37.9	0.00
DIVA	−136.7	279.3	45.6	0.00

Geographical range and diversification

The MCMC chain of the GeoSSE analysis quickly converges and all credible intervals for speciation rate overlap (Fig. 2a), indicating a non-significant difference. Extinction and dispersal rates are nearly indistinguishable between the two areas (Fig. 2). We further found no evidence of a difference in net diversification rate (speciation rate minus extinction rate) between the two regions.

DISCUSSION

Our reconstruction of ancestral areas shows that the Central-Indo Pacific and Central Pacific provinces are the most probable locations of the origin of clownfishes. While most species appeared *in situ*, our analysis suggests that one colonization event along the eastern coast of Africa gave rise to at least seven species that now span the whole range of possible mutualistic interactions with sea anemones (Fig. 3). Because this diversification event happened independently from the main radiation centred on the IAA, we propose that it represents a geographical replicate of the main adaptive radiation of clownfishes that occurred in the Pacific Ocean. Moreover, we cannot distinguish the diversification rates of lineages recovered in the Indian and Pacific Oceans. This similarity suggests analogous evolutionary processes in these areas, but this remains to be formally tested.

Phylogenetic reconstruction

The phylogenetic analysis of the clownfishes that we present in this paper contains samples of several individuals per species and covers nearly all species ranges and colour variation. In this study, we include for the first time sequences of a specimen of *A. tricinctus*. This species, endemic to the Marshall Islands, was originally distinguished from *A. clarkii* on grounds of coloration and slightly greater body depth (Allen, 1972). Our analysis reveals that *A. tricinctus* is in fact paraphyletic with *A. clarkii*, the latter probably representing a complex of several cryptic species. Indeed, *A. clarkii* is one of the most variable species in terms of coloration and spans the largest geographical distribution of any of the clownfishes. In our phylogeny, *A. clarkii* specimens clustered by colour rather than geographical origin (Fig. 1). A melanistic specimen from Bali is more closely related to another melanistic individual originating from Papua New Guinea than to a syntopic orange *A. clarkii*. This intriguing phylogenetic pattern calls for an in-depth analysis of the *A. clarkii* species complex, as its widespread distribution and generalist mutualistic interactions may only be artefacts caused by aggregating the habits of multiple species. Moreover, this finding parallels the case of Caribbean species of *Hypoplectrus*, in which colour-based assortative mating and disruptive selection on colour patterns drive speciation (Puebla *et al.*, 2007, 2008, 2012).

We also sequenced specimens having alternative coloration patterns in *Premnas biaculeatus* (one specimen with green

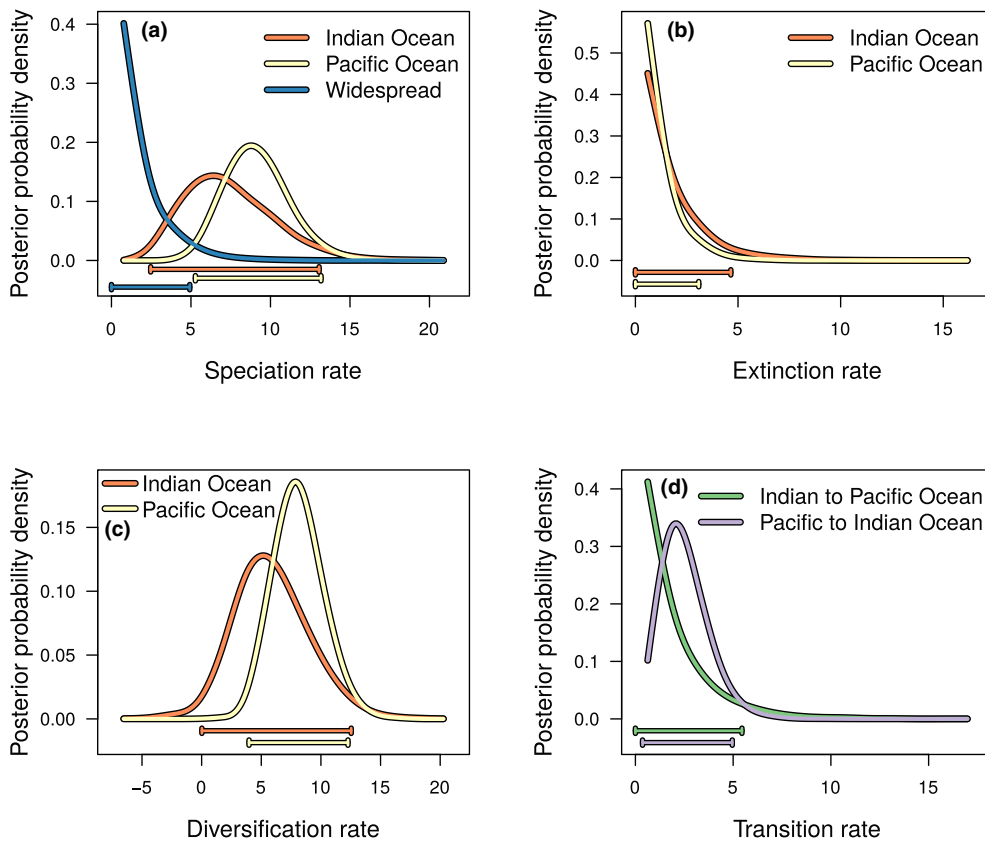


Figure 2 Posterior probability densities of diversification and range expansion parameters of clownfishes from the GeoSSE analysis. The speciation (a), extinction (b), diversification (c) and dispersal (d) rates of clownfishes found in the Indian Ocean are shown in orange, while those of the taxa occurring in the Pacific Ocean are shown in light yellow. Rates are given in number of events per unit of time (tree height rescaled to 1). Note that for widespread species, only the speciation rate is inferred by the model (in blue, panel a). Relevant 95% credible intervals are shown below the curves and are coloured accordingly.

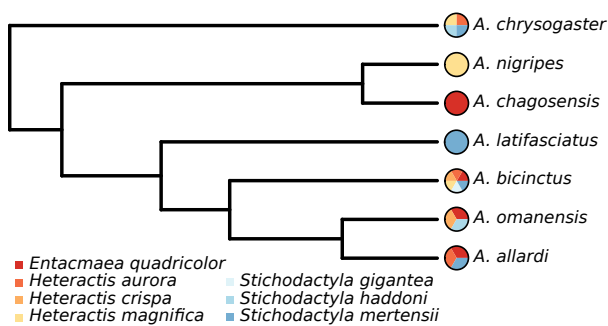


Figure 3 Detail of the phylogeny of clownfishes showing only the east African clade (in the genus *Amphiprion*). Pie charts on the left of the species names are coloured according to the mutualistic sea anemone species. The correspondence between sea anemone species and colour is given in the legend below the phylogeny.

spots on the back and another having yellow coloured stripes, a variety sometimes incorrectly referred to as *P. epigramma*), *A. ocellaris* ('mocha', a brownish variant) and *A. percula* (including the morphs 'picasso' with larger coverage of white bands that form convoluted markings and 'black onyx', in which the black margin of the white bars is wider

and can cover most of the body). Only the 'picasso' variant of *A. percula* showed noticeable genetic differentiation from the other samples for this species. It is still unclear whether or not coloration alone could explain this pattern or whether the collected 'picasso' specimen of *A. percula* is part of a different geographical subclade that is known to exist (Timm *et al.*, 2008) but was not otherwise sampled. The position of *A. chrysopterus* from the Solomon Islands is surprising and most of the nodes that separate this individual from the two other *A. chrysopterus* we sampled are weakly supported. One possible explanation is that the specimen from the Solomon Islands descends from a hybridization event between *A. chrysopterus* and, most probably, *A. akindynos*. Hybridization occurs between clownfishes and has led to the description of two species that are now recognized as hybrids (*A. leucokranos* and *A. thillei*). Further investigation is necessary to understand whether or not *A. chrysopterus* from the Solomon Islands is truly a hybrid or represents an undescribed species.

In general, the topology we recover is inconsistent with the classical taxonomy based on six complexes (Appendix S2; Allen, 1972, 1980). Those complexes represent groups of clownfish that are morphologically similar, such as the

'*A. akallopisos*' complex that groups *A. nigripes* with three other morphologically similar species. Intriguingly, the mutualistic host preferences of *A. nigripes* are highly similar to those of the three other species (Litsios *et al.*, 2012), yet it is distributed in the Maldives where none of the other species occur and is phylogenetically distant from the others in this group. Thus, the appearance of convergent forms throughout the evolution of the clownfish probably explains the discrepancy between the current morphology-based taxonomy and our molecular phylogenetic study. A more surprising result is the discrepancy between the present study and recent phylogenetic analysis (Litsios *et al.*, 2012; Frédérick *et al.*, 2013). A probable explanation is that those studies used primarily mitochondrial and few nuclear markers. We can expect incongruences between mitochondrial and nuclear phylogenies if recurrent hybridization happened during the evolution of the clade under study (Seehausen, 2004). Such a scenario is highly likely in the clownfishes because hybrids occur between distant relatives (Fautin & Allen, 1997). By using only nuclear markers, we removed the bias caused by the mito-nuclear incongruences. Yet, linkage groups with different evolutionary histories may reduce the support of the nodes linking species that have hybridized (Seehausen, 2004).

Biogeographical history

The biogeographical reconstruction we infer shows the Central-Indo Pacific and Central Pacific provinces as the most likely regions of origin of the clownfishes. This is congruent with a broader analysis on the entire Pomacentridae in which the sampling of clownfishes was less extensive (Cowman & Bellwood, 2013). While the majority of clownfishes diversified in the IAA, several species managed to colonize the Indian and central Pacific Oceans. One clade in particular is now distributed along Eastern Africa (including the Red Sea, Maldives and central Indian Ocean; Fig. 1). The node positioned directly below the common ancestor of this East African clade is also reconstructed as being located in the Indian Ocean (area A, Fig. 1), but the probability associated with this reconstruction is low, as is the posterior probability of the node itself (Fig. 1). This suggests that colonization of the Indian Ocean occurred along this branch less than 4 Ma. Clownfishes first colonized the southern tip of India and then crossed the Indian Ocean towards Madagascar. Finally, the Red Sea was only recently colonized by one species, *A. bicintus*. The biogeographical reconstruction (Fig. 1) shows that three other clownfishes reached the Indian Ocean. In the case of *A. clarkii* and *A. sebae*, those events are likely to be range expansions. The Malagasy population of *A. akallopisos* represents a recent colonization event and requires a population genetic study to understand whether *A. akallopisos* populations still exchange genes between the two disjunct areas. Finally, the six areas we used in the biogeographical analysis are large and may not adequately represent species distributions. We exploit the computational capacities of the Bayesian implementation of BayArea

(Landis *et al.*, 2013) to explore the effect of extending the number of areas. Although these results are qualitatively similar to the others, we obtained a more precise description of the colonization of the Indian Ocean by the East African clade, which follows a path that starts around India, then moves to Madagascar and finally includes the Red Sea and Arabian Gulf (Appendix S3). This scenario shows that the colonization of the Western Indian Ocean started when the East African clade was already separated from the lineage leading to *A. akindynos* (Fig. 1). This solution is more parsimonious as it places the common ancestor of the African and *A. akindynos* clades in the Central Indo-Pacific province, which is located between the areas occupied by the extant members of both clades. The distance covered by both clades to reach their present distributions is thus reduced compared with the scenario proposed by the BioGEOBEARS inference.

Replicated adaptive radiation

Adaptive radiation driven by mutualism with sea anemones is likely to be responsible for the diversification of clownfish (Litsios *et al.*, 2012). Many textbook examples of adaptive radiation (e.g. cichlid fishes) consist of replicated instances that occurred simultaneously in multiple isolated areas (Wagner *et al.*, 2012). A similar process has been proposed at the family level in the Pomacentridae (Cooper & Westneat, 2009; Frédérick *et al.*, 2013), but it has been unclear whether this could also be the case in the clownfish radiation. Our biogeographical reconstruction strongly supports the hypothesis that the East African clade diversified independently from the other clownfish lineages and, thus, can be considered as a geographical replicate of the main radiation that occurred in the Central Indo-Pacific region. Indeed, we observe in our phylogenetic tree that seven species of clownfish arose from a common ancestor, which colonized the eastern Indian Ocean. Those species now span the whole range of mutualistic interactions with sea anemones (Fig. 3). Here, we do not explicitly compare the mode of speciation between the East African clade and the main clownfish group. Yet, the diversity in terms of species and mutualistic habits that we observe strongly suggests that the evolutionary processes are similar. Instances of replicated ecological speciation are scarce in the marine environment. A rare illustration of such a process is given by a clade of Neotropical gobies that shows similar ecological divergence among habitats in species separated by the Isthmus of Panama (Taylor & Hellberg, 2005). The new example we present here provides prospects for future studies on ecological speciation and adaptive radiation in the marine realm.

Range expansion and diversification

We use the GeoSSE framework (FitzJohn, 2012) to compare patterns of range expansion and diversification between two putatively independent, geographical radiations of the clownfish. We found that the Indian and Pacific Oceans have

similar rates of speciation, extinction and dispersal. The IAA has often been considered a centre of origin in which speciation occurs at a faster rate than in peripheral areas (e.g. Briggs, 2003). While this may be true for some organisms (Mora *et al.*, 2003; but see Bellwood & Meyer, 2009), our analysis cannot recover this effect in the clownfishes. While it is not possible to link an evolutionary process directly to this rate homogeneity, the obligate dependence on sea anemone hosts is a likely explanation to the decoupling of speciation rates of clownfishes from factors intrinsic to geographical areas. As eight out of the ten potential sea anemone hosts are widespread (Fautin & Allen, 1997), untapped niches could be colonized over the whole range of the group, not only in the IAA. This is likely to account for why we could not detect a significant difference in diversification rates between the two geographical replicates of the clownfishes radiations. The biogeography, phylogeny and diversification of the anemone host species have been only scarcely studied. Analysing this side of the clownfishes radiation could provide useful insight into potential coevolutionary processes between the two mutualistic partners.

While ecological speciation is likely to be the main driver of the diversification of clownfishes (Litsios *et al.*, 2012), it is not the sole factor. Clear geographical subdivisions exist between recently diverged pairs of species, as in the *A. ocellaris/A. percula* species complex (Timm *et al.*, 2008). In this case, the cessation of gene flow between the two incipient species can be explained by geographical causes only. However, our phylogeny shows that the *A. ocellaris/A. percula* species complex together with *P. biaculeatus* forms the most basal group in the phylogeny (Fig. 1). While the other 27 species of clownfish show extensive diversification, this subgroup did not radiate to the same degree. Yet, the proximal causes of the diversification of the clownfishes remain unknown and their identification would require genomic approaches and additional data on population-level divergence.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Samples, primers, molecular clock models and species distributions.

Appendix S2 Species richness map and classical taxonomy.

Appendix S3 Results of the BayArea analysis.

BIOSKETCH

The focus of our research group is on macroevolution, biogeography and computational phylogenetics. We put a strong emphasis on computational and mathematical approaches, mixed with field sampling and molecular sequencing and work on diverse groups of plants including grasses, Polygonaceae, Restionaceae and other angiosperms and animals such as clownfishes. We are particularly interested in understanding how patterns of biodiversity and the underlying macroevolutionary and microevolutionary processes vary across scales and environmental gradients and affect species morphological evolution.

Author contributions: G.L. and N.S. designed the research with the help of P.B.P.; G.L. and N.T. collected the samples; D.L. provided some samples and sequences; G.L. performed all the lab work and analyses; G.L. led the writing with the help of P.B.P., D.L., N.T. and N.S.

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