Molecular Phylogenetics and Evolution 186 (2023) 107827

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

SNP data reveals the complex and diverse evolutionary history of the blue-ringed octopus genus (Octopodidae: *Hapalochlaena*) in the Asia-Pacific

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ARTICLE INFO

Keywords:
Cephalopoda
Species delineation
Phylogenetics
Single nucleotide polymorphism
Systematics

ABSTRACT

The blue-ringed octopus species complex (*Hapalochlaena* spp.), known to occur from Southern Australia to Japan, currently contains four formally described species (*Hapalochlaena maculosa, Hapalochlaena fasciata*, *Hapalochlaena lunulata* and *Hapalochlaena nierstraszi*). These species are distinguished based on morphological characters (iridescent blue rings and/or lines) along with reproductive strategies. However, the observation of greater morphological diversity than previously captured by the current taxonomic framework indicates that a revision is required. To examine species boundaries within the genus we used mitochondrial (12S rRNA, 16S rRNA, cytochrome *c* oxidase subunit 1 [COI], cytochrome *c* oxidase subunit 3 [COIII] and cytochrome *b* [Cytb]) and genome-wide SNP data (DaRT seq) from specimens collected across its geographic range including variations in depth from 3 m to >100 m. This investigation indicates substantially greater species diversity present within the genus *Hapalochlaena* than is currently described. We identified 10,346 SNPs across all locations, which when analysed support a minimum of 11 distinct clades. Bayesian phylogenetic analysis of the mitochondrial COI gene on a more limited sample set dates the diversification of the genus to ~30 mya and corroborates eight of the lineages indicated by the SNP analyses. Furthermore, we demonstrate that the diagnostic lined patterning of *H. fasciata* found in North Pacific waters and NSW, Australia is polyphyletic and therefore likely the result of convergent evolution. Several "deep water" (>100 m) lineages were also identified in this study with genetic convergence likely to be driven by external selective pressures. Examination of morphological traits, currently being undertaken in a parallel morphological study, is required to describe additional species within the complex.

1. Introduction

The blue-ringed octopus genus *Hapalochlaena* was introduced by Robson (1929) and currently comprises four species that are universally accepted and have been adequately described *Hapalochlaena maculosa* (Hoyle, 1883), *Hapalochlaena lunulata* (Quoy and Gaimard, 1832), *Hapalochlaena fasciata* (Hoyle, 1886) and *Hapalochlaena nierstraszi* (Adam 1938; Norman et al., 2016). The genus *Hapalochlaena* is dispersed throughout the Asia Pacific from Temperate Australasia (TAUS) to the Temperate North Pacific (TNP) (Fig. 1) and is identified by distinct iridescent blue rings and/or lines, which advertise toxicity to would-be predators in an aposematic manner (Norman, 2000). All members of the genus thus far studied contain the potent neurotoxin tetrodotoxin (TTX) within their venom and tissues and are the only octopods known to inflict a lethal bite to humans (Flachsenberger and Kerr, 1985; Jacups and Currie, 2008).

The genus *Hapalochlaena* was introduced by Robson in 1929. *Hapalochlaena lunulata*, the greater blue-ringed octopus, previously named *Octopus lunulatus* by Quoy and Gaimard (1832), based on a specimen from Papua New Guinea, was designated the type species. Robson (1929) also included in this genus, *Octopus maculosus* Hoyle 1883 (now known as *H. maculosa*) from "Australia" and its synonym *Octopus pictus*

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https://doi.org/10.1016/j.ympev.2023.107827
Received 20 September 2022; Received in revised form 23 May 2023; Accepted 25 May 2023
Available online 29 May 2023

var. *fasciata* Hoyle, 1886 (now known as *H. fasciata*) from Port Jackson, New South Wales (NSW), Australia. Key diagnostic features used to distinguish these species include morphological characters (size, shape, and placement of rings and/or lines and papillae) along with behavioural and life history traits. *Hapalochlaena lunulata* is the only described member of the genus to exhibit a merobenthic lifestyle producing relatively small eggs (3.5 mm in diameter) with paralarvae able to disperse using water currents (Overath and von Boletzky, 1974). Of the four species it has the largest described range (Central Indo-Pacific). In contrast, both *H. maculosa* and *H. fasciata* are holobenthic, producing relatively larger eggs (7–9 mm and 6–9 mm in diameter respectively) (Norman et al., 2016), which hatch into crawl-away benthic hatchlings (Tranter and Augustine, 1973).

Norman (2000) highlighted the potential existence of five additional putative *Hapalochlaena* species based on morphological and life history traits. One of the proposed new species, *Hapalochlaena* sp. 1, had previously been confused with *H. lunulata* based on general appearance (Norman and Reid, 2000), however in contrast to *H. lunulata*, this species exhibits a holobenthic lifestyle, attains a larger size, has a more muscular form and occupies a different habitat. Additionally, the anti-tropical distribution of *H. fasciata* led to the suggestion that the disjunct 'populations' distributed in NSW, Australia and Japan may represent sister taxa and was therefore noted as requiring further evaluation (Norman, 2000; Norman and Hochberg, 2005). As part of an ongoing morphological study, Finn (2015) reported a total of 12 blue-ringed octopus species occurring in Australia (Finn, 2015). While Finn and Lu (2015) reported that the two species from the Indo-West Pacific, historically treated under the Australian names *H. maculosa* and *H. fasciata* were morphologically distinct from the Australian fauna (Finn and Lu, 2015).

Clarifying the systematics of this genus has been subject to many of the same challenges as other cephalopods, particularly octopods. Upon death, cephalopods exhibit a loss of many physical structures and pigmentation (Bookstein, 1985); this, in combination with few hard body parts and distortion upon preservation (Voight, 1994), can lead to difficulty distinguishing between closely related species (Roper, 1983). Museum collections contain invaluable specimens from a variety of locations across time, however the method of preservation can vary greatly and thus impact species identification and re-evaluation (Roper, 1983).

The phylogenetic status of proposed species within *Hapalochlaena* remains unresolved due to limited examination across the geographic range and/or resolution of markers assessed in previous molecular studies (Acosta-Jofré et al., 2012; Guzik et al., 2005; Lindgren and Anderson, 2018; Takumiya et al., 2005; Tanner et al., 2017). Phylogenetic analysis of partial sequences of two mitochondrial (cytochrome *c* oxidase subunit 3 [COIII] and cytochrome *b* [Cytb]) and one nuclear gene (eukaryotic translation elongation factor 1 alpha 1 [EF-1α]) of a single representative of each of *H. fasciata* (NSW), *H. maculosa* (Victoria) and *Hapalochlaena* sp. 1 (Darwin, Northern Territory NT), in conjunction with 23 other octopod species and four octopod genera supported the monophyletic status of *Hapalochlaena* and found it to be distinct from the genus *Octopus* Cuvier 1797 (Guzik et al., 2005). Whitelaw et al. (2020) presented the genome of *H. maculosa* and estimated that the divergence of this species from *Octopus bimaculoides* occurred around 59 mya. Additional studies analysing partial COI and COIII gene sequences from 36 coleoid cephalopods, including representatives from the *Hapalochlaena* genus identified as *H. maculosa, H. fasciata* and *H. lunulata* in Japan and adjacent waters supported the monophyletic status of the genus (Kaneko et al., 2011).

A large-scale population genetic study using genome-wide SNP markers has been conducted on *H. maculosa* which discovered a clinal species pattern across its geographical range from southern Western Australia to Victoria/ Tasmania (Morse et al., 2018). While genetic

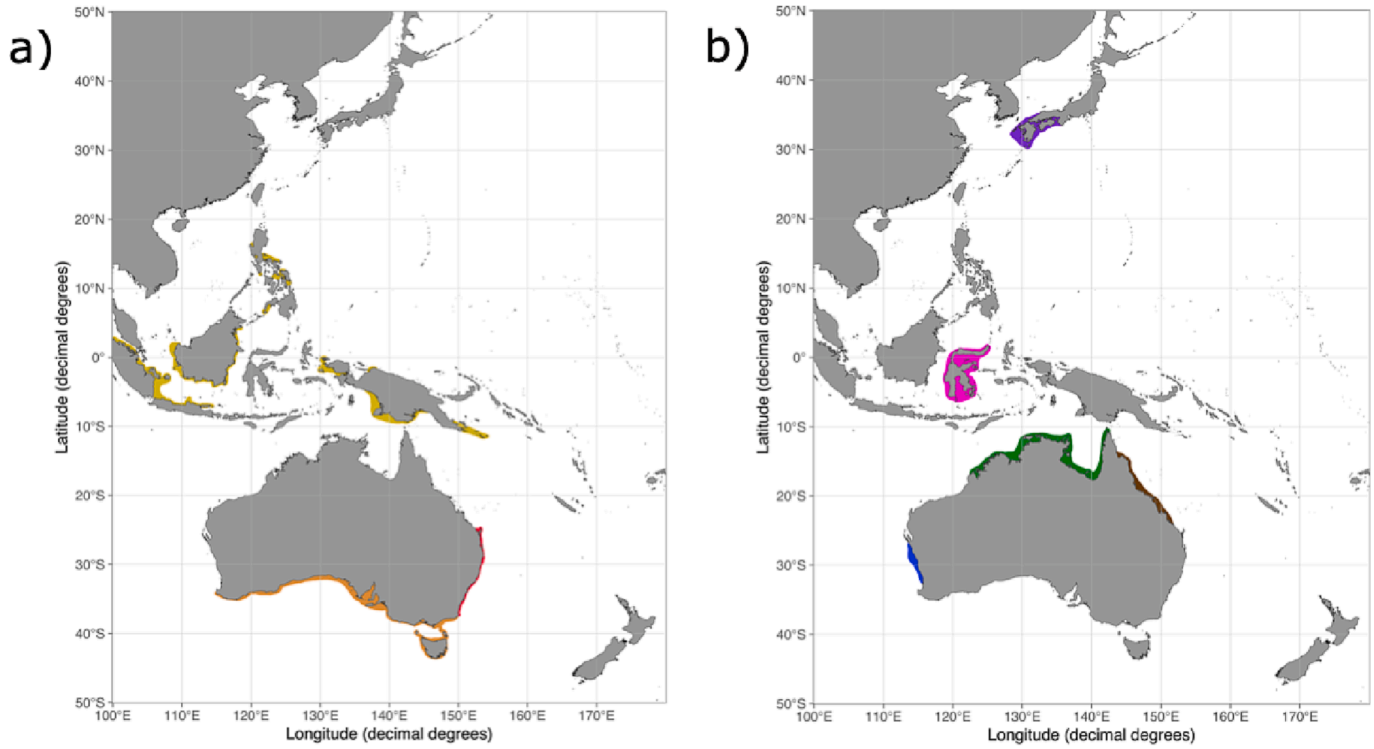

Fig. 1. Published distributions of *Hapalochlaena* species. Distributions of *Hapalochlaena* spp. in the Indo Pacific. **(a)** FAO. Cephalopods of the World. Volume 3. Octopods and Vampire Squids (Norman et al., 2016). *Hapalochlaena* are shown as follows, *H. maculosa* (orange), *H. fasciata* (red) and *H. lunulata* (mustard). **(b)** Cephalopods, a World Guide (Norman, 2000). Additional reported localities, Northern Australia East to Cape York (*Hapalochlaena* sp. 1 [green]), Southern Japan (*Hapalochlaena* sp. 2 [purple]), Subtropical Western Australia (*Hapalochlaena* sp. 3 [blue]), Sulawesi Indonesia (*Hapalochlaena* sp. 4 [pink]) and the Great Barrier Reef Australia (*Hapalochlaena* sp. 5 [brown]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

distances between populations were insufficient to warrant species status, phylogenetic reconstructions including the sister taxon, *H. fasciata* demonstrated genetic distances between distal populations of *H. maculosa* were equivalent to distances between *H. maculosa* and *H. fasciata*. However, no systematic revisions were suggested or as yet implemented as alleles were observed to be shared throughout the species range through occasional migrations among adjacent populations.

Two fatalities from *Hapalochlaena* bites have been reported in Australia, however identification of species involved in these cases lacked clarity due to the lack of taxonomic resolution at the time and the fact that neither victim retained the octopus (Jacups and Currie, 2008; Lane and Sutherland, 1967; White, 2018). Due to the potential risk to human health it is crucial to have clear systematics, which allow for confident identification of individuals involved in both medically significant cases and in academic studies. Misidentification has become a common problem in many sequence databases due to the current state of *Hapalochlaena* systematics. Currently, the observed morphological diversity across their range from southern Australia to Japan is not reflected in their taxonomy (Finn, 2015; Finn and Lu, 2015; Norman, 2000; Norman and Hochberg, 2005) and often leads to misidentification in both academia and popular media. Further confusion is introduced through collection of specimens from markets where the collection location is unknown. *H. maculosa* is particularly prone to this as some specimens in the central and north Pacific have been observed to show a greater level of similarity to *H. maculosa* from southern Australia as opposed to *H. lunulata*. This has led to specimens from these regions being listed in public databases as *H. maculosa* including a specimen from Wuchi market Taiwan (Kaneko et al., 2011) and China (Dai et al., 2012).

The relationship between the geographically distant *H. maculosa* and *H. fasciata* populations in Temperate Australasia (TAUS) and their counterparts in the Temperate North Pacific (TNP) have yet to be thoroughly investigated. To date there has been no comprehensive phylogenetic study of the genus *Hapalochlaena*. Here we utilise genome-wide single nucleotide polymorphisms (SNP) in conjunction with mitochondrial genes to examine the genetic structure, diversification, and putative species boundaries of the *Hapalochlaena* genus. This work elucidates the radiation and evolution of the genus is elucidated with the aim to provide a genetic context for ongoing and future species delineation and descriptions.

2. Methods

2.1. Sample collection

To assess species boundaries within *Hapalochlaena,* samples were sourced throughout their range. As part of an ongoing morphological examination of the group, Julian Finn collected, identified and sampled tissue from representative morphotypes across the range. All individuals used within this study were determined as belonging to the genus *Hapalochlaena* due to the presence of blue rings or lines on the skin, which are indicative of the genus. Morphological characteristics were used to identify individual belonging to the currently described species *H. maculosa*, *H. fasciata* and *H. lunulata* following their species descriptions in Norman et al., (2016). Blue-ringed octopuses were collected by SCUBA diving and intertidal reef-walking during the day and night throughout Australia and Taiwan (MV AEC 12001/1). During collection gloves were worn and no direct contact was made with any blue-ringed octopus, mitigating risk of envenomation. All specimens were collected intertidally using hand nets or subtidally using containers with mesh nets. Tissue samples were lodged in, and obtained from, the collections of Museums Victoria (NMV, n = 35), Western Australian Museum (WAM, n = 7), Museum and Art Gallery of the Northern Territory (MAGNT, n = 2), Queensland Museum (QM, n = 11) and Australian Museum (AM, n = 7). Additional samples collected by Peter Morse from Albany WA were also included (Morse et al., 2018). Storage methods of samples differed across museums and included preservation in 90% ethanol and frozen and maintained at −80 °C. (Supplementary table 1).

2.2. Mitochondrial DNA extraction and amplification

DNA was extracted from muscle tissue for all samples (n = 62) using the "High salt method" (Donnan Laboratories 2001). PCR amplification was used to target the following partial mitochondrial genes based on published primers; 12S ribosomal RNA (12S) (Simon et al., 1991), 16S ribosomal RNA (16S) (Simon et al., 1991), cytochrome *c* oxidase subunit 1 (COI) (Folmer et al., 1994), cytochrome *c* oxidase subunit 3 (COIII) (Guzik et al., 2005) and cytochrome *b* (Cytb) (Guzik et al., 2005). Reactions were composed of 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.1 μL Taq (*Onetaq, New England Biolabs*), 2.5 μL 10 × buffer (Paq5000™), 2 μL dNTP mix (10 μM, *Bioline*), 17.4 μL ddH_2O and 2 μL DNA (diluted to between 1 and 5 ng/μL), totaling to 25 μL. Detailed reaction conditions can be found in Allcock et al. 2008 (Allcock et al., 2008). PCR products were sequenced by Macrogen Inc, Seoul, Korea. Sequences are available on Genbank under the accession XXXXX.

2.3. DNA extraction and genotyping by sequencing

Complete genomic DNA was extracted from all 62 samples using the "QIAamp DNA Micro Kit (Cat No./ID: 56304)" (Qiagen) according to the manufacturer's protocol. Concentration and quality of DNA extracted was assessed by visualisation on a 0.8% agarose gel DNA quantification and using NanoDrop 3300™. DNA extracts were transferred to Diversity Arrays Technology PL, Canberra ACT, Australia who conducted their DArTseq™ pipeline (i.e. restriction enzyme digestion, library preparation, genotype by sequencing (GBS), data generation and QC).

2.4. SNP identification

SNP data returned by Diversity Arrays were imported into R and filtered by loci call rate (>80%) (removes loci), individual call rate (>70%) (removes individuals), repeatability (the proportion of identical genotypes between technical replicates) (0.9) and minor allele frequency (1%) in this order using the dartR package (Gruber et al., 2018). Monomorphic loci resulting from the removal of individuals and/or populations were also removed using the dartR package. Filtering parameters were selected for maximum data retention while minimising errors and missingness due to the age and storage history of museum samples.

2.5. Genetic structure/divergence of SNP data

Genetic clusters were visualised using Principal Coordinates Analysis (PCoA) with the R package dartR (Gruber et al., 2018), and assessed for AIC and BIC from K = 1 to 25 using the snap.clust.choose.k function in Adegenet (Jombart, 2008). K was selected based on the lowest value from both criteria and used to examine admixture of populations in ADMIXTURE v1.3.0. STRUCTURE v2.3.4 (Rosenberg et al., 2001) was run for K values between 8 and 14 and evaluated using the Evanno method implemented in STRUCTURE HARVESTER v0.6.94 (Earl and Vonholdt, 2012). In addition, a Discriminate Analysis of Principal Components (DAPC) (Jombart and Collins, 2015) using the best K was conducted to identify clusters of samples based on genetic similarity. Genetic divergence between populations and putative taxonomic groups was assessed using Weir and Cockerham's unbiased F-statistics (Fst). Pairwise Fst values and their significance were calculated between populations and putative species in R using the Stampp v1.6.1 package (Pembleton et al., 2013).

2.6. Signatures of selection

Loci under putative selection were identified using three methods (PCAdapt v4.3.3 (Luu et al., 2017), OutFLANK v0.2 (Whitlock and Lotterhos, 2015), and Bayescan v2.1 (Foll and Gaggiotti, 2008) to reduce methodological bias. The R package, PCAdapt, (Privé et al., 2020) is able to detect population structure using an initial principal component analysis (PCA) followed by identification of specific markers highly correlated with populations. The analysis was conducted on the full set of 10,346 SNPs. To reduce the impact of linkage disequilibrium (LD) on the detection of population structure a further thinning step was conducted with a window size of 200 and an r2 of 0.1. A scree plot was used to choose the optimal number of principal components (K = 6) and SNPs were considered to be outliers (under selection) if they had a qvalue < 0.01 (estimated false discovery rate of 1%). Bayescan and Outflank required predefined populations and were conducted in R on all populations grouped by location (Supplementary table 1). A further analysis using each method was conducted on populations grouped by location, where appropriate, to minimize the impact of low population sizes (Supplementary table 1). The results were then contrasted to determine which method and data set was able to identify SNPs under selection.

2.7. Putative species boundary estimation using mitochondrial genes

Genetic structuring of locations was examined using five mitochondrial genes (12S, n = 17; 16S, n = 16; COI, n = 36; COIII, n = 18 and Cytb, n = 13) (Supplementary Fig. 4). Samples were obtained throughout the Asia Pacific (this study; see Supplementary table 2). Additional sequences were obtained from NCBI (Supplementary table 2). Alignments for each gene were generated using MAFFT v7.45 (Rozewicki et al., 2019) and a median joining network calculated in POPART v1.0 (Population Analysis with Reticulate Trees) (Leigh and Bryant, 2015).

Species boundaries were estimated using the PTP (Poisson Tree Processes) (Kapli et al., 2017) method on all genes as single-rate, multi-rate and Bayesian. Additionally, sGYMC (single-threshold general mixed yule coalescent) and ABGD (Automated Barcode Gap Discovery) were also run on all genes. All PTP analyses accepted a RAxML tree in Newick format generated in Geneious v10.2.6 from a MAFFT alignment. Both single-rate and multi-rate PTP were calculated using the online server (https://mptp.h-its.org) with a p value of 0.001 specified for single-rate PTP. Specifications for the bPTP run were as follows, a total of 500,000 Markov chain Monte Carlo (MCMC) iterations were run with the first 10% discarded as burn-in and thinned to retain 1 in 100. sGYMC (Pons et al., 2006) was run using the splits v1.0–20 package in R with a Bayesian inference tree generated by BEAST2 using a strict clock model from the same alignment used in Geneious v10.2.6 to generate the input RAXML trees used in previous analyses, with the exception of the model specifications were identical to bPTP.

2.8. Divergence time estimation

Divergence times were estimated using a concatenated alignment of four mitochondrial genes (12S, 16S, COI and COIII) (n = 7) in BEAST2 (Bouckaert et al., 2014) (Cytb was not included as too few sequences were available). The alignment was generated using MAFFT v 1.4 in Geneious v10.2.6 on a subset of *Hapalochlaena* taxa. BEAST2 was run using a calibrated Yule model, relaxed clock log exponential for 50 million generations, burnin of 10% and sampled every 1000 iterations. Additional taxa were included as outgroups (*Argonauta nodosus* [NCBI: MK034303], *Vampyroteuthis infernalis* [NCBI: NC_009689], *Tremoctopus violaceus* [NCBI: KY649286], *Opisthoteuthis massyae* [NCBI: ON367807], *Muusoctopus longibrachus longibrachus* [NCBI: HM572169] and *Muusoctopus longibrachus akambei* [NCBI: HM572177]. Estimations of divergence times were generated using three fossil and one biogeographical calibrations as follows:

1. Cirrata × Incirrata: An exponential prior for the MCRA of the suborders Cirrata (represented by *Opisthoteuthis massyae* [NCBI: ON367807]) and Incirrata (represented by the fossil Styletoctopus annae 95 mya-180 mya)(Fuchs et al., 2009). Prior was set with a minimum age of 95 mya lower with an upper bound of 180 mya.
2. *Vampyroteuthis* × Octopoda: An exponential prior for the divergence of *Vampyroteuthis infernalis* [NCBI: NC_009689] and Octopoda. The lower bound was based on the fossil *Vampyroteuthis rhodanica* dated to a minimum of 162 mya(Fischer and Riou, 2002). Upper bound was based on previous publications (Kröger et al., 2011; Strugnell et al., 2006), which place the divergence of Vampyroteuthis prior to the Permian (250 mya).
3. *Argonauta nodosus* × *Tremoctopus violaceus:* An exponential prior for the divergence of *Argonauta nodosus* × *Tremoctopus violaceus* was based on the fossil *Obinautilus pulcher*. Dated to 29 mya this specimen represents the earliest record of the Argonautidae. The upper bound was set based on ammonite fossils which have not been found to be olde than 64 mya(Kobayashi, 1954). This is an appropriate boundary as no *Argonauta* fossils have been found which predate ammonite records(Strugnell et al., 2008).
4. *Muusoctopus longibrachus longibrachus* × *Muusoctopus longibrachus akambei*: A normal prior was set for divergence of the two *Muusoctopus* subspecies based on a biogeographic boundry created during the Last Glacial Maximum (LGM)(Gleadall, 2013). This places the physical separation of the lineages at 25 ± 5kya.

To include a larger number of samples collected from a greater range of locations the analysis was also conducted using a *Hapalochlaena* dataset composed of only the COI gene (n = 30), for which more data was available. Sequences were also included from the NCBI database resulting in 25 additional sequences (Supplementary table 3).

The coalescent based approach, SVDQuartets (Chifman and Kubatko, 2014) was used to generate species trees based on 10,346 SNP loci and 62 individuals. Populations were renamed using their putative species groups for analysis and input alignments for each method generated using the functions gl2snapp or gl2svdquartets from the dartR package (Gruber et al., 2018). SVDQuartets was run using PAUP v 4.0a (Swofford, 2002) using the following parameters: 100,000 tips assigned to species, randomly sampled quartets, 10,000 bootstraps, multispecies coalescent tree model.

3. Results

3.1. Species boundaries OTU identification

After removal of 1644 SNPs identified as outliers by PCAdapt the remaining 8,702 neutral loci were used to examine population structure. Analysis of SNP data identified supported the existence of 11 genetically distinct clusters. Delimitation analysis of mitochondrial data supported 8 of the 11 clusters. It should be noted that the mitochondrial data set did not contain representatives for three clusters (OTUs C, D and F) identified in the SNP dataset. For the purpose of this study we refer to these as operational taxonomic units (OTU) and have labelled them alphabetically from A-K (Fig. 2).

3.1.1. Temperate Australasia (TAUS): OTU A (H. maculosa)

Individuals found across southern Australia from VIC to southern Western Australia formed a single OTU (A). Fst values within this OTU increase relative to distance, with populations at the extremities of the range showing the greatest genetic divergence (Supplementary table 3). PCoA and DAPC analyses showed no clear structure between populations across southern Australia, likewise STRUCTURE analyses could not distinguish between populations (Fig. 2). High bootstrap support for the clade (100%) was observed in the SVDQuartets phylogeny.

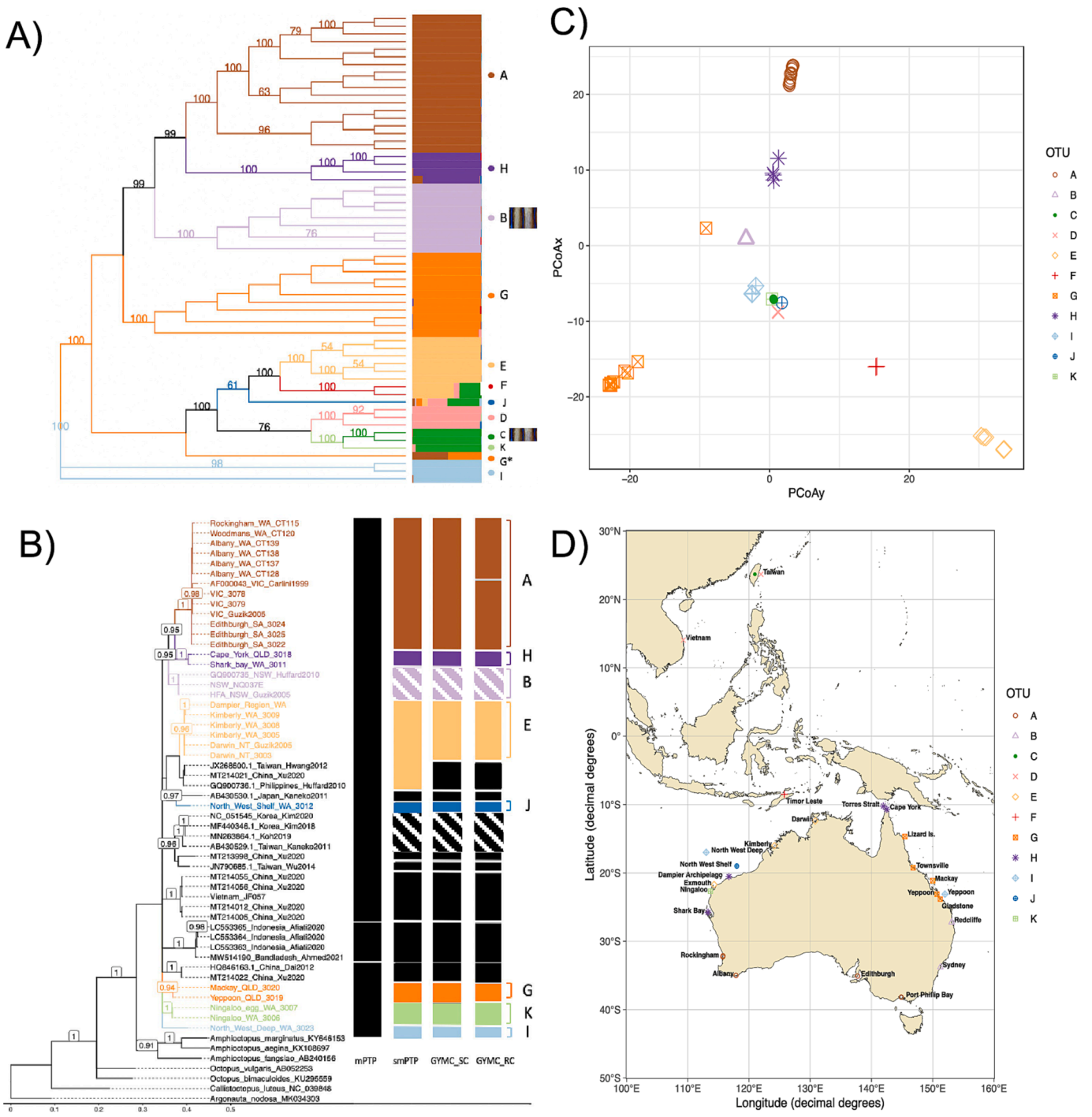

Fig. 2. Delineation of *Hapalochlanea* species boundaries and genetic structure throughout the Indo Pacific using 10,346 SNPs: (a) SVDQuartet phylogeny of *Hapalochlaena* throughout the Indo Pacific generated using 10,346 SNPs, coloured branches represent putative taxonomic units A-K: brown (A/Southern coast of Australia), lilac (B/NSW), apple green (C/Taiwan lined), pink (D/Taiwan ringed), light orange (E/Darwin, NT & Kimberly & Exmouth, WA), red (F/Timor Leste), dark orange (G/G* Great Barrier Reef, QLD), purple (H/Cape York, QLD & Shark Bay, QLD) light blue (I/Deep water Yeppoon, QLD & North West, WA), dark blue (J/North West Shelf, WA) and light green (K/Ningaloo, WA). Posterior support values > 0.90 present on nodes. Bars at terminal branches indicate admixture of OTUs inferred using STRUCTURE, colours approximately correspond to OTUs. Lined box adjacent to OTU indicated lined markings while, OTUs without a box exhibit ringed markings. (b) Species delineation using the mitochondrial COI gene. Bayesian phylogeny (MrBayes) of *Hapalochlaena* throughout the Indo Pacific is coloured according to OTU with black used to represent taxa included from NCBI. Boxes represent putative species in accordance to sPTP, GYMC SC (strict clock) and GYMC RC (relaxed clock) methods. Boxes with diagonal lines represent specimens with lined markings as opposed to rings present in all other specimens. (c) Arrangement of samples according to the first two principal components of a PCoA based on SNP data generated using the dartR package. (d) Map of sample locations coloured by organisational taxonomic units A-K. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Delimitation analysis (ABGD, sPTP and GYMC) for the partial mitochondrial gene COI classified populations across southern Australia (currently classified as *H. maculosa*) as a single unit (OTU A) across all methods with the exception of a relaxed clock GYMC, which identified three distinct genetic groups (VIC, SA and WA). Short branch lengths separating Western and Eastern localities within this OTU and the relationship between Fst and distance suggest that distinctions identified by GYMC may reflect a continuous genetic gradient (isolation by distance) rather than species boundaries.

3.1.2. Lined Blue-ringed octopus: OTU B (H. fasciata), C & D

Hapalochlaena with lined markings from NSW (B) and Taiwan (C) were found to form separate and distinct OTU units. The NSW OTU (B) show the least similarity to other populations with Fst values > 0.78, while individuals with lined markings collected from Taiwan (C) displayed the greatest similarity to Mackay, QLD (0.68). Ringed individuals also sourced from Taiwan (D) did not display high similarity to OTU C (0.88) (Supplementary table 3). Taiwan OTUs C and D are loosely clustered with OTUs I, J, and K in PCoA and DAPC analyses while NSW OTU B formed a tight distinct cluster. STRUCTURE analysis showed little to no admixture in OTU B, similarly OTUs C and D also showed little admixture with each being prescribed its own unit. The SVDQuartet phylogeny displayed high bootstrap support for all OTUs (>90%) (Fig. 2a). Delineation using mitochondrial genes (12S, 16S and COI) supported the OTU status of NSW (B) across methods: ABGD, sPTP and GYMC (strict and relaxed clocks). Comparison of COI between the NSW OTU (B) and NCBI sequences obtained from Japan and Korea identified as *H. fasciata* based on morphological characteristics does not support a sister taxa status (Fig. 2b) suggesting the current description of *H. fasciata* for both northern and southern hemisphere populations may require re-evaluation.

3.1.3. Northwestern Australia and Timor Leste: OTU E & F

Individuals collected from Darwin, NT, Kimberly, WA and Exmouth, WA formed a single OTU (E) spanning across northern Australia. Samples from these locations formed a single defined cluster in both PCoA and DAPC analyses congruent with lower Fst values between populations (<0.12) (Fig. 2c, Supplementary table 3). STRUCTURE analysis indicates OTU E forms a distinct group with traces of admixture to nearby locations (Fig. 2a). Admixture observed in Timor Leste samples (OTU F) suggests that this location may receive gene flow from both northern Australia and the Indo Pacific. This is also reflected in the placement of OTU F within the PCoA plot between OTU E and the J, K, C and D cluster. Furthermore, high bootstrap support for the clade/OTU was observed in the SVDQuartet phylogeny (100%). Mitochondrial sequences across all four genes (12S, 16S, COI and COIII) were examined using delimitation methods ABGD, single rate-PTP and GYMC which all supported the Darwin and Kimberley OTU (E), however the inclusion of a sample from the Dampier archipelago to this group contradicts the SNP analyses. Since SNP and mitochondrial data collected from Dampier Archipelago WA were obtained from different individuals collected at the same location this apparent contradiction could be indictive of sympatry of OTUs H and E.

3.1.4. Great Barrier Reef: OTU G

Individual samples from along the Great Barrier Reef (GBR), QLD; Gladstone, Yeppoon, Townsville, Lizard Island and Mackay comprised a single OTU (G) with the exception of "deep water" Yeppoon samples (OTU I) (Fig. 2). Relatively low Fst values were observed between populations along the GBR (<0.12) with clustering observed in both PCoA and DAPC analyses (Fig. 2c, Supplementary table 3). Overall admixture across the GBR OTU (G) is low with no clear distinction between populations observed, however one individual collected from Mackay does show admixture with OTU A (Southern Australia/*H. maculosa*) (G*) (Fig. 2a). Additionally, placement of this sample within the SVDQuartets phylogeny suggests a distinction from other GBR samples and will warrant further investigation. COI could be successfully sequenced from two individuals collected from the GBR OTU had their COI genes successfully sequenced. Examination of COI genes using the delimitation methods ABGD, sPTP and GYMC unanimously supported the assignment of Yeppoon and Mackay individuals within the same OTU (G).

3.1.5. North QLD and Western Australia: OTU H

Geographically distant populations (>2000 km) from QLD (Torres Strait and Cape York) and central WA (Shark Bay and Dampier Archipelago) were found to form an OTU (H). Due to low sample numbers the Torres Strait + Cape York individuals and the Shark Bay + Dampier Archipelago were examined as two populations. Fst values were relatively low (0.15) congruent with the clustering of these populations observed in PCoA and DPAC analyses (Fig. 2c, Supplementary table 3). Admixture was low within the OTU, the only exception being an individual from Shark Bay, which displayed some admixture with OTU A (southern Australia/*H. maculosa*). SVDQuartets phylogeny exhibited strong support for a single clade comprising OTU J (100% bootstraps) (Fig. 2a). Partial mitochondrial genes were sequenced for the Cape York (12S, COI, COIII and cytb) and Shark Bay (COI, COIII) samples. Delimitation analyses (ABGD, sPTP and GYMC) for COI were congruent with placement of both samples within the same OTU. However, the COIII gene showed greater genetic differences between individuals and each sample was placed in its own unit (Fig. 2b).

3.1.6. "Deep water": OTU I

"Deep water" specimens (>100 m) collected from Yeppoon, QLD and North West (Deep) WA, formed a single OTU (I) despite their large geographic separation (i.e. over ~ 2400 km). Specimens formed a distinct cluster in both PCoA and DAPC analyses (Fig. 2c) with STRUCTURE showing little admixture (Fig. 2a). No mitochondrial genes could be sequenced for "deep water" Yeppoon samples, however partial five mitochondrial genes (12S, 16S, COI and COIII and Cytb) for North West (Deep) WA were analysed. Delimitation methods (ABGD, single rate-PTP and GYMC) placed North West (Deep) WA as a unique unit distinct from all other specimens (Fig. 2b).

3.1.7. Singletons WA: OTU J & K

Singletons collected from North West Shelf, WA and Ningaloo, WA each formed their own distinct OTUs termed J and K respectively. OTU J, represented by a single sample (North West Shelf, WA), exhibits admixture with all adjacent populations (Fig. 2a). Likewise, PCoA and DAPC analyses place the sample in close proximity to OTUs I, K (Ningaloo), C (Taiwan-lined) and D (Taiwan-ringed) (Fig. 2c, Supplementary Fig. 1). Low bootstrap support from the SVDQuartet phylogeny was observed for both singleton OTUs (J & K) (Fig. 2a). Species limits estimated using fragments of five mitochondrial genes (12S, 16S, COI and COIII and Ctyb), ABGD, single rate-PTP and GYMC analyses unanimously supported the classification of both singletons as unique OTU groups (Fig. 2b).

3.2. Divergence time estimation/Ancestral state reconstruction (RASP)

Divergence of the *Hapalochlaena* genus is estimated to have occurred in the early Oligocene- early Pliocene between 4.3 and 33.4 mya based on four concatenated mitochondrial genes (Supplementary Fig. 2). A separate genealogy of the COI gene, which was able to include a greater coverage of *Hapalochlaena* populations concurred with the topology of shared samples, however divergence time estimates may suggest an older origin with the most recent common ancestor (MRCA) predicted to have emerged between 49.5 and 126.3 mya (Fig. 3a).

Radiation of the genus based on the COI gene is estimated to have occurred in the Central Indo-Pacific (CIP). However, this result is approximate and additional samples are required to identify the location and time with greater confidence and specificity. RASP analysis using

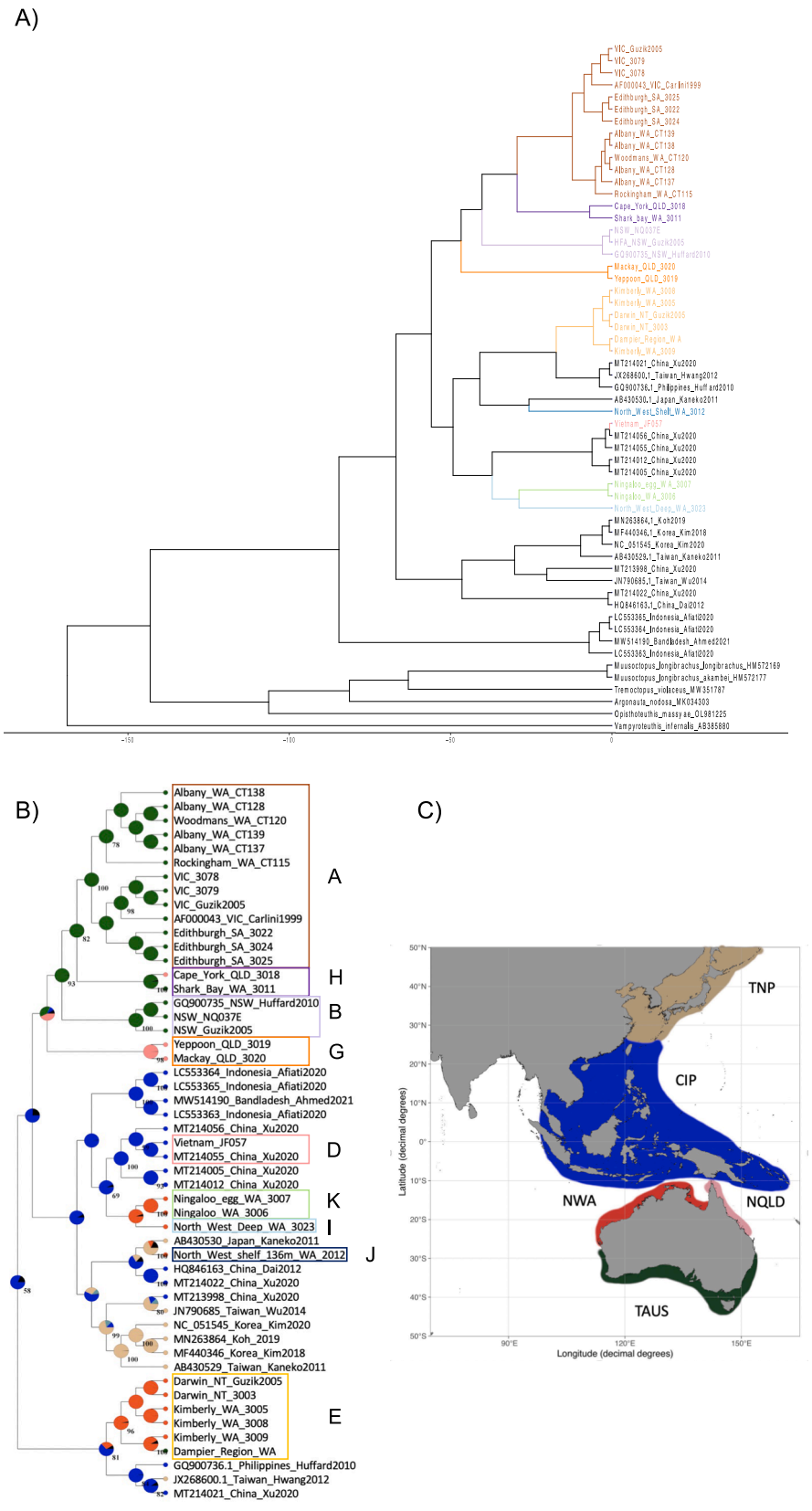

Fig. 3. Divergence and radiation of *Hapalochlaena* throughout the Asia Pacific: a) Divergence time estimates of *Hapalochlaena* throughout the Indo Pacific generated using Bayesian methods, coloured branches represent putative taxonomic units A-K: brown (A/Southern coast of Australia), lilac (B/NSW), apple green (C/Taiwan lined), pink (D/Taiwan ringed), light orange (E/Darwin, NT & Kimberly & Exmouth, WA), red (F/Timor Leste), dark orange (G&G*/ Great Barrier Reef, QLD), purple (H/Cape York, QLD & Shark Bay, QLD) light blue (I/Deep water Yeppoon, QLD & North West, WA), dark blue (J/ North West Shelf, WA) and light green (K/Ningaloo, WA). Bars (blue) represent standard error within a 95% confidence interval; b) Reconstructed Ancestral State Phylogeny constructed using RASP BBM. Most likely points of origin inferred for each node in the tree are shown as pie charts coloured according to major geographic regions using the colour scheme shown in c; c) Map of oceanic zones: temperate Australasia (TAUS) = Green, North East Australia (N_QLD) = Pink, North West Australia (NW_AUS) = Red, Central Indo Pacific (CIP) = Blue and temperate north Pacific (TNP) = beige. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the full set of sequences present in the phylogeny indicates colonisation of northern Australia may have occurred twice with OTU E and OTUs J and K representing separate events (Fig. 3b). A RASP analysis conducted using a single representative from each OTU in conjunction with unique locations display a largely congruent topology (Supplementary Fig. 6).

4. Discussion

Using SNP and mitochondrial data from specimens across the Asia Pacific we show that the genus *Hapalochlaena* is highly diverse, encompassing between 8 and 11 genetically distinct lineages supported by a combination of Bayesian, maximum likelihood and delimitation analyses. Support was present for the current species assignments of *H. maculosa* across southern Australia and also *H. fasciata* located in NSW, Australia with each forming a single OTU. A greater diversity of OTUs than encapsulated in the current systematics were identified throughout the Central Info Pacific (CIP) and Temperate North Pacific (TNP). Divergence time estimates suggest major lineages within *Hapalochlaena* diversified between ~ 25–50 mya. These results indicate current systematics are not sufficient to describe the diversity of the genus *Hapalochlaena* and a revision is currently being conducted in a parallel study.

4.1. Species boundaries and OTUs

Greater morphological variation within the *Hapalochlaena* genus, which extends beyond the currently accepted species, has been recognised in several publications documenting differences in size, the nature of patterning of iridescent blue rings and/or lines, variability in the functionality of their ink sac and reproductive strategy (holobenthic/merobenthic) (Finn, 2015; Finn and Lu, 2015; Norman et al., 2016). Norman (2000) proposed a putative species for specimens found between Darwin (NT) and Cape York (QLD), termed *Hapalochlaena* sp. 1. This is corroborated by our analyses which showed a distinction between specimens collected from Darwin, Kimberly and Exmouth (OTU E) compared with Timor Leste (OTU F) specimens sequenced in this study and believed to be *H. lunulata* (Quoy and Gaimard 1832), based on collection locality and external morphology (J. Finn pers. comm.). Low genetic similarity between OTU E and OTU H (Cape York, Torres Strait) suggests that the described range for *Hapalochlaena* sp. 1 is likely to contain two sympatric lineages. Norman (2000) also proposed a putative species for the Great Barrier Reef (GBR) blue-ringed octopus (*Hapalochlaena* sp. 5), which exhibits a functional ink sac and a holobenthic lifestyle distinguishing it from currently accepted species (Norman, 2000). Evidence was found for the presence of two distinct lineages (OTUs G and I) along the GBR collected between Lizard Island and Mackay, "shallow water" (0–50 m) and "deep water" (>100 m). As morphological features were not examined in this study; we cannot confirm the relationship to the previously reported putative species. *Hapalochlaena* collected from depths > 100 m prior have not been previously described. "Deep water" Yeppoon specimens were genetically distinct from adjacent shallow water populations while displaying high similarity to a distant singleton collected from northwest Australia (OTU J) and Yeppoon samples collected from a similar depth. The impact of depth on reproductive isolation in this genus has not been investigated and due to the difficulty in identifying and obtaining specimens from depths > 100 m, this also means that the range, distribution and effective population sizes of lineages from these depths have not been established.

Of the samples included in this study two were unique specimens with no replicates suitable for genetic analyses. Rarity in systematics is common with 13% of invertebrates identified from single specimens in American Museum of Natural History publications (Lim et al., 2012). Singletons within studies can result from difficulty in obtaining samples and/or preservation methods used (Roper, 1983). Preservation methods can greatly impact both morphological and genetic studies, this is a particular issue when examining comprehensive historic repositories for species delimitation (Appleyard et al., 2021). Formalin is a common preservative used most prominently on older samples for preservation of morphological traits (Jeon et al., 2011; Roper, 1983). However, formalin preservation inhibits genetic analyses through three main mechanisms i) facilitating binding of DNA to itself or proteins, ii) fragmentation and iii) base modification (Quach et al., 2004; Wiegand et al., 1996). With the advent of next generation sequencing in conjunction with the growing concern around the safety of formalin use an increasing number of specimens are being preserved using DNA friendly methods (ethanol/freezing) (Spencer et al., 2013). Conversely, ethanol preservation and freezing frequently results in the loss of some morphological features, particularly in octopus, which exhibit few hard body parts/diagnostic features (Lefkaditou and Bekas, 2004).

Low numbers of *Hapalochlaena* specimens preserved appropriately for molecular work has strong implications for delimitation using genetic information as common methods such as AGBD, PTP and GYMC examine differences between intra and interspecific variation to establish species boundaries (Lim et al., 2012). In particular, ABGD generates pairwise comparisons and examines their distribution to determine the "gap" between inter and intra specific divergences (Puillandre et al., 2012). In contrast GYMC examines lineages through time and identifies the increased rate of divergence indictive of allele coalescence within a single species, assuming that a speciation event occurs at a much slower rate. The speciation event can then be inferred as occurring prior to the rapid radiation (Fonseca et al., 2021). Similar to GYMC, PTP models substitutions on a phylogenetic tree, however, it does not require time calibration (Zhang et al., 2013). These methodologies can be sensitive to sample sizes and prone to bias when applied to low and non-representative sampling (Puillandre et al., 2012). As a result, the relationship of singletons (OTU J and OTU K) could not be definitively established with each placed within its own OTU. Observed taxonomic uncertainty could be due to multiple factors including being represented by a single specimen, relatively long divergence times and incomplete sampling of close relatives (Joly et al., 2014).

Identification of *Hapalochlaena* species for use in genetic studies is often conducted based upon the iridescent blue lines and/or rings patterning the mantle and arms in conjunction with location (Kim et al., 2018). This can be misleading as it does not account for convergent evolution of morphological traits nor compare patterns between different population in Australia and the NTP. Currently, *H. fasciata* is the only accepted species which exhibits lined patterning and has been documented from NSW, Australia. However, "lined" *Hapalochlaena* have also been documented in Japanese waters leading to the proposal of an anti-tropical distribution for this species (Norman and Kubodera, 2006). By definition anti-tropical distributions are characterised as one contiguous range disrupted due to a range of factors (climatic shift, fluctuating sea levels etc) resulting in contraction to the extremities of the range (Briggs, 1987). While many marine taxa do exhibit true anti-tropical distributions (*Lobelia, Mytilus* spp., *Dasyatis*) (Hilbish et al., 2000; Kokubugata et al., 2012; Le Port et al., 2013) some species, particularly those with limited distinct morphological characteristics and incomplete systematics can be mischaracterized (Grant et al., 2005). A morphological revision of the labrid *Bidianus vulpinus* contradicted the previous anti-tropical status for the species, suggesting a complex containing a minimum of four species (Norman and Kubodera, 2006). Likewise, morphological distinctions have been observed with specimens collected from Japan given the putative species assignment of *Hapalochlaena* sp. 2 (Norman, 2000). Molecular methods can aid in the evaluation of anti-tropical taxa with limited diagnostic characters (Norman and Kubodera, 2006). Analysis of SNP data in this study indicates the morphological similarity between these groups is likely the result of convergent evolution as lined specimens from NSW and from Japan did not form a monophyletic group (Fig. 2). Similarly, allozyme and mitochondrial gene analyses contradicted the previous anti-tropical distribution (bipolarity) hypothesis for the anchovy genus (*Engraulis*)

(Grant et al., 2005). Examination of molecular markers also revealed the squid *Ommastrephes bartramii* (Lesueur 1821), which was previously believed to exhibit an antitropical distribution was likely composed of four significant "species" (Fernández-Álvarez et al., 2020). Cryptic species are becoming increasing relevant in cephalopod systematics where morphological characteristics can be complicated by condition of the specimens examined as well as environmental factors (Otilio et al., 2020). Several previous phylogenetic studies of *Hapalochlaena* included a single representation of each species and did not sample "lined" octopus from the North Pacific and NSW, Australia. As a result, this study represents the first genetic comparison of these two morphologically similar groups.

Examination of *H. maculosa* specimens across southern Australia (OTU A) using SNP data was congruent with previous work by Morse et al. (2018) suggesting a clinal species complex based on greater genetic divergence between locations at the extremities of their range (Morse et al., 2018). Mitochondrial delimitation methods (ABGD, mPTP, bPTP & GYMC) indicate divergence between *H. maculosa* locations was insufficient to warrant species status, however when GYMC was run using a relaxed as opposed to a strict clock model, locations in the West (WA) and East (VIC and SA) were delimited as separate species. An inability to successfully distinguish between morphologically distinct groups using molecular methods has been observed in Sepiolidae. It has been postulated that rapid speciation followed by strong drift of morphological feature may result in genetically similar and morphologically distinct species (Fernández-Álvarez et al., 2021). An in-depth morphological examination of *H. maculosa* across southern Australia may aid in resolving contradictions in the molecular signals observed.

4.2. Dating and diversification of the genus

The *Hapalochlaena* species complex is estimated to have diverged from the lineage containing *Amphioctopus* during the early Eocene era approximately ~50 mya based on divergence time estimates using partial COI sequences. This estimate is corroborated by previous divergence time estimates between octopod genomes, which indicated *H. maculosa* diverged from *O. bimaculoides* ~59 mya (Tanner et al., 2017; Whitelaw et al., 2020). A notable radiation of the genus occurs post the Eocene-Oligocene extinction event (~33 mya) between ~50 and 25 mya, which gives rise to lineages in the Central Indo Pacific (CIP) and temperate Australasia (TAUS). This suggests the point of origin for the genus is likely to have occurred in the CIP, potentially within the Indo Australian archipelago (IAA) a known hotspot for biodiversity and speciation at this time (Yasuhara et al., 2017). Composed of broad shallow water areas with complex reef systems interconnecting a large geographical area (Briggs and Bowen, 2013), the IAA currently contains the greatest species richness for coastal cephalopods globally (Rosa et al., 2019). Patterns of low endemism within the IAA coinciding with shared taxa in TAUS and the temperate northern Pacific (TNP) indicate the IAA as a point of origin for many cephalopod species including *Hapalochlaena* in the North Western Pacific. Three main hypotheses have been suggested for the patterns of species richness in the IAA i) overlapping distributions of taxa each dispersing outward from their point of origin (centre of overlap) ii) centre of diversification/radiation (centre of origin) and iii) refugia for species during climatic change (centre of accumulation/survival) (Briggs, 1999; Hughes et al., 2002; Jokiel and Martinelli, 1992; Rosa et al., 2019; Woodland, 1983). There is evidence to suggest multiple hypotheses may be contributing to the patterns observed as opposed to a single driver (Mironov, 2006). Transition of the global biodiversity hotspot (hopping hotspot) from the Tethys/Arabian to the IAA during the late Eocene to early Oligocene (~33 mya) extinction event was observed in patterns of four reef associated fish lineages, which showed a rapid diversification correlated with reef habitat in the IAA (Cowman and Bellwood, 2013; Gaboriau et al., 2018). Furthermore, a higher proportion of reef associated taxa indicated a greater resilience to extinction event allowing for a greater than expected retention of species diversity (Cowman and Bellwood, 2011). Heterogeneity of the IAA reefs is believed to be an important factor in retaining diversity through events of climatic upheaval providing a range of refugia compared to homogeneous habitats (Pellissier et al., 2014). Additionally, heterogeneous and complex benthic habitats may aid in facilitating sympatric and/or allopatric speciation of benthic octopods and nektobenthic cuttlefishes and sepiolids when compared to pelagic squid (Rosa et al., 2019).

Fluctuating sea levels during the Pleistocene lead to the exposure of the Sundra and Sahul shelves during glacial periods. Low sea levels during the last glacial maximum led to approximately 90% loss of shallow water coastal habitat up to 60 m deep throughout the Pacific (Ludt and Rocha, 2015). Restricted water flow throughout the Indo Pacific and loss of coastal habitat during these periods greatly influenced marine species composition (Rocha and Bowen, 2008). Genetic signatures have been identified in olive sea snake (*Apiysurus laevis*) populations across Northern Australia consistent with contractions to refugia during low sea levels. Subsequent colonisation events from western to eastern populations were identified based on diversity indices indicating refugia in WA during the Pleistocene retained populations that were lost in the east (Lukoschek et al., 2008,2007). Distributions of *Hapalochlaena* also show a distinction between east and west population across Northern Australia, which may be indicative of isolation to refugia during the Pleistocene (Ludt and Rocha, 2015).

4.3. Mechanisms of divergence and speciation

Dispersal ability plays an integral role in the distribution and population dynamics of marine organisms (Higgins et al., 2013). Coleoid cephalopods utilise two main reproductive strategies holobenthic and merobenthic (Doubleday et al., 2008). Holobenthic cephalopods exhibit a reduced dispersal ability relative to merobenthic species as the offspring hatch well-developed and assume a benthic lifestyle immediately. In contrast, merobenthic species have a pelagic phase allowing for dispersal on water currents post hatching before settling to a benthic lifestyle (Ibáñez et al., 2018; Villanueva et al., 2016). A merobenthic strategy has been proposed as the ancestral state for members of the family Octopodidae with the holobenthic life history believed to be associated with colonisation of temperate and deep-water environments (Ibanez et al., 2014). Radiation of *Hapalochlaena* is predicted in this study to have occurred in the tropical Indo Australian Archipelago (IAA) during the ~25–50 mya. The subsequent colonisation of temperate zones including temperate Australasia (TAUS) are associated with the shift to a holobenthic strategy for *H. maculosa* and *H. fasciata* followed by minimal diversification relative to tropical zones. *H. lunulata* retains the predicted ancestral merobenthic strategy, occupying shallow tropical waters (Ibáñez et al., 2014). A degree of reproductive plasticity in some cephalopod species or genera, where by differences in egg size, shape and number have been observed and are often associated with environmental conditions (Laptikhovsky, 2006; Laptikhovsky et al., 2009). Egg size was demonstrated to decrease from the Eastern to Western Mediterranean for the mysopsid squids (*Loligo* and *Alloteuthis*), an octopod (*Eledone cirrhosa*) and an argonaut (*Argonauta argo*). However, cuttlefish and sepiolid squids examined did not display any notable difference in egg size along the same gradient, it should be noted this does not take into account seasonality (Laptikhovsky et al., 2009). Cooler and less productive waters have been suggested to favour larger eggs and consequently larger yolks resulting in larger more robust offspring (Alekseev, 1981). The comparatively complex trophic structures found in tropical waters with high predator diversity may support the quantity of offspring over other adaptive strategies (Alekseev, 1981). Conversely putative *Hapalochlaena* species identified from warmer waters, such as Darwin ([*Hapalochlaena* sp. 1]) and the GBR ([*Hapalochlaena* sp. 5]) exhibit larger eggs, however it should be noted that the life history of these specimens have not been fully elucidated (Norman, 2000). Additionally, many of the putative species identified in this study

have completely unknown life histories. Therefore, further investigation is required to fully elucidate the evolution of reproductive strategies within this genus and the resulting impact of genetic structure and speciation.

5. Conclusion

This study presents the first large scale genetic analyses of the *Hapalochlaena* genus and demonstrates that the level of species diversity exceeds current systematics for the genus. Previously described morphological diversity within the genus is supported by the genetic analyses conducted in this study including a distinction between lined *Hapalochlaena* populations within NSW, Australia and the Northern Pacific. This provides a greater understanding of species diversity within the genus and has implications for how future studies should be conducted and compared based on genetic distances observed across locations. Detailed investigation of morphological features of *Hapalochlaena* specimens, currently being undertaken in a parallel study, is required to officially describe species within the genus.

CRediT authorship contribution statement

Brooke L. Whitelaw: Writing – original draft, Formal analysis, Methodology, Visualization, Investigation. **Julian K. Finn:** Resources, Supervision, Conceptualization, Writing – review & editing, Funding acquisition. **Kyall R. Zenger:** Writing – review & editing, Methodology. **Ira R. Cooke:** Writing – review & editing, Methodology. **Peter Morse:** Resources, Writing – review & editing. **Jan M. Strugnell:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Museums Victoria (NMV), the Western Australia Museum (WAM), Australian Museum (AM) and the Museum and Art Gallery of the Northern Territory (MAGNT) for allowing us access to their extensive collections. Julian Finn would like to thank the following people for assisting with the collection of blue-ringed octopuses in Australia and Taiwan, and for providing access to specimens in museum collections: Mark Norman (NMV / Parks Victoria), Chung-Cheng Lu (NMV / National Chung Hsing University), Colin Silvey (NMV), Chris Rowley (NMV), Melanie Mackenzie (NMV), John Ahern (University of Melbourne), Mandy Reid (AM), Alison Miller (AM), John Healy (QM), Darryl Potter (QM), Richard Willan (MAGNT), Corey Whisson (WAM), Clay Bryce (WAM), Shirley Slack-Smith (WAM), Nick Chun-Yen Lin (National Tsing Hua University), Mong-Fong Lee (National Penghu University of Science and Technology), Wensung Chung (University of Queensland), Christine Huffard (Monterey Bay Aquarium Research Institute), Roy Caldwell (Berkeley University of California), Robert Condon (University of North Carolina at Wilmington), Koji Nakamura (Japan Underwater Films), Nobuhito Yamashita (JUF), Yasushi Okumura (JUF), Masahiko Sakata (JUF). Specimens collected as part of the CRC-REEF C1.1.2 Seabed Biodiversity on the Continental Shelf of the Great Barrier Reef World Heritage Area (GBRWHA) (Principal Investigators C. Roland Pitcher (CSIRO), Peter Doherty (AIMS), Neil Gribble (QDPI-F), Peter Arnold (QM) and John Hooper (QM)) were essential to this study. We also thank Colin Silvey and Erin Morgan for initial DNA extractions and PCRs which contributed to this study.

Funding Sources

This work was supported by an Australian Biological Resources Study (ABRS) research grant awarded to Julian Finn, Mark Norman, Jan Strugnell and Chung-Cheng Lu (ref: RF211–41) administered by Museums Victoria. While Brooke Whitelaw was based at Melbourne Museum under the co-supervision of Julian Finn (Oct 2018 to Nov 2021) all associated cost were covered by Museums Victoria.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107827.

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