THREE ROUTES TO CRYPSIS: STASIS, CONVERGENCE, AND PARALLELISM
IN THE *MASTIGIAS* SPECIES COMPLEX (SCYPHOZOA, RHIZOSTOMEAE)

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ABSTRACT

Evolutionary inference can be complicated by morphological crypsis, particularly in open marine systems that may rapidly dissipate signals of evolutionary processes. These complications may be alleviated by studying systems with simpler histories and clearer boundaries, such as marine lakes—small bodies of seawater entirely surrounded by land. As an example, we consider the jellyfish *Mastigias* spp. which occurs in two ecotypes, one in marine lakes and one in coastal oceanic habitats, throughout the Indo-West Pacific (IWP). We tested three evolutionary hypotheses to explain the current distribution of the ecotypes: (H₁) the ecotypes originated from an ancient divergence; (H₂) the lake ecotype was derived recently from the ocean ecotype during a single divergence event; and (H₃) the lake ecotype was derived from multiple, recent, independent, divergences. We collected specimens from 21 locations throughout the IWP, reconstructed multilocus phylogenetic and intraspecific relationships, and measured variation in up to 40 morphological characters. The species tree reveals three reciprocally monophyletic regional clades, two of which contain ocean and lake ecotypes, suggesting repeated, independent evolution of coastal ancestors into marine lake ecotypes, consistent with H₃; hypothesis testing and an intraspecific haplotype network analysis of samples from Palau reaffirms this result. Phylogenetic character mapping strongly correlates morphology to environment rather than lineage ($r = 0.7512$, $p < 0.00001$). Considering also the deeper relationships among regional clades, morphological similarity in *Mastigias* spp. clearly results from three separate patterns of evolution: morphological stasis in ocean medusae, convergence of lake morphology across distinct species and parallelism between lake morphologies within species. That three evolutionary routes each result in crypsis illustrates the challenges of interpreting evolutionary processes from patterns of biogeography and diversity in the seas. Identifying cryptic species is only the first step in understanding these processes; an equally important second step is exploring and understanding the processes and patterns that create crypsis.

Keywords: cryptic species, evolution, homoplasy, island biogeography, jellyfish, marine biodiversity, marine lakes, morphology, phylogeography, radiation, speciation

Running Title:

Stasis, convergence, and parallelism

1. Introduction

Reconstructing ancestral phenotypes and patterns of descent is a difficult problem in evolutionary biology (Losos, 2011a). Phylogenetic inference can be complicated by extremes in morphological variance that conceal links between appearance and genetic relationships. The difficulties caused by such phylogenetically uninformative or misleading morphological variance are acute in marine environments where cryptic species are common (Bickford et al., 2007; Knowlton, 2000). Estimates of the proportion of marine species that are cryptic (i.e., morphologically difficult to distinguish (Appeltans et al., 2012; Knowlton, 2000) and thus taxonomically classified together) range as high as 55% for some taxa (Appeltans et al., 2012), possibly a result of the prevailing physical structure of marine environments (Hamner, 1995) and the primarily non-visual mating cues used by many marine species to recognize conspecifics (Knowlton, 2000). Consequently, morphological crypsis can mask species boundaries, despite deep evolutionary divergences (Bickford et al., 2007; Goetze, 2003; Shenkar and Swalla, 2011; Singhal and Moritz, 2013).

The difficulties of evolutionary reconstruction raised by a high incidence of cryptic species have been compounded by uncertainty about the geographic distribution of variation in marine systems. A dearth of clear geographic discontinuities in the ocean led to the conclusion that marine species generally have high dispersal, relatively rare population differentiation, and an elevated proportion of ecological speciation via adaptation to local environments (Bowen et al., 2013; Carr et al., 2003; Palumbi, 1994). However, potentially important roles for limited dispersal have been emphasized (Buonaccorsi et al., 2004; Cowen and Sponaugle, 2009; Cowen et al., 2000), and there are many short-range endemics (Bird et al., 2007; Froukh and Kochzius, 2007; Wood and Gardner, 2007). These mixed results likely indicate that multiple mechanisms and patterns of evolution occur on various scales in the oceans (Bowen et al., 2013; Dawson and Hamner, 2008; Palumbi, 1994).

These challenges for evolutionary inference embodied in cryptic species may be overcome in three ways. First, molecular analyses have been used as an important, albeit also imperfect (Wheeler et al., 2013), complement to morphological data for reconstructing lineages and their histories (Losos, 1999). Second, the clearest geographic situations have been sought out for study, oftentimes on terrestrial islands and in freshwater lakes, where the evolutionary systems are clearly bounded (Baldwin et al., 1990; Grant and Grant, 2009; Losos, 2009; Losos et al., 1998; Meyer, 1993; Schluter and Nagel, 1995; Seehausen, 2006). Third, studying incipient species has provided opportunities to explore factors influencing divergence of lineages on micro-evolutionary timescales, when patterns have been least obscured by the passage of time (Lynch, 1989; Via, 2009). Molecular analyses of species inhabiting relatively young islands, therefore, may provide particularly clear situations—to the extent that islands are microcosms of more complex mainland systems (MacArthur and Wilson, 1967)—for studying the factors that influence morphological evolution.

Marine lakes—small bodies of seawater surrounded entirely by land—may provide this kind of opportunity for studying marine organisms. Marine lakes act as ‘islands’ to an oceanic ‘mainland’ (Dawson and Hamner, 2005), serving as natural laboratories inhabited by a suite of marine coastal organisms (Dawson et al., 2009). Like their freshwater lake and terrestrial island counterparts, replication of these marine islands provides a clear framework for testing specific hypotheses (Dawson et al., in press).

Here, we explore a case study: the morphological evolution of a scyphozoan jellyfish, *Mastigias* spp. (Rhizostomeae, Mastigiidae). *Mastigias* medusae are found throughout the Indo-

West Pacific (IWP; Kramp, 1961) and occur as two ecotypes: one primarily in coastal waters including semi-protected coves and lagoons (hereforth called “ocean”), and the other in marine lakes (“lakes”) (Dawson et al., 2009). Despite multiple described species of *Mastigias* in the IWP (Kramp 1961; Mayer 1910), most recent observations are ascribed to *M. papua* (e.g. Becking et al., 2015; Dawson, 2005b; Lewis et al., 2008; Saravanan et al., 2013) irrespective of ecotype or geographic location. Adding to the taxonomic uncertainty, *Mastigias* from disparate ocean locations appear predominately similar (Fig. 1a, c, e), as do *Mastigias* from disparate lake locations (Fig. 1b, d). Moreover, each *Mastigias* ecotype occurs proximate to the other ecotype throughout the IWP—for example, both are found in Palau, in Papua, and in Berau (Fig. 1; Dawson et al., 2009). Thus, in addition to taxonomic uncertainty, this pattern also raises the question: how did the morphologically most distinct medusae become the geographically most proximate (and vice versa)? Scyphozoans, like many other marine invertebrates, show high incidence of cryptic species (Appletans et al., 2012; Dawson, 2004; Dawson and Jacobs, 2001; Holland et al., 2004), but case studies for the taxon are sparse. Preliminary molecular analyses of *M. papua* in Palau suggest repeated evolution of the marine lake ecotype from an ocean ancestor since the Last Glacial Maximum (LGM; Dawson and Hamner, 2005), when modern marine lakes formed (Dawson et al., 2009; Smittenberg et al., 2011), and suggest evolution of morphologically distinct ecotypes consistent with subspecies (Dawson 2005b). It is unknown, however, if the morphological similarity within each *Mastigias* ecotype across the IWP is a plesiomorphic consequence of shared ancestry or a homoplastic result of convergent or of parallel evolution.

To examine these questions, we combine molecular phylogenetic and phylogeographic analyses of *Mastigias* spp. with descriptions of their morphological variation across the IWP and within the Palau archipelago. We test three hypotheses which could explain the morphological similarity within, and disparity between, ecotypes of *Mastigias* spp. across the IWP (Fig. 2).

H₁: The lake and ocean ecotypes are from an ancient divergence; potentially they may even be separate species of *Mastigias*. In this scenario, the two types diverged at least prior to the LGM, with the lake ecotype subsequently colonizing multiple marine lakes. Although geologic evidence suggests the current marine lakes did not exist prior to the LGM (Smittenberg et al., 2011), refugia lakes could have existed (e.g. see Grigg et al., 2002). The lake ecotype might have survived the LGM by living in refugia lakes and subsequently dispersed into newly formed, modern-day marine lakes as the refugia lakes became inundated with the rising sea-level.

H₂: The two *Mastigias* ecotypes are separated by one single, post-glacial transition between ecotypes. In this scenario, the lake ecotype arose from a recent colonization event from the ocean into one modern, post-glacial marine lake, followed by divergence into the lake ecotype and its subsequent dispersal to other lakes throughout the IWP. The long-lived pelagic medusa stage which brood planula larvae until the larvae are near ready to settle on the benthos (Uchida, 1926) provides a possible mechanism for successful long-distance dispersal and colonization.

H₃: The lake ecotype evolved by repeated and independent colonization of lakes across the IWP from adjacent coastal populations, resulting in multiple transitions between ecotypes. As the marine lakes were formed, each one was independently colonized from the adjacent source population. As conduits into the lakes closed off or lakes became otherwise isolated, the jellyfish in each lake independently diverged into the lake ecotype. Lake ecotypes would not occur in the ocean nor disperse between lakes.

To distinguish among these hypotheses, we map morphological variation among *Mastigias* onto their molecular phylogeny. We aim to identify the best supported hypothesis of

morphological evolution in *Mastigias*, and to consider whether this result may provide some insight into patterns of evolution in the seas.

2. Methods

2.1 Study Region & System

Marine lakes occur throughout the world in coastal regions where karst topography is present, with a large assemblage in the Indo-West Pacific including Palau (Dawson et al., 2009; Hamner and Hamner, 1998). Sedimentation rates and ¹⁴C-dated sediment cores in Palau, and lake bathymetry more broadly, indicate modern marine lakes formed after the LGM as rising sea levels flooded through cracks into low-lying areas within islands (Dawson et al., 2009; Smittenberg et al., 2011). Modern marine lakes vary in the timing and amplitude of tides relative to the ocean illustrating different degrees of connectivity (Hamner and Hamner, 1998) which, combined with varying depths, cause marine lakes to exhibit a variety of physical environments, from holomictic and ocean-like to stratified (e.g. increasing dysoxia with depth) to meromictic i.e. stratified with an anoxic layer below a sharp chemocline (Becking et al., 2011; Cerrano et al., 2006; Hamner and Hamner, 1998).

2.2 Molecular analyses: Extraction, Amplification, Sequencing & Alignment

For phylogenetic analyses, tissue was biopsied from the oral arms of 60 *Mastigias* and two of its sister taxon *Phyllorhiza punctata* (Bayha et al., 2010) (Table 1, Fig. 3). For a detailed intraspecific analysis, tissue samples were biopsied from an additional 157 medusae from 15 locations in Palau (Table 1); 15 specimens or the highest number available were used per location. All tissues were preserved in 95% ethanol and stored at -20°C.

DNA was extracted using a CTAB-phenol/chloroform protocol (Dawson and Jacobs, 2001) and used in 50 µl polymerase chain reactions (PCRs) to amplify five molecular markers: mitochondrial cytochrome *c* oxidase subunits I (COI) and III (COIII), 16S rDNA, and nuclear histone 3 (H3) and 28S rDNA; preliminary analyses indicated 28S rDNA contained only rare autapomorphies within *Mastigias* spp. and therefore was not analyzed further. COI was amplified using LCOj (Dawson and Hamner, 2005) paired with MphCO (Dawson and Hamner, 2005), HCO2198 (Folmer et al., 1994) or Aa_HCOI_12582 (Bayha and Dawson, 2010). 16S was amplified with 16S-L (Ender and Schierwater, 2003) and Aa_H16S_15141H (Bayha and Dawson, 2010); H3 was initially amplified with Histone 3 F Deg and Histone 3 R Deg (Colgan et al., 1998) until specific primers H3_Mp_9F (CGACCGGAGGAAAGGCACCC) and H3_Mp_327R (CGTTTTGCGTGGATGGCGC) were designed. COIII was initially amplified with COIIIA and COIIIB (O’Foighil, unpublished) until specific primers were designed which paired COIII_Mp_6670b (ACGTAGCGTCGTGTGGAAC) with either COIII_Mp_7339Rdeg (ATAYCAACTGGCTGCYTCAAACC) or COIII_Mp_7339R (ATACCAACTGGCTGCCTCAAACC). All PCR reactions were composed of 0.5 µM primers, 5.0 µl 10x PCR buffer, 3 mM MgCl₂, 0.2 mM dNTPs, 0.1 µl Taq polymerase (Applied Biosystems, Foster City, CA), and 1 µl sample and run on an Applied Biosystems 2720 thermal cycler. All PCRs of COIII and H3, and occasional PCRs of COI and 16S, also included 0.2 mM Bovine Serum Albumin (BSA). All PCRs included at least one initial hold of 94°C of 3–5 min, then between 33–39 cycles that included denaturation at 94°C for 30–45s, annealing between 50–56°C for 30–60s, and extension at 72°C for 60–90s, before a final hold of 72°C for 10 min.

Primer sequences and thermocycles specific for each locus and primer pair are given in supplemental material (S1).

Amplicons of COIII, during primer development, and H3, throughout the study, were cloned using pGEM-T vector (Promega, CA) and One Shot TOP10 competent cells (Invitrogen, Life Technologies Corp.) using the pGEM-T protocol (Promega, CA) and DNA was purified using Invitrogen's PureLink MiniPrep Kit after cultures were grown in TB overnight. DNA then was sequenced, or for amplicons of all other markers, cleaned with ExoSap and sequenced, at the University of Washington's High-Throughput Genomics Unit, at the University of California, Berkeley's DNA Sequencing Facility, or at Macrogen.

Sequences were trimmed of primers, assembled into contigs, and inspected visually to check base calls and open reading frames using Sequencher v. 4.1 (Gene Codes Corporation, Michigan, USA). Sequences were confirmed to have high sequence similarity with *Mastigias* or other scyphozoans by BLASTn (Altschul et al., 1997). All loci were then aligned in MUSCLE (Edgar, 2004) using default parameters and checked by eye. 16S was aligned using three additional strategies: using MUSCLE (Edgar 2004) with six combinations of gap opening and extensions penalties ranging from -1,000 to -100 and -16 to -0.05 respectively; in MAFFT (Katoh et al., 2005) with the E-INS-i algorithm using default parameters for the offset and gap opening values set to either default, 1.0, or 3.0; and in TCOFFEE using default parameter settings (Notredame et al., 2000). All 16S alignments listed above were compared in GBLOCKS using default parameters and ambiguously aligned regions were excluded from further analyses, leaving only regions that were aligned robustly across alignments (Castresana, 2000; Talavera and Castresana, 2007). Sequences were deposited in GenBank (XXXX – XXXX).

2.3 Molecular Analyses: Phylogenetic Reconstruction & Intraspecific Networks

Initial gene tree analyses showed locus H3 sequences form two distinct clusters and BLASTn results suggested two paralogous H3 loci, which we denoted as H3a and H3b and analyzed separately. Trees were reconstructed with *Phyllorhiza*—the sister taxon to *Mastigias*—as the outgroup to polarize character evolution, and also without *Phyllorhiza* in case of effects of long-branch attraction, per Bergsten (2005). The best fitting model of sequence evolution for each of the mtDNA and nDNA markers (HKY+I for all loci except HKY+Γ for H3a and HKY for H3b) was selected using corrected Akaike Information Criterion and Bayesian Information Criterion delta scores in jMODELTEST (Posada, 2008). Maximum likelihood gene trees for each locus were constructed and bootstrapped 1,000 times on the CIPRES portal (Miller et al., 2010) using GARLI 2.0 (Zwickl, 2006). A majority-rule consensus of bootstrap trees from GARLI was compiled in PAUP* v. 4.0b10 (Swofford, 2002). Population expansion in each mitochondrial clade was tested using Extended Bayesian Skyline Plots prior to Bayesian gene tree construction in BEAST v. 1.7.5 (Drummond and Rambaut, 2007), which used priors from jMODELTEST. Bayesian trees were constructed with all assumptions of clocks and population growth combinations, and then examined in Tracer v. 1.5 (Rambaut and Drummond, 2007) to determine that the assumptions of a relaxed clock with a logistically growing population returned the most resolved tree. Bayesian Markov Chain Monte Carlo methods were run for 100 million iterations, with samples taken every 1,000 steps and the first 10,000 trees discarded as burn-in. All Bayesian chains were run at least twice; there was no difference in topology or supported branches' lengths between runs. Sample trees were re-sampled using LogCombiner in the BEAST package, excluding burn-in, to produce a final amount of 10,000 trees. Convergence was assessed using Tracer v. 1.5 (Rambaut and Drummond, 2007).

Species trees were reconstructed using Bayesian and maximum likelihood analyses of two datasets: (1) partitioned matrices, with models of evolution specified for each locus, and (2) from unlinked, individual gene trees. Partitioned trees, for (1), were generated (a) using maximum likelihood on CIPRES' GARLI 2.0 (Miller et al., 2010; Zwickl, 2006) with a majority-rule consensus of 1,000 bootstrap trees compiled in PAUP* (v.4.0b10 Swofford, 2002) and (b) using Bayesian analyses in BEAST v.1.7.5 (Drummond and Rambaut, 2007). Two species trees, for (2), were constructed using Bayesian analysis in *BEAST (Drummond and Rambaut, 2007) for (a) a dataset of only loci with all specimens and (b) a dataset of only specimens with data for all loci. All Bayesian analyses were each run twice, for 100 million iterations with samples taken every 1,000 iterations. The resulting trees were thinned in LogCombiner after removing a 10,000 iteration burn-in to produce the final 10,000 trees. Convergence was tested before and after the thinning using Tracer v.1.5; there was no difference between runs in the final tree's topology or supported branches' lengths.

Agreement in tree topology between reconstruction methods within loci and between loci within methods was assessed using COMPARE2TREES v. Sept 2011 (Nye et al., 2006) using trees with only supported nodes with ≥ 70 posterior probability or bootstrap support. Overall topological similarity was measured by calculating a percentage of matched branches between two compared trees (Nye et al., 2006).

Detailed intraspecific analyses were performed on an expanded Palau dataset since it could provide greater resolution between locations within a species and region, due to larger sample size of many ocean and lake locations, than the phylogenetic analyses. Intraspecific relationships within Palau were represented using statistical parsimony analyses of COI and COIII loci independently, as well as concatenated, in TCS (Clement et al., 2000). Population differentiation was estimated by calculating Φ_{ST} between locations using Arlequin v. 3.5.1.2 (Excoffier et al., 2005).

2.4 Morphological analyses

Specimen collection: Our morphological dataset included a total of 262 medusae. Existing data from 183 *Mastigias* from Palau (Dawson, 2005a) were combined with new data from seventy-nine *Mastigias* medusae ranging between 20–160 mm bell diameter (per Dawson, 2005a) collected from a total of 16 locations across the IWP (Table 1, Fig. 3). Whole animals were fixed in 4% formalin with seawater. Additionally, 116 *Mastigias* (Table 1, Fig. 3), including some at locations from which preserved medusae were unavailable, were measured from photographs taken of medusae *in situ* with the oral-aboral axis perpendicular to the photographer. Overall size was measured *in situ* on 33 medusae from ocean locations in Palau for comparison to existing population size data from lake locations. Comparable data for the sister taxon *Phyllorhiza* were collected from one specimen and five photographs.

Morphological measurements: Morphological features described by Dawson (2005), except characters on coloration and mass (*f1* to *f8*), were measured on preserved specimens. Photographed individuals allowed measurement of only six externally visible continuous morphological features (*f9-f12*, *f14-f15* from Dawson, 2005a) using JMicroVision v. 1.2.7 (Roudit), but did enable scoring of additional coloration and meristic features (*f1-f7*; Dawson, 2005a). One character, mean terminal club length, was correlated with the terminal club number; a complete absence of terminal clubs would result in “missing data” for length, thus creating a bias for any analyses which would exclude any specimens with missing data. To correct for this, we calculated a new metric by multiplying terminal club number by mean terminal club length.

Since photographs had different resolutions and magnifications, we standardized all quantitative measures that are isometric with size (Dawson, 2005a) as ratios of the bell diameter. Each character was tested for normality and homoscedasticity in R v. 2.11.1 (R Development Core Team, 2010) or, for small samples ($n < 5$), the median and mean were compared and small differences taken as a rough approximation of normality. All samples met the assumptions of normality and homogeneity of variance.

Construction of data matrices: The fragility of jellyfish makes preservation difficult, and many specimens had some damage which necessitated coding one or more traits as missing data. To maximize inclusion of specimens in non-metric Multidimensional Scaling (nMDS) analyses, which do not permit missing data, we reduced the number of traits in the matrix. To do so objectively, we initially performed a Principal Component Analysis (PCA) on those individuals without any missing data to determine which of the 35 non-color related characters contained the most informative, non-redundant data (R v. 2.11.1, `prcomp()`, stats package, R Development Core Team, 2010). We then grouped characters within 5-degree arcs around the PCA origin into clusters. Any character which fell in more than one cluster was assigned to the cluster to which it was closest. Finally, the character with the strongest signal in each cluster was considered relative to other clusters; only those that were >40% of the strongest overall—which was a natural gap between strong and weak signals—were included in the final matrix for nMDS analyses. To assess the impact of this data reduction step on subsequent inferences, we conducted sensitivity analyses by repeating the procedure using 2.5-degree or 10-degree arcs and using 30% or 50% cutoffs for signal strength. In all exploratory tests, these sensitivity analyses showed that samples occupied the same relative positions on nMDS plots and that the final matrix (5-degree, 40% cutoff) was statistically indistinguishable from those using more characters and alternative groupings. We performed this data-reduction on a dataset containing specimens from across the IWP and on another containing specimens from only Palau, generating two matrices for nMDS analyses: (1) *IWP1* which has 13 independently informative characters for 203 individuals across the IWP, and (2) *PW1* which has 15 independently informative characters for 136 specimens in Palau. Subsequently, we also performed the character-reduction including photographed specimens with only external characters, and thus representing every location, resulting in two additional matrices: (3) *IWP2* which had five independently informative characters for 250 IWP specimens, and (4) *PW2* which had five characters for 169 Palau specimens.

nMDS analyses: nMDS analyses were performed in Primer v. 5.2.9 (Clarke and Gorley, 2001) using Bray-Curtis similarities. Each nMDS analysis used ten randomly seeded starts to assess repeatability of the results (Clarke, 1993) and minimum stress results used to assess the data representation quality. Agreement between analyses of the paired datasets (*IWP1* and *IWP2*, *PW1* and *PW2*) was assessed by the similarity in clustering, as well as PERMANOVA calculations of nested environment and location variables (R v. 2.11.1, `adonis()`, vegan package; Oksanen et al., 2011). Palau had the most complete sampling coverage from multiple cove and lake locations, so we additionally performed post-hoc Tukey tests on the nMDS distributions along each axis from *PW2* to determine the specific locations driving the overall pattern (R v. 2.11.1, `TukeyHSD()`, stats package, R Development Core Team, 2010). P-values within each analysis set were considered after Sequential Bonferroni correction (Rice, 1989).

An estimate of overall phenotypic similarity among samples across the IWP was calculated as the distance on the *IWP2* nMDS plot of each datapoint from the minimum X and Y values on the plot (i.e. $\sqrt{(x_p - x_{\min})^2 + (y_p - y_{\min})^2}$). Thus, points with a similar distance were closer

together on the nMDS and therefore phenotypically similar; boxplots show the variation in overall similarity within a site.

2.5 Hypothesis Testing, Character State Reconstruction, and Phylogenetic Contrasts

To test consistency of the species tree with each of our three alternative hypotheses, we compared the overall topological similarity of the species tree with each of the representative hypothesis trees drawn in Fig. 2. Since the hypothesis trees tips are identified only by ecotype and region, we reclassified specimens in the species tree similarly. Then, within each region and ecotype pair, we randomly assigned on each tree an additional identifier per individual to create a unique name (e.g. Region1-Lake-F, Region2-Ocean-D). Branch lengths were generated for each hypothesis tree by scaling the tree to the species tree's molecular clock based on the positioning of the hypothesized divergence relative to the Last Glacial Maximum. Each hypothesis tree was compared to the species tree using COMPARE2TREES v. Sept 2011 (Nye et al., 2006) to generate an overall topological similarity score.

We inferred ancestral morphological states across the species tree using squared-change parsimony reconstruction in Mesquite v. 2.75 (Maddison and Maddison, 2011). Since only the genetic samples from Raja Ampat and Japan had corresponding samples in the morphological dataset, the character mean value for each location was used. Categorical values were converted to 0 or 1 binomials or hierarchical number classes to calculate a mean. Overall phenotypic similarity was calculated as the mean values of the linearized distance on nMDS analyses for each location. Those overall mean values, as well as mean values of each categorical and continuous character individually (regardless of coverage), were mapped across the species tree. We estimated correlation between phenotypic similarity and environment type (ocean/lake) using Felsenstein's Independent Contrasts executed in the PDAP package of Mesquite (Midford et al., 2010) after reducing the degrees of freedom due to uncertainty at nodes with low support (Purvis and Garland, 1993). P-values across these ancestral state reconstructions were considered after Sequential Bonferroni correction (Rice, 1989).

3. Results

3.1 Phylogenetic Analyses

All gene tree reconstructions except that for locus H3, which had low overall resolution (supplemental material S2), and all species trees grouped *Mastigias* specimens into three, strongly supported, deeply divergent clades: one "China Seas clade," including Japan, Berau and Vietnam; a second "Pacific Islands clade," including Palau, West Papua and Enewetak; and a third "Solomon Sea clade," exclusive to Tufi (Fig. 4; supplemental material S2). The general topology of gene trees was consistent between methods within loci (overall topology similarities = 100%) and between loci (overall topology similarities $\geq 70\%$). The same topology was produced in the Maximum Likelihood and Bayesian partitioned dataset species trees and with the Bayesian species tree (overall topology similarities $\geq 98\%$; Fig. 4). The consensus topology for each gene tree and the species tree suggests that the Solomon Sea clade branches basally relative to the other two main clades and that there is considerable divergence between all three clades (Fig. 4; species tree with *Phyllorhiza* outgroup, supplemental material S3).

There was greater divergence between clades than between ecotypes. The between clade mean estimated divergence (mean substitutions per site) of the partitioned dataset was 0.071 and 0.067 between Solomon Sea clade and the China Seas clade and the Pacific Islands clade, respectively. The divergence between the China Seas and Pacific Islands clades was 0.049. The

within-clade mean distance, which includes the difference from ocean and lake ecotypes, is decidedly less, with the Solomon Sea clade at 0.005, the China Seas clade at 0.010, and the Pacific Islands clade at 0.006. Between clade mean estimated divergence in COI and COIII datasets ranged between 0.071–0.124. With the exclusion of the Solomon Seas clade, which was comprised of only two Tufi samples, the remaining clades included both lake and cove locations within a given region.

Within Palau, both COI and COIII haplotype networks show central and diverse ocean haplotypes with lake haplotypes on the periphery of the network (supplemental material S4), as does the concatenated mitochondrial network (Fig. 5). Lake locations typically have only one to three unique haplotypes each, whereas cove locations are more diverse and share common haplotypes across locations (Fig. 5); the exception to this pattern is Ongeim'l Tketau (OTM) which has multiple rare haplotypes. Calculation of Φ_{ST} between locations reveals that ocean sites, Mekeald Lake (MLN) and Tketau Lake (TLM) have zero or weak genetic differentiation (supplemental material S5). In contrast, comparisons between all ocean locations with each meromictic lake and with T-Lake (TLN) and Ongael Lake (OLO), as well as TLN, OLO and meromictic lakes with each other, have $\Phi_{ST} > 0.2$ and $p \leq 0.0028$.

3.2 Morphological Analyses

The nMDS analyses of the *IWP1* and *IWP2* grouped specimens by environment (ocean vs. lake) regardless of region (Fig. 6a, supplemental material S6), with stress scores indicating the plot represented the respective data matrix well (*IWP1* stress = 0.07; *IWP2* stress = 0.02). Additionally, PERMANOVA calculations show a significant ($p < 0.001$) clustering by environment in each matrix. For *IWP1*, environment (ocean vs. lake) explained 15% of the total variance, and locations within environments another 51%; for *IWP2*, the variance explained by environment is 47% and locations within environments 33%. Estimates of overall phenotypic similarity display the two ecotypes distinct from each other, although with a degree of variation within ecotypes (Fig. 6b).

Generally, medusae with the ocean ecotype display a high degree of pigmentation in the bell, oral arms and terminal clubs, including an abundance of colored spots on the bell and flecks in the canals. Medusae with the ocean ecotype are typically large (median bell diameter 187.5 mm, maximum bell diameter 230 mm in Palau ocean locations) and generally have all of the 8 terminal clubs (median = 8, mean = 7.5) which are just over half the length of the bell diameter. They are also fairly robust, with the oral pillars, oral disc and bells that are between 15 and 30% thicker and a canal system that has 2.5–5 times more anastomoses than the lake ecotype medusae. In addition, medusae with the lake ecotype are typically smaller (Dawson, 2005b) and have lost most or all of the pigmentation found in the ocean medusae. They have a reduction in the number, length and thickness of the terminal clubs, with a median 7 of 8 terminal clubs (mean = 6.3), although older lakes often have medusae with no terminal clubs, and when present, the terminal clubs are only about 0.17 of the bell diameter in length.

The nMDS analyses of all Palau medusae show meromictic lake locations cluster together, as do holomictic and 'stratified' lakes (stratified, but not meromictic) with ocean locations (*PW1* stress = 0.08; *PW2* stress = 0.02; supplementary material S6). The PERMANOVA tests indicate that specimens within each nMDS clustered significantly by environment ($p < 0.001$). *PW1* had 14% of the variance explained by environment (ocean versus lake) and 50% explained by locations within environments; *PW2* had 51% of the variation explained by environment and 30% by locations within environments. Post-hoc tests of the *PW2* nMDS analysis indicated that

the majority of the difference between locations was on Dimension 1 (although variance on Dimension 2 is greater in lakes, the means are statistically indistinguishable for all but one pair of meromictic lakes; supplemental material S7). On Dimension 1, pair-wise comparisons were significantly different ($p < 0.0003$) in 15 of 16 paired comparisons between ocean sites and meromictic lakes, 4/4 of comparisons between holomictic and meromictic lakes, 3/4 of the comparisons between ‘stratified’ and meromictic lakes and 4/6 of comparisons between different meromictic lakes; comparisons between ocean sites ($n = 6$ pairs), between ocean sites and holomictic and ‘stratified’ lakes ($n = 8$) and between holomictic and ‘stratified’ lakes ($n = 1$) were never significant ($p > 0.001$) (supplemental material S7).

3.3 Hypothesis Testing, Character State Reconstruction and Phylogenetic Contrasts

The overall topological similarity of the species tree to H_1 was 36.4%, to H_2 was 35% and to H_3 was 82.7%. The scores are not a probability of the veracity of the hypothesis, but rather indicative of its value only relative to the other hypotheses; H_3 was more than twice as good as either other hypothesis. Character state reconstruction indicates that both lake and ocean ecotypes are found in each well-sampled clade of the tree (i.e., excluding the Solomon Sea clade, which has only two individuals). The lake ecotype (dark shades; Fig. 6c) is found multiple times across the tips of the phylogenetic tree; the ocean ecotype (light shades) is found throughout the tree, from tips through to deeper nodes. The ancestral morphological state is reconstructed unequivocally as an ocean ecotype. Not only is the ocean ecotype reconstructed as the ancestral morphological state for the entire tree, but also within each of the principal clades. The lake ecotype only occurs at the tips of the tree and the character state reconstruction switches to an ocean ecotype usually (6/7 times) within two nodes back on the tree.

Phylogenetic contrasts indicate a significant correlation between overall mean phenotypic similarity (Fig. 6b) and environment ($r = 0.7512$, $p = 6.514 \times 10^{-9}$; Fig. 6c). Morphology and environment were significantly correlated in 19 of 48 individual characters ($-0.4 \geq r \geq 0.4$ and $p < 0.0013$; supplemental material S8). Nine of 16 categorical morphological characters significantly correlated with environment; significant characters included those related to pigmentation. Ten (of a total possible 32) continuous characters were significant, including terminal club length, metrics regarding internal canal complexity and oral pillar dimensions. Oral arm length was marginally significant across all regions ($p = 0.017$, but not within the Sequential Bonferroni correction).

4. Discussion

4.1 Hypothesis Evaluation

Mastigias medusae occur as morphologically distinct ‘ocean’ and ‘lake’ ecotypes in geographically proximate locations across the Indo-West Pacific. *Mastigias* in the IWP also form a complex of at least three regionally distinct and reciprocally monophyletic clades, which are separated by genetic distances equivalent to species-level differences in some other jellyfishes (Bayha and Dawson, 2010; Ortman et al., 2010). Thus, the two ecotypes are not restricted to distinct clades and are not distinguished by a single ancient nor a single recent divergence, refuting our hypotheses H_1 and H_2 .

Rather, both ecotypes occur within multiple species-level lineages; each well-sampled species contains lake and ocean ecotypes, with each lake population most closely related genetically to its nearby ocean population (Fig. 4). The transitions between ocean and lake ecotypes occur near the tips of the species tree, indicating multiple recent transitions (Fig. 6c).

This interpretation is supported by detailed evidence from Palau that shows lake ecotypes are recently derived from ocean ecotypes, have low genetic diversity, and low gene flow (Fig. 5), and supported by the species tree having greatest similarity with the H₃ hypothesis tree. The multiple transitions between closely-related ocean and lake populations is strong evidence supporting H₃: recent, repeated, independent evolution from coastal waters into marine lakes. The broader phylogenetic analysis that shows at least two reciprocally monophyletic lineages of *Mastigias* across the IWP, both with ocean and lake ecotypes, indicates modern *Mastigias* became morphologically cryptic via three routes: stasis, convergence and parallelism.

4.2 Three routes to crypsis

The high incidence of cryptic species in marine environments may confound attempts to infer patterns of descent from morphology and thus inhibit understanding of evolutionary patterns and processes in the seas (Bickford et al., 2007; Knowlton, 2000). In *Mastigias* spp., morphological similarity within habitat types could be a plesiomorphic consequence of shared ancestry, attributable to synapomorphies, or a homoplastic result of convergent or of parallel evolution. Ancestral state reconstruction reveals that crypsis in *Mastigias* is a result of both retention of ancestral characteristics and *de novo* evolution of homoplasies. While these, like all ancestral character reconstructions, are hypotheses, they are robust in that characters are mapped across strongly supported nodes in the species tree and most individual characters show the same pattern as the overall morphology (Fig. 6c). Plesiomorphic features and stasis characterize ocean ecotypes along species lineages; lake ecotypes are characterized by homoplasious *de novo* evolution, either through convergent evolution between species or through parallel evolution within species.

Stasis, parallelism, and convergence are relative terms. In the context of this study, they describe the broad patterns present in *Mastigias* and which could be present in other marine organisms. While there is discussion about how, when, and at what scale the terms apply (e.g. Arendt and Reznick, 2008), we find that distinguishing between the terms is useful when setting up different evolutionary questions as long as the scale is specified (Elmer and Meyer, 2011). In this sense, we use the terms to distinguish patterns of morphological evolution relative to the underlying phylogeny. Stasis is used to mean the retention of an ancestral phenotype despite the accumulation of genetic differentiation; parallelism is repeated independent evolution of a phenotype within a lineage; convergence is independent evolution of a phenotype in different lineages. Critically, parallelism evinces repeated evolution of a trait state from one recent common ancestor, whereas convergence requires traits evolve after divergence from the most recent common ancestor and establishment of the lineages being compared. That ‘lineage’ can apply at various scales on the tree of life is consistent with the idea that all scales of comparisons are valid (Elmer and Meyer, 2011; Lescak et al., 2015; Westram et al., 2014) and thus we apply stasis, parallelism, and convergence at the intrageneric scale to patterns of morphological evolution along lineages within *Mastigias* as an example of the kinds of evolution one can see in marine taxa.

4.2.1 Crypsis through stasis

Morphological stasis in *Mastigias* is displayed in the similarity of ocean species across the IWP. The morphology of ocean ecotypes was statistically indistinguishable across clades and grouped tightly on the *IWP1* and *IWP2* nMDS analyses. Critically, phylogenetic character reconstruction indicates that the common ancestor of the three *Mastigias* species lineages had an

ocean ecotype. Assuming approximately 0.65% sequence evolution per million years for medusozoan COI (e.g. Govindarajan et al., 2005), which is within the general range for COI across taxa (Lessios, 2008)), the genetic differences between the three major clades of *Mastigias* indicate relative morphological stasis over ~6 million years of independent evolution.

Morphological similarity of ocean ecotypes relates to several obvious characters that are statistically distinct from the lake ecotype and that may relate to the environmental conditions in the ocean. Ocean ecotypes are colored, most obviously with blue pigment in the bell, which also is adorned with abundant yellow or white spots; these colors also may appear in the oral arms, terminal clubs and internal canals (see Fig. 1). Similar blue pigmentation in other rhizostome jellyfish has been hypothesized to be photoprotective for symbiotic *Symbiodinium* (Blanquet and Phelan, 1987), a function also tentatively attributed to *Mastigias* (Dawson and Hamner, 2003). Photoprotective pigment may benefit the ocean medusa-zooxanthellae holobiont that daily inhabits the high-light near-surface waters, a situation that contrasts with the lake environment and medusae (Dawson and Hamner, 2003; see next paragraph). Additionally, ocean medusae are generally large and have eight long thick terminal clubs; the clubs are large relative to medusa size and considerably exceed the size of clubs of the lake ecotype. The ocean ecotype's large size and long terminal clubs, which may aid in swimming quickly and efficiently in straight lines (Dawson and Hamner, 2003; Dawson, 2005a), may benefit ocean medusae which have to maintain cohesive populations in the face of a tendency to be dispersed or advected away by tidal currents. Similar environmental conditions, e.g. high water clarity, advective currents, and visual predators, occur in all ocean locations across regions and thus may create conditions for morphological stasis despite genetic divergence.

4.2.2 *Crypsis through convergence*

The lake ecotypes are morphologically similar across the IWP (Fig. 6a) despite a deep evolutionary divide between lake ecotypes in different species (e.g. Palau vs. Berau) and despite the most recent common ancestor of each species being an ocean ecotype (Fig. 6c). Thus, morphological similarity of the lake ecotype across the IWP, at least between species, arose via convergence.

Within meromictic lakes across regions, the suite of similar morphological characters is the converse of the characteristic ocean ecotype: lake ecotypes *generally* exhibit a reduction in pigmentation, in the number, length and thickness of terminal clubs, and in overall size (see: Dawson, 2005a; Fig. 1), although, there is greater variation in phenotype in the lakes than in the ocean ecotype (Fig. 6a) due to both greater variation within populations (Fig. 6a, b) and the larger number of isolated populations in the lake ecotype (Fig. 5, Fig. 6c). The reduction or loss of pigmentation in Palau lake medusae has been linked with the evolution of behavioral photoprotection in higher turbidity lakes (Dawson and Hamner, 2003), which was hypothesized to possibly allow finer and more immediate control of sunlight exposure, although the exact sequence of evolution of the two is unclear (Dawson and Hamner, 2003). The reduction in size and in terminal clubs, combined with a broader, slightly flattened bell shape, should increase drag from a less streamlined body that could explain reduced efficiency and speed of swimming in the lake ecotype (see Dawson and Hamner, 2005), and may be related to the lack of a selective force to maintain cohesive populations in lake environments (Dawson, 2005a; Dawson and Hamner, 2003). Oral arm length is marginally significantly correlated with environment across regions and highly significant within Palau, indicating an effect of environment, at least when

areas are studied in detail, or an effect of non-random gene flow into a novel environment (Edelaar and Bolnick, 2012).

While the phylogeographic patterns are most detailed for Palau, similar processes seem to be shaping *Mastigias* in other regions. Deep meromictic lakes in Berau and in Palau show morphotypes that are far diverged from the adjacent ocean forms (see Fig. 1). Relatively shallow holomictic lakes in Raja Ampat and Palau (and for available qualitative measurements, Vietnam) show medusae morphology and genetics that are less diverged from the adjacent ocean (see also: Dawson, 2005a; Dawson and Hamner, 2005). Deeper meromictic lakes also occur in Raja Ampat and harbor medusae that also exhibit the more diverged lake morphology (Becking et al., 2015), but samples were not available for this study.

4.2.3 *Crypsis through parallelism*

While the similar morphology of lake ecotypes *across* species demonstrates convergent evolution, *within* species it evinces parallelism. Within both the Pacific Islands and the China Sea clades, lake populations do not group together in a single monotypic clade, but have different independent origins, predominantly from an ancestral ocean population. For example, in the China Sea clade, the population in Hang Du Lake I in Vietnam is derived from an ocean ancestor independent of the divergence of medusae in Berau lakes from ocean ancestors; the same is true in Palau, in which lake populations cluster together morphologically on the *PW1* and *PW2* nMDS plots, but genetically show signatures of independent colonizations from the ocean (Fig. 5) and low gene flow. In Palau, the morphological evolution and distinct genetics of the more isolated lakes which do not exhibit any secondary contact with the ocean has been interpreted as incipient speciation (Dawson, 2005b). Thus, the lake ecotype morphology has arisen in parallel in each lake within *Mastigias* species.

The same kinds of morphological changes — loss of pigmentation, reduction in number, length and thickness of terminal clubs, reduction in oral arm length and overall medusa size — occur in populations in older and isolated lakes, particularly meromictic lakes (Dawson, 2005a). As noted in section 4.2.2, these similarities may be attributable to behavioral adaptation in photoprotection and landlocked habitat. In addition, oral arm length is significantly smaller in lakes than in the ocean within Palau, which may also pertain to swimming efficiency in landlocked environments (see section 4.2.2), or may be related to decreased reliance on *Symbiodinium* to meet metabolic needs (McCloskey et al., 1994), as the oral arms are the tissue that has a large concentration of the symbiotic *Symbiodinium*.

However, lake populations are not monotypic but show morphological variations on the ecotype. Even though morphology is similar within ecotype based on the nMDS, there is still a significant amount of variation between locations within ecotype. Previous studies with aquarium-raised medusae in Palau indicate that, while potentially slightly plastic with environmental conditions, most of the location-specific morphological differences are heritable and not primarily eco-phenotypic (Dawson, 2000; Dawson, 2005a). Most holomictic lake medusae have morphotypes similar to the ocean ecotype, while meromictic lake populations vary in morphology (Fig. 6a, b, Dawson, 2005a), and vary in other aspects such as behavior (Dawson and Hamner, 2003; Hamner and Hauri, 1981). Indeed, the different ages of lakes and the degrees of differentiation of independently evolving lake medusa populations suggest a progressive reduction in features from ocean to lake ecotype (Fig. 6b; Dawson and Hamner, 2005). Early stages in evolution may be indicated by the holomictic lake population in OLO, which is intermediate between the clusters of ocean and of meromictic lake medusae in the nMDS

analyses (Fig. 6a, b). OLO and also TLN exhibit significant amounts of isolation, similar to meromictic lakes. That said, whether a lake is holomictic, stratified but not meromictic, or meromictic may not be as important in determining morphological evolution as the degree and duration of isolation or biotic factors; morphologies in holomictic lakes range across the nMDS dimensions (e.g. in Fig. 6a, DCG in Raja Ampat cluster near oceanic locations; OLO in Palau is transitional and DKK in Berau clusters with meromictic lakes in Palau and Berau). Depending on the matrix used (and the features included within each), the exact amount of variance ascribed to between-environments or within-environment-between-locations changes, indicating that different features respond in different ways or at different rates to the various environments.

4.3 Implications for understanding crypsis in the seas

Cryptic species are common in the ocean (Appeltans et al., 2012; Bickford et al., 2007; Knowlton, 2000). However, many are “pseudo-sibling species” that can be distinguished using biochemical, genetic or robust quantitative morphological analyses (Knowlton, 1993). Application of these tools has resulted in an increase in distinct, but morphologically similar, species identified in many taxa, including scyphozoans (Bickford et al., 2007; Dawson, 2005a; Dawson and Hamner, 2005; Knowlton, 1993, 2000; Xavier et al., 2010, this study). Using these approaches could increase the number of known species by as much as 55% in some marine taxa and 4.0–15.5% overall (Appeltans et al., 2012). Cryptic species distinction is a key step in better understanding the magnitude and distribution of marine biodiversity (Bickford et al., 2007).

Yet, distinguishing cryptic species, by itself, may not dramatically increase our understanding of evolutionary processes, biogeographic patterns, or patterns of adaptation in the seas (Bickford et al., 2007; Knowlton, 2000). The majority of publications distinguish cryptic species but not the mechanism or process causing crypsis in distinct species (Lee and Frost, 2002; Witt et al., 2003). This failure to identify the causes of crypsis is understandable given the complexity of these mechanisms in marine environments; indeed, our results for *Mastigias* suggest that too often we do not know whether crypsis is due to stasis, convergence, or parallelism. Yet, understanding both 1) whether phenotypes are the result of external or internal constraints or the result of convergent or parallel adaptations and 2) the mechanisms causing adaptation has important evolutionary implications.

Understanding the patterns and processes generating crypsis should be the essential second step following distinction of cryptic species. The patterns and mechanisms which drive adaptation are fundamental to understanding when, where and how diversity arises, persists or is extirpated (Sanford and Kelly, 2011). Thus, the first step of identifying cryptic species remains important for elucidating biodiversity and identifying the evolutionarily significant units that may be affected by changing environments. However, the second step is equally important for understanding the mechanisms that generated extant biodiversity and how evolutionarily significant units may respond to future changes.

Crypsis through stasis, convergence, or parallelism could be caused by many processes involved in shaping dispersal and habitat partitioning (Bowen et al., 2013; Palumbi, 1994), by genetic or morphological constraints (Erwin, 2007; Losos, 2011b), or by the similarity in environment (Conover et al., 2009; Edwards et al., 2012; Eldredge et al., 2005; Hamner, 1995; Losos, 2011b). All three routes to crypsis—stasis, convergence, parallelism—are illustrated in the morphological evolution of *Mastigias*. Their relative frequencies in other marine taxa are unknown. Each may be commonplace, given the prevalence of crypsis (Hamner, 1995; Lee and Frost, 2002; Norris and Hull, 2012). Common selective and/or constraining forces could limit

changes in morphology (Bickford et al., 2007) and create crypsis through stasis in marine organisms that experience strong selection from similar environments, as hypothesized by Hamner (1995) and shown in Lee and Frost (2002). Selective forces from similar and physically constraining environments also were hypothesized to produce a prevalence of convergence in ocean systems (Hamner, 1995). Although marine examples of parallelism and convergence are lacking, the few existing studies have challenged assumptions about the time and geographic scales at which evolution occurs in the seas (e.g. Dawson and Hamner, 2005; Ravinet et al., 2016). Increasing recognition of the possibility of island and island-like marine environments and widespread phylogeographic structure in marine systems (Dawson, 2015; Gillespie and Clague, 2009; Hachich et al., 2015; Vermeij, 2004) should increase the awareness of researchers of opportunities for independent evolution—convergent, parallel, or divergent.

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Figure Legends

Fig. 1. *Mastigias* occurs in two ecotypes across the Indo-West Pacific. The left column shows individuals with the ocean ecotype, whereas the right shows individuals from marine lakes. The ocean ecotype is similar across regions, whereas the similarity of the lake ecotype depends both on the habitat and the physical isolation of the lake from the ocean. Similarity between regions within an ecotype may be cryptic. *Mastigias* from (a) Ngerchaol Cove, Ngerchaol, Palau, a semi-enclosed ocean site that experiences full tidal exchange with the surrounding lagoon, (b) from Ongeim'l Tketau, Mecherchar, Palau, a meromictic lake that is hydrographically isolated from the ocean and formed approximately 10,000 years before present, (c) coastal waters surrounding Komodo, Indonesia (photo courtesy of R. Olsen), (d) Haji Buang, Berau province, Indonesia, a meromictic lake possibly younger in approximate age (based on depth) and less isolated (based on tidal dampening relative to the ocean) than OTM (Becking et al., 2011; Dawson, unpublished data, Hamner and Hamner, 1998; photo courtesy of L. Bell), (e) coastal waters surrounding Waigeo, West Papua, Indonesia, and (f) Lake A, Gam, West Papua, Indonesia, a shallow and holomictic lake with high tidal exchange. The 'typical' lake ecotype, as seen in (b) and (d) also occurs in at least one lake in Misool, West Papua, Indonesia (Becking et al., 2015; Dawson et al., 2009), but is not shown because specimens were not available for this study.

Fig. 2. The pattern of *Mastigias* morphology seen across the IWP may be explained by one of three evolutionary hypotheses. **H₁** shows an ancient divergence between lake and ocean ecotype clades, and gene flow between regions within each ecotype. In this scenario, the Last Glacial Maximum (LGM) would occur after the divergent event between ecotypes. **H₂** indicates a eurymictic ocean population with one recent colonization event into a lake and subsequent divergence therein. The LGM would have occurred prior to the divergent event in this scenario. In both **H₁** and **H₂** regional phylogeographic structure may exist within ecotypes although this is not depicted. **H₃** depicts regional isolation in ocean ecotypes and multiple colonization events in each region from coastal ocean waters into marine lakes. The LGM would have occurred prior to the divergent event in this scenario. For each hypothesis, a schematic of proposed geographic pattern of evolution is on top and the resulting phylogenetic hypothesis is below. Different shades denote different regions. Open circles denote ocean ecotypes; solid circles, lake ecotypes. Dashed lines indicate dispersal between ocean sites; dotted lines dispersal between lakes (via the ocean). Solid lines indicate a post-glacial colonization event from the ocean to a lake and subsequent divergence. Black squares on phylogenetic trees indicate the node in which the most recent common ancestor between lake and ocean ecotypes occurs.

Fig. 3. Map of the Indo-West Pacific showing sampled locations. Symbols denote the sample type available from each location. The exact location for the photo from the Philippines is not known. The inset shows the fourteen locations in Palau used in phylogeographic analyses. Details of location and sample sizes for each data type are in Table 1; samples labeled as "Lagoon" in Table 1 are not shown as they came from two sites within the ocean lagoon, one near CLM and the other near OTM.

Fig. 4. Bayesian species tree constructed via tree congruence of gene trees. Branches are labeled with support indices Bayesian posterior probability (BPP; as a percentage) and maximum likelihood bootstrap percentage (MLB) in the following order: species tree of all loci BPP first,

then the BPP species tree of all specimens, followed by the BPP/MLB from the Bayesian and maximum likelihood partitioned species tree. Symbols for support values are: * ≥ 95 , 95 > + ≥ 90 , 90 > ^ ≥ 80 , 80 > ° ≥ 70 , all other values < 70 are marked with a hyphen; a ‡ indicates support not applicable due to missing specimens in that tree. Dotted lines connect the branch and its support values if the latter could not fit in the space above the branch. Grey branches appeared in Bayesian trees but not in the Maximum Likelihood tree. Vertical bars at the tips indicate environment type: open bars are ocean locations, filled bars are lakes.

Fig. 5. Haplotype network of concatenated COI and COIII sequences for locations in Palau. Each branch corresponds to one nucleotide difference; unsampled haplotypes are indicated by a black dot. Circle area is proportional to the number of individuals with that haplotype, as shown by the scale in the center. Sites are identified by the last three letters of site codes provided in Table 1; shading and patterns indicate the locations at which individuals were sampled. Ocean locations are denoted by striped patterns on a white background, lakes by solid shading and two well-connected lakes by dotted patterns.

Fig. 6. Morphological variation of *Mastigias* across the IWP. **(a)** nMDS analysis of the IWP2 data matrix. Symbols used in the plot are shown with site acronyms in the Bayesian species tree in (c); full site details are given in Table 1. Specimens from the Pacific Islands & South Philippine Sea (PISPS) clade are in black, China Sea in dark grey and Solomon Sea in light grey. Environment type and clades for each symbol are given below the nMDS plot, with ocean locations designated by open, holomictic lakes by crossed and meromictic lakes by closed symbols. Within the PISPS clade, further distinction is made for specimens from Raja Ampat (RA) and Palau (PW). **(b)** Linearized overall phenotypic similarity. Boxes enclose the inner quartile range of values with the heavy line denoting the median. Dashed lines extend to the minimum and maximum values, excepting outliers indicated by open circles. Open boxes are from ocean locations; shaded boxes from lakes. Lakes are ordered from left to right by increasing depth, which is a proxy for duration of isolation (per supplemental material S4). **(c)** Bayesian species tree with posterior probability as a percentage for each node given as * ≥ 95 , 95 > + ≥ 90 , 90 > ^ ≥ 80 , and 80 > ° ≥ 70 ; branches with less than 70% posterior probability in the species tree were collapsed. Only enough tips from each location to preserve the topology are shown. The double slash at the terminus of the ingroup indicates the distance has been truncated; the full distance of the branch is given below. Shades indicate the mean distance on the nMDS from (minX, minY), and should be interpreted only in that light colors are similarly close to (minX, minY); whereas dark values are far from (minX, minY). Shades at the tip are the states assigned to the tree; other shading of internal branches indicates maximum parsimony ancestral state character reconstruction. Vertical bars adjacent to leaves indicate the environment type; open bars are ocean locations, shaded bars are lakes. Phylogenetically independent contrasts show overall phenotypic similarity is correlated with environment ($r = 0.7956$, $p < 0.0001$) after adjusting the degrees of freedom for soft polytomies.

986 **Table 1.** The region, location, sites, and sample sizes for analyses using photographs, whole specimens, and tissues for phylogenetic
987 analyses. Italicized portion of acronyms are used as site identifiers for analyses of only Palau specimens. For COI and COIII, total
988 numbers are given for the phylogenetic analyses before the slash and after the slash for phylogeographic analyses. Habitat type
989 denotes either an ocean location or a lake; lakes are further differentiated into broad categories of either holomictic (mixed),
990 ‘stratified’ (stratified with gradually decreasing hypoxia, but not meromictic) or meromictic (stratified with anoxic conditions below a
991 chemocline). * Hang Du I, Vietnam has a variable rainwater surface layer associated with monsoonal rains, but is otherwise is iso-oxic
992 with depth and thus classified as holomictic (Cerrano et al., 2006). † 30 specimens from Dawson (2005) were combined with 16 new
993 specimens.
994

Species	Region	Locality	Site	Abbreviation	Habitat	Lake Type	Morphology		Loci				
							Specimen	Photograph	COI	COIII	16S	H3a	H3b
<i>Mastigias</i> spp.	Papua	Tufi		IDORTUF	Ocean			1	2	2	2	2	2
			Raja Ampat	<i>Unknown</i>	Ocean			3					
			Mastigias Papua Cove	IDWPMPC	Ocean		4		5	5	5	3	5
			Gam Bay	IDWPGAM	Ocean		2		5	5	5	2	5
			DAG Lagoon Reef Flat	IDWPDGL	Ocean		1		1	1	1		1
			Danau Lake a Gam	IDWPDAG	Lake	Holomictic	1		2	2	2	1	2
			Danau Ctenophore Gam	IDWPCDG	Lake	Holomictic	1		1	1	1		1
			Rewo Bay		Ocean			1					
	Indonesia	Komodo		IDNTKOM	Ocean			2	1	1	1		1
			Haji Buang	IDBEHBM	Lake	Meromictic	15	14	2	2	2	2	2
			Tanah Bamban	IDBETBM	Lake	Holomictic	19	4	1	1	1	1	1
			Kakaban	IDBEDKK	Lake	Holomictic	12	8	2	2	2		
	Philippines	<i>Unknown</i>			Ocean			5					
	China	Hong Kong			Ocean			2					
	Micronesia	Chuuk			Ocean			1					
		Marshall Islands	Bikini Atoll		Ocean			2					
			Enewetak	MHRHEWE	Ocean			2	1	1	1	1	1
		Palau	Ngermeuangel Cove	PWKONCK	Ocean		8	2	5 / 15	5 / 15	5		5
			Ngerchaol Cove	PWKONCN	Ocean		12	9	1 / 15	1 / 15	1		1
			Risong Cove	PWKORCA	Ocean		20	1	5 / 15	5 / 15	5		5
			Ongael Lake	PWKOOLO	Lake	Holomictic	27	13	4 / 15	4 / 15	4	1	3
			Goby Lake	PWKOGLK	Lake	Meromictic	30	4	4 / 15	4 / 15	4		4
			Clear Lake	PWKOCLM	Lake	Meromictic	30	7	4 / 15	4 / 15	3		
			Ongeim'I Tketau	PWKOOTM	Lake	Meromictic	46 [†]	9	5 / 15	5 / 15	5	1	5
			Ngermeuangel Lake	PWKONLK	Lake	Meromictic	20	3	6 / 15	6 / 15	6		6
			CRRF Dock	PWKOCRF	Ocean				0 / 9	0 / 9			
			Little Risong Cove	PWKOLCU	Ocean				0 / 13	0 / 13			
			Tab Ketau Cove	PWKOTKC	Ocean				0 / 7	0 / 7			

			Mekeald Lake	PWKOMLN	Lake	Holomictic			0 / 14	0 / 14					
			Tketau Lake	PWKOTLM	Lake	Stratified	3	1	0 / 12	0 / 12					
			T-Lake	PWKOTLN	Lake	Stratified		4	0 / 14	0 / 14					
			Lagoon	PWKOLGN	Ocean		3		0 / 2	0 / 2					
	Vietnam	Hang Du I		VNHDHDL	Lake	Holomictic*		2	1	1	1	1	1	1	1
	Japan	Sukumo Bay		JPKISKM	Ocean		8	12	2	2	2	2	2	2	2
		Shirahama			Ocean			1							
		Unknown			Ocean			3							
<i>Phyllorhiza</i>	Australia	Unknown			Ocean			4	2	2	2	2	2	2	2
	Borneo	Unknown			Ocean			1							
	Mexico	Gulf of California	La Paz	MXBSBAP	Ocean		1								

Figure 1

Ocean Ecotype

Lake Ecotype

Figure 2

H₁: Ancient Divergence and Dispersal

H₂: Recent Divergence and Dispersal

H₃: Parallel Colonization & Divergence

Figure 3

Figure 4

Figure 5

Concatenated Mitochondrial

Figure 6