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Evolution and Population Genomics of Loliginid Squids

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Evolution and Population Genomics of Loliginid Squids

A dissertation submitted in partial satisfaction of the

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in Biology

by

Samantha Hue Tone Cheng

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ABSTRACT OF THE DISSERTATION

Evolution and Population Genomics of Loliginid Squids

by

Samantha Hue Tone Cheng

Doctor of Philosophy in Biology

University of California, Los Angeles, 2015

Professor Paul Henry Barber, Chair

Globally, rampant harvesting practices have left vital marine resources in sharp decline precipitating a dramatic loss of the biodiversity and threatening the health and viability of natural populations. To protect these crucial resources and ecosystems, a comprehensive assessment of biodiversity, as well as a rigorous understanding of the mechanisms underlying it, is urgently needed. As global finfish fisheries decline, harvest of cephalopod fisheries, squid, in particular, has exponentially increased. However, while much is known about the evolution and population dynamics of teleost fishes, much less is understood about squids. This dissertation provides a robust, in-depth examination of these mechanisms in commercially important squids using a novel approach combining genetics and genomics methods. In the first chapter, a suite of genetic markers is used to thoroughly examine the distribution and evolution of a species complex of bigfin reef squid (*Sepioteuthis* cf. *lessoniana)* throughout the global center of marine biodiversity, the Coral Triangle, and adjacent areas. Phylogenetic analyses and species delimitation methods unequivocally demonstrate the presence of at least three cryptic lineages

sympatrically distributed throughout the region. While these putative species are reciprocally monophyletic, they are difficult to distinguish morphologically and little is known about how they differ in life history and ecology. To this end, in chapter 2, patterns of population structure over the Coral Triangle and adjacent regions were examined using genetic and genomic methods to identify important processes shaping both genetic and demographic connectivity in two of these cryptic species. Using both mitochondrial DNA (cytochrome oxidase subunit 1) and genome-wide single nucleotide polymorphisms (generated from restriction site associated digest (RAD) sequencing), we find strong, but discordant, patterns of population structure between these sympatric sibling taxa suggesting contrasting dispersal life histories. Moreover, detection of putative outlier loci highlights the possible role of selective pressures from regional environmental differences in shaping ongoing divergence. Given the fine-scale resolution achieved in chapter 2 with using RAD sequencing, in chapter 3, we apply these methods to examine potential population structure in the highly valuable market squid fishery (*Doryteuthis opalescens*) in California. This fishery has long been hypothesized to be two separate stocks due to different spawning peaks and areas. Using genome-wide SNPs and a rigorous temporal sampling scheme, we determined that northern and southern regions do not represent two distinct spatial stocks. Rather, complex patterns of temporal population structure lend support to continual spawning of genetically distinct cohorts at both sites throughout the 2014 harvest season. Collectively, these results demonstrate that squid biodiversity and population structure is much more complex than previously thought. Through the use of genetic and genomic technologies, we can delineate populations and identify the mechanisms driving connectivity to provide key information for fisheries management and conservation.

The dissertation of Samantha Hue Tone Cheng is approved.

Frank E. Anderson

Howard Bradley Shaffer

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EPIGRAPH

We need the tonic of wilderness…At the same time that we are earnest to explore and learn all things, we require all things be mysterious and unexplorable, that land and sea be infinitely wild, unsurveyed and unfathomed by us because unfathomable. We can never have enough of Nature.

– *Walden; or, Life in the Woods*, Henry David Thoreau

My soul is full of longing for the secrets of the sea, and the heart of the great ocean sends a thrilling pulse through me.

– *The Secret of the Sea*, Henry Wadsworth Longfellow

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First, and foremost, I would like to thank my advisor, mentor, and friend, Paul Barber, for always believing in me and encouraging me to pursue my interests. Since I began as a summer intern in 2007, he has always been there for me – whether helping me to hone my research ideas, work on my writing, or find myself as a scientist. He's been my constant cheerleader in graduate school, letting me explore my interests and, equally as important, insisting that the best things learned are often outside of the lab, with your fellow graduate students, over beers. For all the wisdom and encouragement, I am incredibly grateful.

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and N. Rodriguez conducted all sample collections and helped develop the project scope. P.H. Barber helped develop the project scope and helped to revise the manuscript.

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INTRODUCTION

Marine resources, such as fisheries, support global livelihoods and food security. Despite management efforts, fisheries have drastically declined in the past thirty years (Myers and Worm 2003). These crashes highlight major gaps in information for maintaining sustainable resources. In particular, accurate identification of harvested species is crucial to successful management. While such information seems fundamental, increasingly, genetic evidence is demonstrating that many harvested marine species are composed of multiple different species, prompting serious concern that we are "managing in the dark" (Bickford et al. 2007). This lack of information can lead to serious miscalculations and inferences about the health and abundance of harvested populations.

While correct species identification lays the groundwork for successful marine resource management, in order to manage populations, we need information regarding where and when and how to protect vulnerable populations. Spatial marine management is a conservation practice where resource managers and conservationists delineate specific areas to focus specific conservation efforts. However, this management practice requires robust and well-validated information on species connectivity patterns, or how individuals and genes exchange among populations and geographic areas (Sale et al. 2005). Variations in connectivity influences how populations change in abundance and diversity over time. This variation is important to understand, as connectivity is essential for maintaining viable population sizes and increase genetic diversity, which together allow populations to be more resilient to environmental and anthropogenic change.

As global finfish fisheries have declined, exploitation of cephalopod species has exponentially risen (Caddy and Rodhouse 1998). Currently, cephalopod species comprise a substantial portion of global landings, often dominating regional fisheries economics (e.g. the Agulhas Current, Patagonian Shelf) (Hunsicker et al., 2010), however, much less is understood about their population dynamics and evolution than of teleost fishes (Piatkowski et al., 2001). Thus, this dissertation focuses on using genetic and genomic tools to resolve issues of species identity (chapter 1) and to delineate spatial patterns of population connectivity (chapters 2 and 3) in two valuable species of squids. The first, the bigfin reef squid, *Sepioteuthis* cf. *lessoniana*, is the most understudied, yet one of the most heavily harvested species in the tropical and subtropical Pacific and Indian Oceans, Mediterranean and Red Seas. Mounting evidence indicates that this widely distributed squid species is likely composed of multiple unidentified species (termed a "species complex") (Segawa et al., 1993). However, little is known about how many species exist, where they occur, how they differ. The second, market squid, *Doryteuthis opalescens*, ranges from Alaska to Baja, and is the largest and most valuable fishery in California (Vojkovich, 1998; Zeidberg, 2013). The market squid fishery has suffered from a few major collapses in the past few decades, prompting concern that current management is insufficient. In particular, concentration of fishing efforts over spawning areas has raised questions regarding the level of connectivity between distinct spawning grounds. The results from these studies are providing comprehensive information and tools that can and are being used in designing current management strategies.

In chapter 1, I use traditional phylogenetic methods to determine the extent and distribution of the *S. cf. lessoniana* species complex using a multi-gene dataset generated from almost 400 individuals collected from locations throughout the Indian and Indo-West Pacific

Oceans. Using both maximum likelihood and Bayesian inference along with rigorous validation methods (species tree estimations and species delimitation), the dataset indicated three reciprocally monophyletic lineages within the species complex with no distinct morphological differences. There is no evidence that these putative species are geographically separated on a broad spatial scale (1000s of km). Moreover, different putative species were often brought in from the same reef fishing area, indicating widespread sympatric occurrence at fine and broad spatial scales. This adds significant resolution to the decades-long debate about species identity in this group and raises questions about how these different species co-occur.

In chapter 2, I use a comparative phylogeographic approach to compare patterns of population structure and connectivity in sympatric species of bigfin reef squid, *S.* cf. *lessoniana* (lineages B and C, (Cheng et al. 2014)) to assess potential barriers to gene flow in a vital fisheries species that is not under any specific management regime except in Japan and Thailand, despite being harvested in over 20 countries (Jereb and Roper 2006, FAO). Although most studies on marine connectivity use one or two molecular markers (Hellberg et al. 2002), I employ next-generation sequencing because it reflects small genetic changes happening over shorter and more ecological relevant time scales, allowing for more accurate estimates of connectivity (Allendorf et al. 2010). Specially, I examined possible barriers to gene flow over the dynamic Coral Triangle region and adjacent areas using a combination of mitochondrial CO1 data (from 165 and 498 individuals from lineages B and C, respectively) and genome-wide single nucleotide polymorphisms. Using a restriction site associate digest (RAD) sequencing method (Wang et al. 2012), I generated ~2,000 genome-wide single nucleotide polymorphisms from 53 and 116 individuals of each sympatric species (lineages B and C) over a subset of locations over the region. Patterns of strong, but discordant, genetic structure were found in both

species that do not precisely correspond to previously hypothesized barriers to gene flow in the area (e.g. Pleistocene glaciation cycles (Ludt and Rocha 2014) and prevailing oceanographic currents (Barber et al. 2006)). These results also highlight likely differences in dispersal life history between the two cryptic species, with lineage B showing patterns more similar to restricted dispersers (e.g. benthic and strongly reef associated organisms) (Barber et al. 2006; DeBoer et al. 2008) and lineage C showing patterns corresponding to wide dispersal capacity similar to pelagic organisms such as tunas (Jackson et al. 2014). Indications of strong limitations to demographic connectivity suggest that these reef squid should be managed on a local scale, rather than a broad regional scale. Moreover, as these cryptic species are sympatric and often harvested together, the most conservative approach would be to manage harvest of *S.* cf. *lessoniana* species based on limited dispersal ability.

In chapter 3, I use genome-wide SNPs to determine whether any spatial or temporal population structure exists in the market squid (*Doryteuthis opalescens*) fishery in California. Market squid are currently managed as one population in California despite distinct peaks of abundance north and south of Point Conception. It has been theorized that these peaks could indicate two separate populations with different spawning times, however studies have been inconclusive (Reichow and Smith 2001). Using the same RAD sequencing technique from chapter 2, I specifically test whether separate northern and southern populations exist to determine if there is a need to reorganize existing management. This project is in collaboration with California Fish and Wildlife (CDFW) who are responsible for management. Examination of temporally paired replicates through five months of the 2014 harvest season failed to find clear patterns of spatial structure. However, pairwise examination of sampled replicates indicates a complex pattern of temporal structure suggesting that spawning individuals recruiting to

different spawning grounds at different times are genetically distinct. This lends preliminary support to the existence of smaller distinct cohorts constantly recruiting in California (Jackson & Domeier 2003) rather than two major spawning aggregations (Hixon 1983; Spratt 1979). Moreover, this study demonstrates the utility of genome-wide SNPs add fine-scale resolution to investigating population structure in squid populations where previous markers have not been able to.

Collectively, the results from this dissertation highlight the complexity of patterns of population structure and processes driving gene flow and evolution in commercially valuable neritic squid species. Generally, these results add to the growing compendium of evidence that pelagic and neritic squids do not have as wide-ranging dispersal capacity as has been long assumed (Aoki et al. 2008; Brierley et al. 1995; Buresch et al. 2006; Sin et al. 2009; Thorpe et al. 1986). Rather, using genome-wide scale analyses, I observe complex patterns of connectivity in both the *S. cf. lessoniana* species complex and in *D. opalescens*. Evidence of limited dispersal and complex temporal population structure in lineage B and *D. opalescens* suggests that availability of spawning sites may be an important driver of population divergence, as has been theorized for some squids (Brierley et al. 1993; Buresch et al. 2006), and in a number of other marine organisms, such as salmon (Seeb et al. 2011) and other migratory fish (Adams et al. 2006; Skjæraasen et al. 2011). Moreover, this study provides a practical demonstration of both the utility of mitochondrial DNA for illustrating broad-scale patterns of population connectivity and the insights that can be gained through the use of genome-wide SNP data. The results from this dissertation illustrate that harvested squid populations have unexpectedly complicated patterns of spatial and temporal population structure that need to be taken into account for planning effective conservation and management.

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CHAPTER 1

Molecular evidence for co-occuring cryptic lineages within the Sepioteuthis cf. lessoniana species complex in the Indian and Indo-West Pacific Oceans

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CEPHALOPOD BIOLOGY AND EVOLUTION

Molecular evidence for co-occurring cryptic lineages within the *Sepioteuthis* cf. *lessoniana* species complex in the Indian and Indo-West Pacific Oceans

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Abstract The big-fin reef squid, *Sepioteuthis* cf. *lessoniana* (Lesson 1930), is an important commodity species within artisanal and near-shore fisheries in the Indian and Indo-Pacific regions. While there has been some genetic and physical evidence that supports the existence of a species complex within *S.* cf. *lessoniana*, these studies have been extremely limited in scope geographically. To clarify the extent of cryptic diversity within *S.* cf. *lessoniana*, this study examines phylogenetic relationships using mitochondrial genes (cytochrome oxidase *c*, 16s ribosomal RNA) and nuclear genes (rhodopsin, octopine dehydrogenase) from nearly 400 individuals sampled from throughout the Indian, Indo-Pacific, and Pacific Ocean portions of the range of this species. Phylogenetic analyses using maximum likelihood methods and Bayesian inference identified three distinct lineages with no clear geographic delineations or morphological discriminations. Phylogeographic structure analysis showed high levels of genetic connectivity in the most widespread lineage, lineage C and low levels of connectivity in lineage B. This study provides significant phylogenetic evidence for cryptic lineages within this complex and confirms that cryptic lineages of *S.* cf. *lessoniana* occur in sympatry at both small and large spatial scales. Furthermore, it suggests that two closely related co-occurring cryptic lineages have pronounced differences in population structure, implying that

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underlying differences in ecology and/or life history may facilitate co-occurrence. Further studies are needed to assess the range and extent of cryptic speciation throughout the distribution of this complex. This information is extremely useful as a starting point for future studies exploring the evolution of diversity within *Sepioteuthis* and can be used to guide fisheries management efforts.

Keywords Cryptic diversity · Marine · Myopsidae · *Sepioteuthis* · Squids · Phylogenetics

Introduction

Tropical coral reefs contain approximately one-third of all described marine organisms. However, it is widely acknowledged that biodiversity counts in marine environments are grossly underestimated (Sala & Knowlton, 2006; Bickford et al., 2007) and only 10% of existing reef species (~ 93,000 species) have been discovered and described (Reaka-Kudla, 1997). In part, this underestimation is due to high levels of cryptic/sibling species (Knowlton, 1993, 2000). This presents a huge challenge to cataloguing marine biodiversity as such species complexes lack traditional morphological differences and may differ in physiology, behavior, or chemical cues (Knowlton, 1993) which would only be obvious with sufficient observation and comparison. In particular, the global epicenter of marine biodiversity, a region called the Coral Triangle (Fig. 1) comprises just 1% of global ocean area yet contains the highest number of described species in the marine realm (Briggs, 1999), including many cryptic and/or endemic species (Allen & Werner, 2002; Allen, 2008; Anker, 2010). Furthermore, strong genetic breaks across many taxonomic groups (e.g., Carpenter et al., 2011) including damselfish (Drew et al., 2008; Leray et al., 2010), giant clams (DeBoer et al., 2008; Nuryanto & Kochzius, 2009), gastropods (Crandall et al., 2008), seastars (Williams & Benzie, 1997), pelagic fishes (Fauvelot & Borsa, 2011), benthic crustaceans (Barber & Boyce, 2006; Barber et al., 2006, 2011), and neritic reef fishes (Planes & Fauvelot, 2002; Ovendon et al., 2004; Gaither et al., 2009, 2011), indicate a potential for many more cryptic taxa. However, most of the above studies have focused on benthic invertebrates. Considerably less is known about larger organisms, especially those of commercial importance such as pelagic fishes and cephalopods.

Recent revisions within the Indo-West Pacific region (including the Coral Triangle) documented numerous new cephalopod species particularly among Sepiidae (cuttlefishes), Loliginidae (neritic myopsid squids), and littoral octopods (Natsukari et al., 1986; Norman & Sweeney, 1997; Norman & Lu, 2000; Okutani, 2005). Genetic evidence for cryptic species complexes in cuttlefishes (Anderson et al., 2011), myopsid squids (Vecchione et al., 1998; Okutani, 2005), and neritic loliginid and cuttlefish species (Yeatman & Benzie, 1993; Izuka et al., 1994, 1996a, b; Triantafillos & Adams, 2001, 2005; Anderson et al., 2011) suggests that the diversity of cephalopods in the Indo-Pacific exceeds current taxonomic delineations and needs to be explored further. However, to date, many studies suggesting cryptic diversity within these groups have been limited in geographic scope and use different sources of information to infer cryptic species, making comprehensive diversity assessments very difficult.

One nominal cephalopod species in particular, the big-fin reef squid *Sepioteuthis* cf. *lessoniana*, Lesson, 1830 is a common, commercially harvested squid throughout the Indo-Pacific region. Substantial morphological and genetic evidence indicate that extremely high levels of cryptic diversity exist within this taxon. A taxonomic revision by Adam (1939), synonomized 12 Indo-West Pacific *Sepioteuthis* species into *S.* cf. *lessoniana*, relegating any noted differences to geographic variability. *S.* cf. *lessoniana* ranges from the central Pacific Ocean (Hawaii) to the western Indian Ocean (Red Sea) and into the eastern Mediterranean via Lessepsian migration (Salman, 2002; Mienis, 2004; Lefkaditou et al., 2009). Evidence for cryptic species in *S.* cf. *lessoniana* first arose in Japan, with the recognition of three distinct color morphs and corresponding isozyme differentiation in *S.* cf. *lessoniana* harvested from around Ishigakijima in Okinawa Prefecture in Southwestern Japan (Izuka et al., 1994, 1996a). These delineations have been furthered corroborated by differences in egg case morphology and size (Segawa et al., 1993a), chromatophore arrangement (Izuka et al., 1996b), and temperature-mediated distributions (Izuka et al., 1996a). Triantafillos & Adams (2005) found similar allozyme evidence for two cryptic species within Western Australia, and theorized that evident spatial and temporal heterogeneity of growth and life history may maintain species

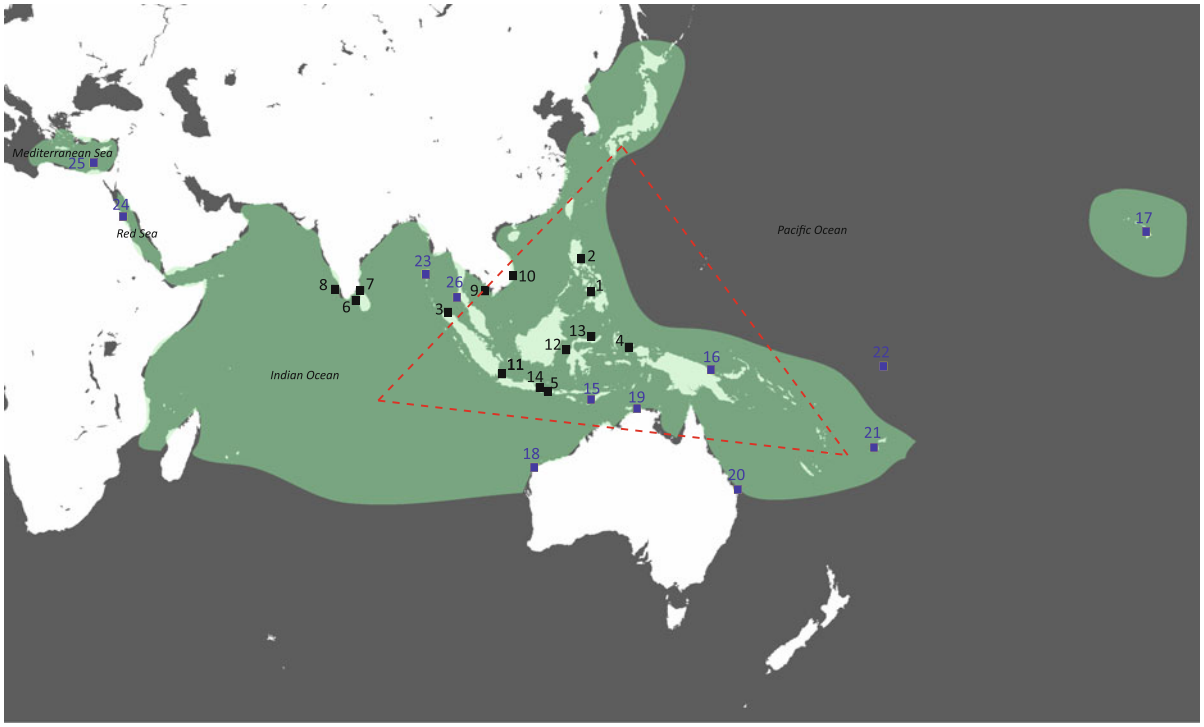

Fig. 1 Range of *Sepioteuthis* cf. *lessoniana* and sampling localities for this study. *Areas highlighted in green* represent the reported range of *S.* cf. *lessoniana* (Jereb & Roper, 2010). *Black squares* indicate localities with the numerals corresponding to those in Table 1. The *red dotted lines* indicate the Coral Triangle region (Conservation International, 2012)

boundaries (Jackson & Moltschaniwsky, 2002). However, these studies only document cryptic diversity in two point locations within a very broad range.

Despite their importance in sustaining local economies and food security of Indo-West Pacific communities, near-shore cephalopods, including *S.* cf. *lessoniana*, within the Coral Triangle and the surrounding Indo-West Pacific are relatively understudied. This study aims to provide an in-depth assessment of *S.* cf. *lessoniana* cryptic diversity in the region. Given the wide applicability of molecular methods for cryptic species detection, both mitochondrial and nuclear DNA evidence will be used to provide a phylogenetic assessment of the species complex.

Methods

Collection localities and sampling techniques

A total of 377 juvenile and adult *S.* cf. *lessoniana* specimens were collected from local fish markets and via hand jigging (day and night) from 14 locations throughout Indonesia, the Philippines, Vietnam, and Southern India (Fig. 1; Table 1) from 2011 to 2012. Fishing localities of most samples were not more than ∼10–20 km offshore judging from interviews with fishermen and type of vessel and motor used. Samples obtained from the markets had been collected primarily using hand jigs over reef and sea grass beds and occasionally via beach seines in sea grass habitats (Ticao, Luzon, Philippines). Fishing for *S.* cf. *lessoniana* mostly took place at night, with the exception of samples caught in Pulau Seribu, Indonesia. Mantle tissue was preserved in 95% ethanol. Voucher specimens were preserved in 10% formalin when available.

DNA extraction and amplification

Total genomic DNA was extracted from 1 to 2 mg of ethanol-preserved mantle tissue using Chelex (Walsh et al., 1991). Two mitochondrial and two nuclear genes were amplified in this study: mitochondrial cytochrome oxidase subunit 1 (CO1 or *cox1*), 16s ribosomal RNA (*rrnL*), rhodopsin, and octopine dehydrogenase (ODH). From the inferred

Table 1 Collection localities and sample sizes

	Locality	Country	*n*	Time	Locality code
1	Dumaguete, Negros Oriental	Philippines	27	July 2011	DUM
2	Ticao, Luzon	Philippines	74	July 2011	TIC
3	Banda Aceh, Sumatra	Indonesia	6	July 2011, May 2012	SUM ACEH
4	Raja Ampat, West Papua	Indonesia	36	July 2011, June 2012	RAJ
5	Bali	Indonesia	40	September 2011	BAL
6	Pambam, Gulf of Mannar	India	5	March 2012	GM
7	Mandapam, Palk Bay	India	2	March 2012	PB
8	Mangalore	India	2		MNG
9	Phu Quoc Island	Vietnam	17	April 2012	VN001-017
10	Nha Trang	Vietnam	53	April 2012	VN067-150
11	Pulau Seribu, Java	Indonesia	22	June 2012	PSE
12	Donggala, Sulawesi	Indonesia	41	August 2012	DON
13	Manado, Sulawesi	Indonesia	44	August 2012	MAN
14	Muncar, Java	Indonesia	4	August 2012	MUN
		Total	377		

mitochondrial lineages, two nuclear genes were amplified from a subset of individuals. The mitochondrial genes were amplified together using a multiplexing approach using a Qiagen Multiplex kit following the standard published protocol for 25 µl reactions. CO1 and 16s were amplified using universal HCO-2198 and LCO-1490 primers (Folmer et al., 1994) and 16sAR and 16sBR primers (Kessing et al., 1989), respectively. Rhodopsin and ODH were amplified using cephalopod specific primers (Strugnell et al., 2005) in 25 µl Amplitaq Hotstart PCR reactions. PCR thermal cycling parameters were as follows: initial denaturation for 94°C for 120 s, then cycling 94°C for 15 s, 36–68°C for 30 s, and 72°C for 30 s for 25–30 cycles, following by a final extension step of 72°C for 7 min. The following annealing temperatures were used: 36°C for CO1, 42°C for 16s, 55°C for rhodopsin, and 68°C for ODH. The resulting amplified products were cleaned up and sequenced at the UC Berkeley DNA Sequencing Facility. Samples collected from India were amplified and sequenced at the Anderson lab at Southern Illinois University-Carbondale following the protocols outlined in Anderson et al. (2011).

Data analysis

DNA sequences were edited and checked in Sequencher v 4.1 (Gene Codes, Ann Arbor, MI, US). Sequences for each gene region were aligned using the CLUSTALW plug-in implemented in Geneious. As the 16s alignment was characterized by a number of large gaps ($n = 5$), the initial alignment was realigned to an invertebrate 16s structural constraint sequence (*Apis mellifera*) with RNAsalsa (Stocsits et al., 2009) using default parameters. Unique haplotypes were identified among the sampled individuals for all gene regions using the DNAcollapser tool in FaBox (Villesen, 2007). For each individual gene dataset, Kimura-2-parameter distances and haplotype diversity (with standard error) were calculated within and between each lineage in Arlequin 3.5.1.2 (Excoffier & Lischer, 2010).

Individual gene alignments were first analyzed with both maximum likelihood methods (RAxML 7.3.2) and Bayesian inference (MrBayes 3.1.2) on the CIPRES Science Gateway (Miller et al., 2010) using sequences of *Sepioteuthis australis*, *Loligo bleekeri*, and *Sthenoteuthis oualaniensis* obtained from GenBank (Table 2) as outgroups. Total sequence data from 87 representative individuals were then concatenated in Mesquite into three datasets—mitochondrial genes, nuclear genes, and all genes. After comparing the results from individual and concatenated datasets, the mitochondrial dataset were run using 70 additional individuals that were not amplified for nuclear genes to assign lineage identity. *Sepioteuthis sepioidea* and *S. australis* sequences from GenBank were used as

Table 2 GenBank accession numbers and outgroups used in this study to infer phylogenetic relationships within the *S.* cf. *lessoniana* species complex

Species	16s	CO1	Rhodopsin	ODH	References
Sepioteuthis australis	AF110087	AF075401	AY616917	AY616901	Anderson (2000), Strugnell et al. (2005)
Loligo forbesi	AF110075	AF075402	AY545184	AY545136	Anderson (2000), Strugnell et al. (2004)
Sthenoteuthis oualaniensis	AB270958	AB270943	AY545185	AY545137	Wakabayashi et al. (2012), Strugnell et al. (2004)
Sepioteuthis sepioidea	AF110090	AF075392			Anderson (2000)
Moroteuthis robusta	EU735241	AB264116			Lindgren (2010), Wakabayashi et al. (2012)
Loligo bleekeri	AF110074	AB573754			Anderson (2000), Iwata et al. (2010)

outgroups (Table 2). Each concatenated dataset was partitioned into individual genes. RAxML was run for single and combined datasets using the GTR-GAMMA model for both bootstrapping and maximum likelihood search. Node support was estimated using 1,000 rapid bootstrap replicates and nodes with greater than 50% support were used to construct the final consensus tree. Bayesian phylogenetic analyses were implemented in MrBayes 3.1.2. Default values for prior parameters were used in the analysis. Each dataset was run for 5,000,000–7,500,000 generations after which the average standard deviation of split frequencies fell below the stop value of 0.01. Datasets were run with a mixed model and gamma rate distribution (+G). The final consensus tree was constructed using the fifty-percent majority rule and resulting posterior probability values for each node were used as estimates of clade support.

The previous methods estimated gene trees, indicating the history of that particular gene, and not necessarily the lineages or species. Incomplete lineage sorting can result in discordance between inferred gene trees and between the gene trees and species trees, resulting in incorrect inferences about phylogenetic relationships (Maddison, 1997; Nichols, 2001). All four genes were concatenated into a partitioned dataset and analyzed using a multi-locus, multi-species coalescent framework employed in *BEAST (Heled & Drummond, 2010) to estimate the most likely species tree. Each of the 87 individuals was assigned to one of three distinct lineages (lineage A, B, or C) identified in the concatenated datasets. Nucleotide substitution model parameters were estimated for each individual gene dataset using jModelTest (Guindon & Gascuel, 2003; Darriba et al., 2012). Models with the best AIC score that could be implemented in *BEAST were chosen. Each MCMC analysis was conducted for 100,000,000 generations (sampling every 1,000 steps with a 15% burn-in determined from trace plots and estimated samples sizes). A Yule process tree prior and an uncorrelated lognormal relaxed clock branch length prior were used for all analyses. All other priors were left at default values. Convergence of the posterior and parameters was assessed by examining likelihood plots in Tracer v1.4 (Rambaut & Drummond, 2007). Final best tree was determined from 255,000 combined post-in samples from the three runs.

Validation of lineages

To explore the validity of lineages inferred from the previous gene and species tree techniques, we used a Bayesian species delimitation method implemented through the Bayesian Phylogenetics and Phylogeography (BP&P version 2.2) program (Yang & Rannala, 2010). This coalescent-based method is designed to estimate the posterior distribution for different species delimitation models using a reversible-jump Markov chain Monte Carlos (rjMCMC) algorithm on a user-provided guide tree with priors for ancestral population size (θ) and root age (τ_0). At each bifurcation of the guide tree, the rjMCMC algorithm estimates the marginal posterior probability of speciation (from here on, termed "speciation probability"). We invoked the 50% majority rule for inferring the likelihood of a speciation or splitting event.

We used BP&P to estimate the posterior probabilities of splitting events to validate the lineages inferred from

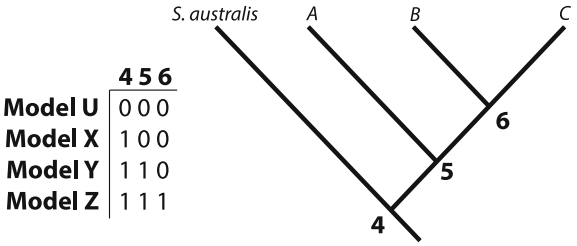

Fig. 2 Species delimitation models on a fully resolved guide tree generated from *BEAST. Each model represents a tree with different nodes bifurcated (denoted by *1*) or collapsed (denoted by *0*). For example, *model X* retains the bifurcation at *node 4*, but has *nodes 5* and *6* collapsed, indicating that lineages *A*, *B,* and *C* all represent one species, while *S. australis* is distinct (from Yang & Rannala, 2010)

our phylogenetic analyses using all combined loci. The rjMCMC analyses were each run for 300,000 generations (sampling interval of five) with a burn-in period of 24,000, producing consistent results for all separate analyses. For each analysis, we used algorithm 0 with fine-tuning to achieve dimension matching between delimitation models with different numbers of parameters. Each species delimitation model (Fig. 2) was given equal prior probability (0.25). We modified the species tree from *BEAST by removing two of the outgroups (*Sthenoteuthis oualaniensis* and *Loligo forbesi*) to use as the guide tree for BP&P. We removed these two distant outgroups as we were not delimiting species from different genera. Within this tree, we treat *S. australis* as a type of "control," as it is a morphologically and phylogenetically valid species (Anderson, 2000; Jereb & Roper, 2006), thus it should be delimited from the *S.* cf. *lessoniana* complex.

Assignment of prior distributions can significantly affect the posterior probabilities calculated for each model (Yang & Rannala, 2010). Thus, we tested the influence of different divergence scenarios by considering different combinations of the priors for ancestral population size (θ) and root age (τ_0). The combinations of priors assumed three scenarios: (1) large ancestral population size $\theta \sim$ G(1,10) and deep divergences $\tau_0 \sim$ G(1,10), (2) small ancestral population size $\theta \sim$ G(2,2000) and shallow divergences $\tau_0 \sim$ G(2,2000), and (3) large ancestral population sizes $\theta \sim$ G(1,10) and shallow divergences $\tau_0 \sim$ G(2,2000). This third combination represents the most conservation combination of priors that should favor models with fewer species (Yang & Rannala, 2010).

Phylogeographic patterns within lineages

Genetic diversity and differentiation were investigated between populations within lineages B and C to assess likely factors influencing genetic structure. Lineage A was excluded from phylogeographic analyses as this clade only contained 20–24 individuals and the sample sizes were too small to conduct tests with any reliability. For lineages B and C, populations with $n < 10$ were omitted from analyses. Five localities were also omitted from analysis due to small sample sizes (all three sites from India and Banda Aceh, Sumatra and Muncar, Java in Indonesia). Diversity indices (haplotype diversity, nucleotide diversity, and associated standard deviations) were calculated each locality for each lineage using Arlequin 3.5.1.2 (Table 4). Patterns of phylogeographic structure among sampled localities were assessed. Percent variation within and among localities in each lineage, f-statistics (ϕ_{ST}), and locality pairwise comparisons (ϕ_{ST}) were calculated using an analysis of molecular variance (AMOVA).

Results

Sequence variation

Screening of mitochondrial DNA regions CO1 (675 bp) and 16s (549 bp) in 379 and 371 individuals, respectively, yielded 157 and 89 unique haplotypes. From the subset of 267 and 282 individuals screened for nuclear DNA regions rhodopsin (542 bp) and ODH (866 bp) 27 and 158 unique haplotypes were found, respectively. The gene regions analyzed were very different in terms of variability. Mitochondrial CO1 and 16s and nuclear ODH had a higher proportion of variable sites than nuclear rhodopsin.

Phylogenetic relationships

Phylogenies recovered from all analyzed datasets resolved the same set of monophyletic lineages in both maximum likelihood and Bayesian inference methodologies. Three well-supported lineages (average support values—lineage A [BPP 0.997, MLB 96.33%], lineage B [BPP 0.983, MLB 93.67%], and lineage C [BPP 0.813, MLB 69.00%] where BPP is Bayesian posterior probability and MLB is maximum likelihood

bootstrap support) were resolved in each dataset, comprising the same individuals in each analysis. Topologies resolved between mitochondrial and nuclear datasets were not always concordant. In both maximum likelihood and Bayesian consensus trees for the all gene dataset and the mitochondrial gene dataset, lineages B and C were sister to each other (Figs. 3, 5). However, in the nuclear genes dataset, lineages A and C were sister to each other (Fig. 4). However, for all datasets, the support values for that particular node are low (BPP 0.51–0.89, MLB 49–72%), indicating that the relationship between those lineages is not well-resolved and further statistical assessment is needed. The maximum clade credibility species tree topology (determined from 255,000 post burn-in tree topologies) is concordant with the hypothesis of three distinct lineages within *S.* cf. *lessoniana* (Fig. 6). Similar to the maximum likelihood and Bayesian analyses of the concatenated datasets, each lineage had very high support values and large divergences between lineages.

The results from the Bayesian species delimitation for this species complex show variable support for three independent lineages (Fig. 7). When assuming large ancestral population sizes and deep divergences or large ancestral population sizes and shallow divergences, there was strong support for the bifurcation between lineages A and B (posterior probability >0.50) as well as between lineages A and C (posterior probability >0.50) (Fig. 7). Our outgroup, *S. australis*, consistently demonstrated independence with strong support (posterior probability $= 1.00$). However, under a divergence scenario with small ancestral population sizes and shallow divergence, we did not have any support for bifurcation of any nodes on the guide tree, including between the *S.* cf. *lessoniana* species complex and *S. australis* (posterior probability $= 0.00$, Fig. 7). Previous simulations with variations in the θ prior indicate that BP&P is particularly sensitive to small ancestral population sizes, and thus this outcome is likely a result of this (Leaché & Fujita, 2010; Yang & Rannala, 2010). The topology of the guide tree also influenced the number of evolutionary lineages inferred, for example, our second and third guide trees support three independent lineages (Fig. 7). Placement of divergent lineages as sister taxa can inflate what BP&P regards as a speciation event (Leaché & Fujita, 2010). This emphasizes the need for a reliable guide tree for accurate species delimitation estimates as random rearrangements of the tips impacts how many species are delimited. BP&P supports two to three evolutionary lineages depending on the guide tree topology.

While all three lineages were consistently recovered from all individual and concatenated genetic datasets, average divergence between each lineage varied depending on the gene region in question. In all datasets, distance between lineages was always much higher than intra-lineage values (Table 3). However, K2P distances between mitochondrial lineages were much greater than the distance separating the same lineages in the nuclear datasets (Table 3). This is reflected in the deeper phylogenetic relationships that were resolved in the mitochondrial trees (Fig. 5). Furthermore, genetic substructuring within the mitochondrial lineages was present and absent in the nuclear lineages (Table 3). Comparing lineages, haplotype diversity was comparatively similar in lineages B and C in all genes analyzed. Lineage A had comparatively much lower haplotype diversity than lineages B and C in all genes, except CO1 (Table 4). In the analyses with representative individuals, lineage C seemed to be the most commonly found among all sampled sites (Fig. 8). When examining the frequency of each lineage in all sampled individuals (including individuals with identical haplotypes), the representations of lineages B and C were much more common than lineage A (Fig. 8; Table 5).

Distribution and phylogeography

Examining the distribution of each lineage throughout the sampled localities, it is clear that geography does not delimit these cryptic lineages (Fig. 8). Lineages B and C are present in nearly all sampled localities, while lineage A seems to be only found in central Indonesia (Donggala, Manado, Raja Ampat) and in Banda Aceh. Lineage B was predominant in samples from both Vietnamese locations (Nha Trang and Phu Quoc Island), in southern (Bali) and central Indonesia (Manado), and in the northern Philippines (Ticao). Lineage C was predominant throughout most Indonesian sites (Pulau Seribu, Muncar, Donggala, Raja Ampat), southern India, and in the central Philippines (Dumaguete). In terms of abundance, lineage C was most commonly sampled followed by lineage B with lineage A being rare (Table 5). However, these results must be taken purely at descriptive value and not as a

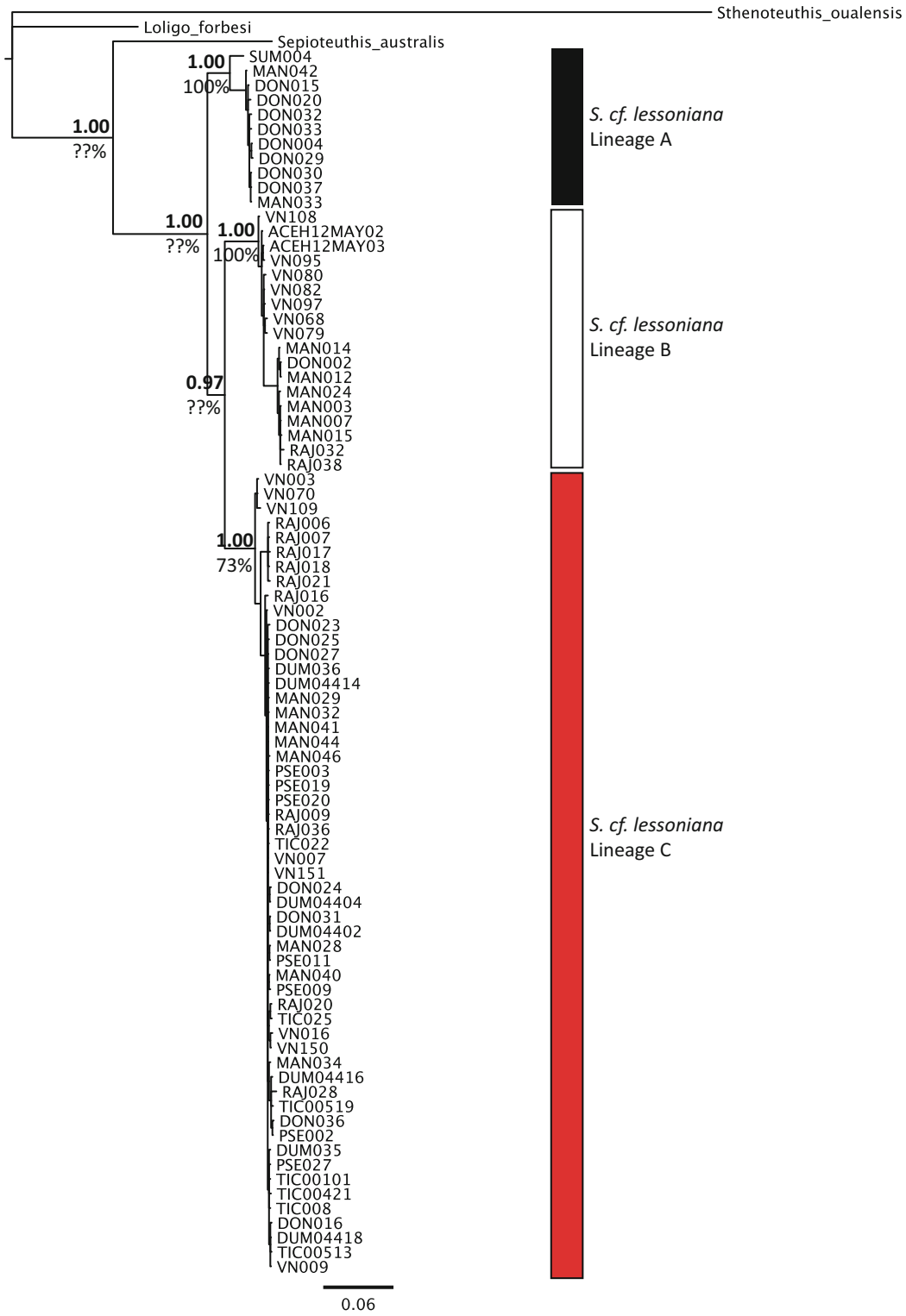

Fig. 3 All gene markers concatenated. Values above the node are Bayesian posterior probabilities and values below are bootstrap support from RAxML. Codes represent sampling locality and individual number (Table 1)

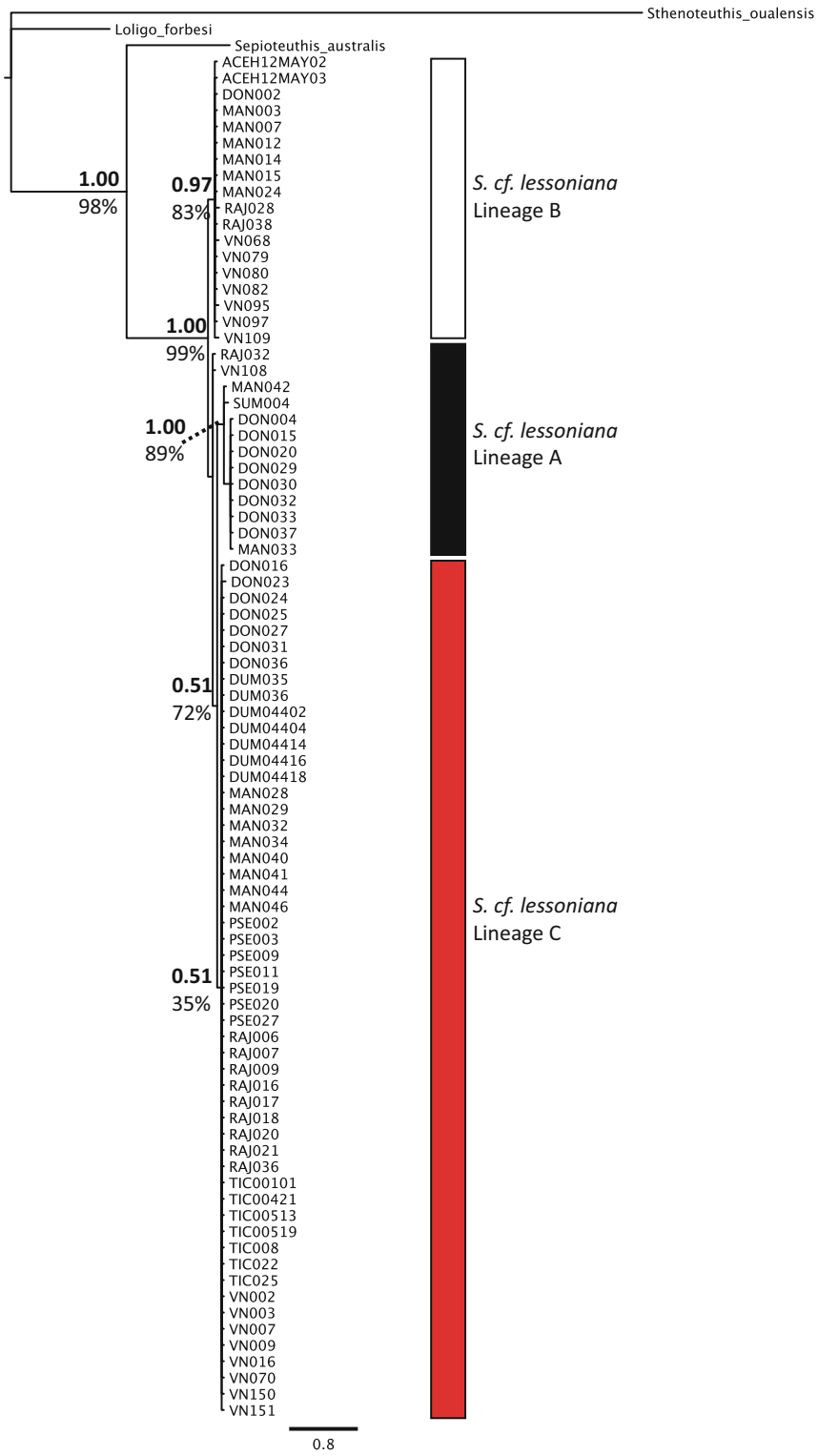

Fig. 4 Rhodopsin (Rhd) + octopine dehydrogenase (ODH) gene tree. *Values above the node* are Bayesian posterior probabilities and *values below* are bootstrap support from RAxML. *Codes* represent sampling locality and individual number (Table 1)

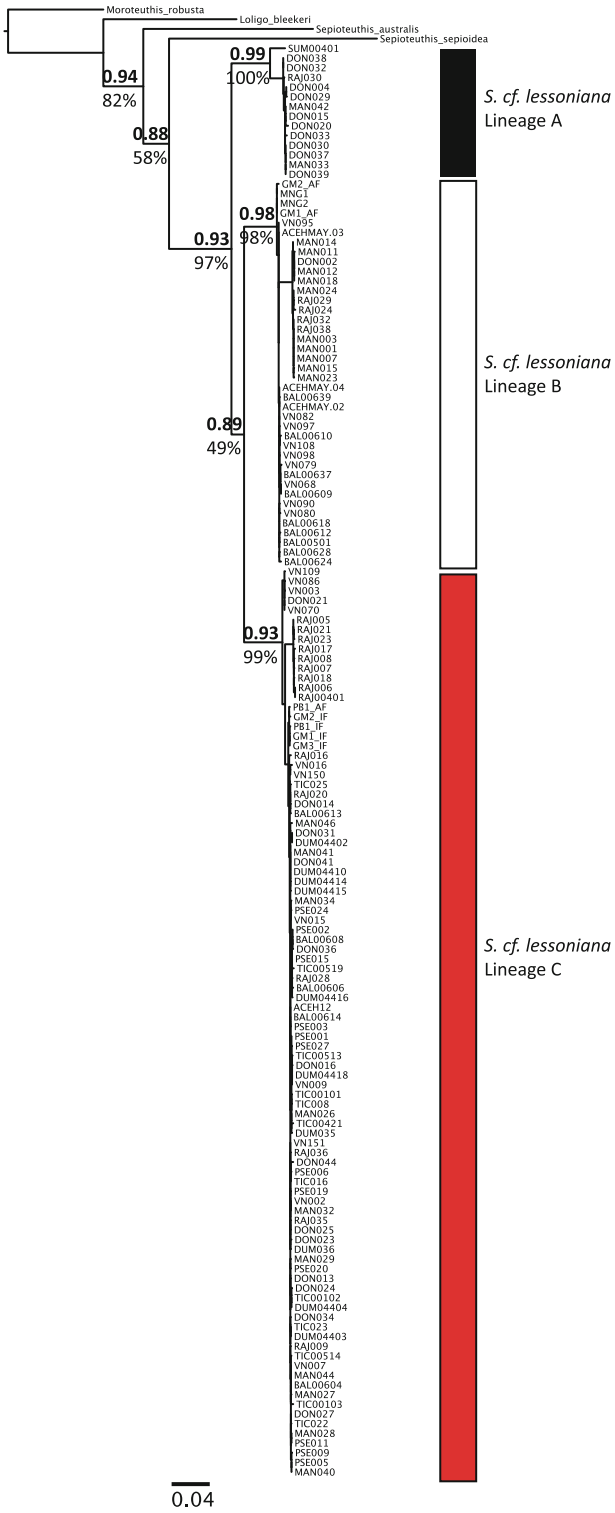

Fig. 5 Mitochondrial CO1 (CO1) + 16s rRNA (16s) gene tree. *Values above the node* are Bayesian posterior probabilities and *values below* are bootstrap support from RAxML. *Codes* represent sampling locality and individual number (Table 1)

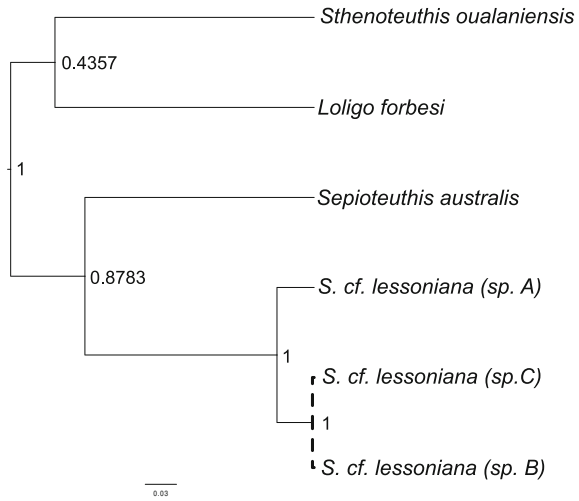

Fig. 6 Maximum clade credibility species tree determined from 255,000 post burn-in topologies from *BEAST. *Values at the nodes* are posterior probabilities

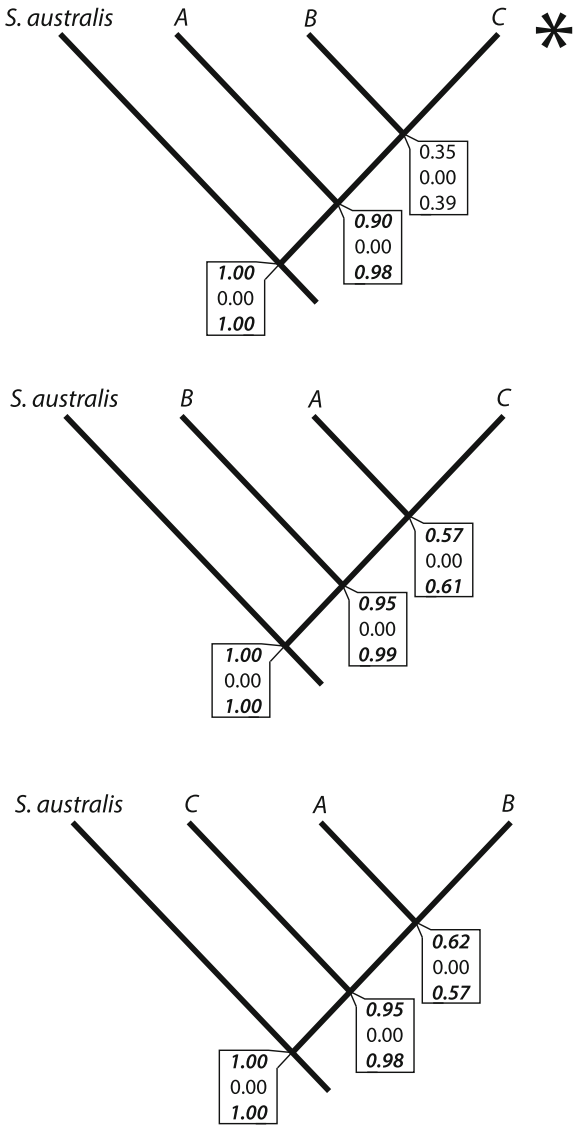

Fig. 7 Bayesian species delimitation results for the *Sepioteuthis* cf. *lessoniana* species complex assuming four species guide trees with the species tree from *BEAST denoted with a *asterisk*. Speciation probabilities from each combination of priors for θ and τ_0 are provided for each node with probabilities greater than 0.50 highlighted in *bold* and *italics*. *Top* θ and $\tau_0 \sim$ G(1,10); *middle* θ and $\tau_0 \sim$ G(2,2000); *bottom* $\theta \sim$ G(1,10); and $\tau_0 \sim$ G(2,2000). Different arrangements of taxa on the tips results in different speciation probabilities for one of the nodes within the complex

true indicator of occurrence as sample sizes between locations are extremely varied (Table 5). Haplotype diversity within each lineage was very high for the mitochondrial genes and for ODH (Table 5). There was not a clear pattern of difference between each lineage in terms of haplotype diversity as they were all similarly high. Rhodopsin had much lower levels of haplotype diversity, particularly for lineages A and C (Table 5). This corresponded to very low levels of variation between individuals seen in this gene region.

AMOVA results suggest that there is significant genetic substructuring between the three localities (Bali, Manado and Nha Trang) sampled in lineage B. Overall ϕ_{ST} was very high for all gene regions for lineage B with the majority of variation explained by among population differentiation. Comparing the population pairwise ϕ_{ST} values from all gene regions, samples from Manado were significantly strongly differentiated from Bali and Nha Trang (Table 6). There was low differentiation between Bali and Nha Trang, but this value was not significant. Haplotype diversity of each population from the mitochondrial data indicated that diversity is comparably high in Manado and Bali, but slightly lower in Nha Trang (Table 5). Nucleotide diversity is low for all localities.

Conversely, AMOVA results for lineage C suggest limited genetic substructuring between eight geographic localities sampled (Donggala, Dumaguete, Manado, Nha Trang, Phu Quoc, Pulau Seribu, Raja Ampat, and Ticao). Overall ϕ_{ST} was fairly low for all gene regions with the majority of variation explained by within locality differentiation (Table 6). Among population variation is driven by high levels of genetic

Table 3 Pairwise differences within and between lineages of *S.* cf. *lessoniana* complex based on Kimura-2-parameter values

Mitochondrial CO1				Mitochondrial 16s rRNA			
	A	B	C		A	B	C
A	*8.26*	85.54	88.30	A	*1.34*	18.92	21.96
B	77.28	*8.26*	79.97	B	17.07	*2.37*	17.28
C	82.05	73.72	*4.25*	C	20.87	15.78	*0.83*
Nuclear ODH				Nuclear rhodopsin			
	A	B	C		A	B	C
A	*0.19*	6.36	3.51	A	*0.58*	2.87	1.85
B	5.32	*1.89*	5.04	B	2.55	*0.07*	1.06
C	3.11	3.79	*0.60*	C	1.55	1.02	*0.005*

Average within-lineage pairwise difference on the diagonal (X), average number of pairwise between lineages above diagonal (Y) and average corrected between lineage pairwise difference below diagonal ($Y - (Y + X)/2$). All data values are significant ($P < 0.01$)

differentiation in mitochondrial gene regions between both Nha Trang and Raja Ampat and all localities. This pattern was not observed in the ODH dataset, rather only Raja Ampat was significantly differentiated from all localities save Nha Trang and Phu Quoc. All other localities at all gene regions had very low levels of differentiation in relationship to each other (Table 5). All populations were characterized by high haplotype diversity and low nucleotide diversity in both mitochondrial genes and the ODH gene (Table 5). Rhodopsin had very low levels of haplotype and nucleotide diversity (Table 5) and was found to contain numerous individuals with identical sequences.

Discussion

In lieu of obvious and observable morphological differences, use of molecular genetic tools has proved to be a powerful way to detect cryptic species via the phylogenetic species concept (rev. Bickford et al., 2007). Using this technique, many marine species that were once considered cosmopolitan are actually comprised of many genetically distinct species (Solé-Cava et al., 1991; Knowlton et al., 1992; Colborn et al., 2001; Westheide & Schmidt, 2003; Anderson et al., 2011). The data collected in this study allowed for a robust and extensive phylogenetic assessment of the *S.* cf. *lessoniana* complex. Analyses of both mitochondrial and nuclear genes consistently recovered three well-supported reciprocally monophyletic lineages from across the Coral Triangle (Figs. 3, 4, 5, 6). Deep divergences among lineages were found in mitochondrial (73–82% for CO1, 16–21% for 16s based on Kimura 2-parameter (K2P) distances) genes. Shallower divergences were observed in nuclear genes (4–5% for ODH and 1–3% for Rhd). The divergences found between lineages exceed values commonly observed for congeneric species (Avise, 2000) and similar very deep divergences in mitochondrial gene regions have been reported in cephalopods (Strugnell & Lindgren, 2007) including the *Sepia pharoanis* (Anderson et al., 2011) and *Pareledone turqueti* (Allcock et al., 2011) complexes. Furthermore, greater inter-lineage versus intra-lineage K2P distances along with reciprocal monophyly of each lineage provides extremely strong evidence that each of these lineages represents independent evolutionary trajectories typical of species and higher taxa that have evolved over long periods of time (Donoghue, 1985). All four gene trees, mitochondrial, and nuclear

Table 4 Haplotype diversity within resolved lineages of *S.* cf. *lessoniana* complex

Mitochondrial CO1				Mitochondrial 16s rRNA			
	A	B	C		A	B	C
Hap. Div	0.947 ± 0.034	0.847 ± 0.032	0.935 ± 0.011	Hap. Div	0.884 ± 0.36	0.893 ± 0.014	0.870 ± 0.016
Sample size	20	115	244	Sample size	24	118	229
Nuclear ODH				Nuclear rhodopsin			
	A	B	C		A	B	C
Hap. Div	0.763 ± 0.130	0.994 ± 0.004	0.974 ± 0.005	Hap. Div	0.442 ± 0.133	0.763 ± 0.038	0.392 ± 0.045
Sample size	20	69	193	Sample size	20	65	182

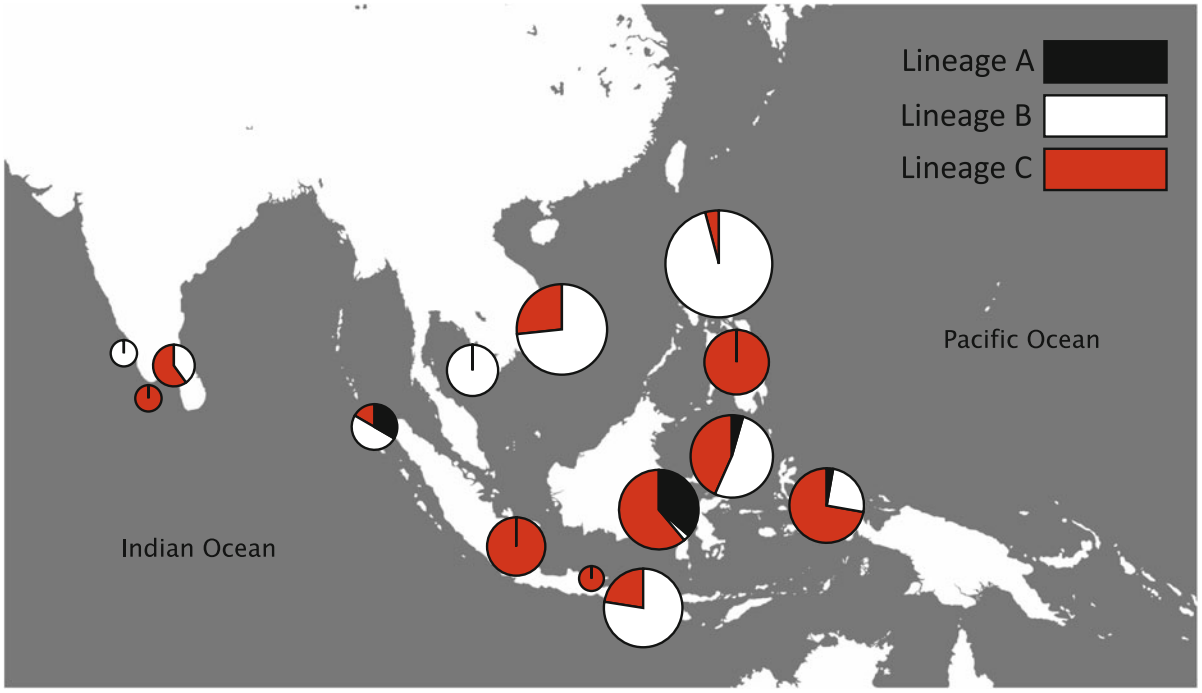

Fig. 8 Distribution of cryptic lineages throughout sampling range. Size of pie diagrams corresponds to sample size from that locality

datasets produced concordant topologies indicating the absence of hybridization or introgression and the presence of reproductive isolation (Mayr, 1942; Dobzhansky, 1970) despite co-occurrence at multiple sites. Inferences from species tree estimations and species delimitation methods support at least two to three evolutionary independent lineages (Figs. 6, 7) under a conservative approach. Fulfillment of multiple species concept criteria lends very strong support for species status to these cryptic lineages of *S.* cf. *lessoniana.*

Of all Loliginidae, *S.* cf. *lessoniana* has the largest distribution. Within this genus, *S. sepioidea* is restricted to the Caribbean and *S. australis* to southern Australia and New Zealand (Jereb & Roper, 2010). Past biogeographic hypotheses for *Sepioteuthis* indicate the migration of a Tethyan relic following the break-up of Pangaea from the Atlantic into the Pacific Ocean (Anderson, 2000). For *S.* cf. *lessoniana*, Anderson (2000) hypothesized that it diverged from its Caribbean sister taxon and spread through the Pacific through the Indian Ocean and eventually to the Red Sea and Mediterranean, subsequently becoming extinct in the East Pacific (2000). With a nearly cosmopolitan distribution, one would assume that *S.* cf. *lessoniana* must have much greater dispersal potential than its sister taxa. However, characteristics of paralarval and adult life history indicate that *S.* cf. *lessoniana* is likely the least dispersive of all Loliginidae. Hatchlings of *S.* cf. *lessoniana* are among the largest (4–10 mm) (Segawa, 1987; Jivaluk et al., 2005) in the family. These large hatchlings possess well-developed nervous systems (Shigeno et al., 2001) and are strong swimmers (Kier, 1996; Thompson & Kier, 2001), thus highly unlikely to behave as a "passive" disperser. In fact, even among other loliginid species with much smaller sizes at hatching (e.g., *Loligo opalescens* 3–4 mm; Fields, 1965) there is strong evidence for advanced paralarval navigation in order to entrain themselves in favorable oceanographic areas (Okutani & McGowan, 1969; Zeidberg & Hamner, 2002). Specifically, paralarval *S.* cf. *lessoniana* orient over dark areas indicating active navigation to reef habitat (Lee et al., 1994) and demonstrate active hunting and shoaling behavior shortly after hatching (Choe, 1966; Sugimoto & Ikeda, 2012).

Similarly, the movement of adults is restricted to coastal habitats, possibly extending no more than 16.2 km (*S.* cf. *lessoniana*—Ueta & Jo, 1990) to 35 km (*S. australis*—Pecl et al., 2006) along the coast during its short lifetime. *S.* cf. *lessoniana* commonly

Table 5 Diversity indices (haplotype diversity (hap. div.) and nucleotide diversity (nuc. div.) for each locality and lineage using four gene regions

	Locality	*n*	Marker	Lineage B			Lineage C		
				n.p.	Hap. Div	Nuc. Div	n.p.	Hap. Div	Nuc. Div
1	Dumaguete, Negros Oriental	27	mtCO1	–			27	0.93 ± 0.04	0.003 ± 0.002
	Philippines		mt16s	–			30	0.45 ± 0.10	0.001 ± 0.001
			nucODH	2	*	*	29	0.74 ± 0.07	0.001 ± 0.001
			nucRhd	–			20	0.00 ± 0.00	0.000 ± 0.000
2	Ticao, Luzon	74	mtCO1	4	*	*	71	0.89 ± 0.03	0.002 ± 0.002
	Philippines		mt16s	3	*	*	52	0.58 ± 0.07	0.001 ± 0.001
			nucODH	4	*	*	59	0.74 ± 0.03	0.0003 ± 0.0004
			nucRhd	3	*	*	53	0.00 ± 0.00	0.000 ± 0.000
4	Raja Ampat, West Papua	36	mtCO1	9	*	*	26	0.95 ± 0.03	0.014 ± 0.007
	Indonesia		mt16s	9	*	*	27	0.68 ± 0.06	0.006 ± 0.004
			nucODH	7	*	*	25	0.82 ± 0.07	0.001 ± 0.001
			nucRhd	6	*	*	26	0.08 ± 0.07	0.0002 ± 0.0003
5	Bali	40	mtCO1	31	0.62 ± 0.10	0.001 ± 0.001	9	*	*
	Indonesia		mt16s	31	0.53 ± 0.10	0.001 ± 0.001	9	*	*
			nucODH	1	*	*	–		
			nucRhd	1	*	*	–		
9	Phu Quoc Island	17	mtCO1	–			17	0.73 ± 0.11	0.004 ± 0.003
	Vietnam		mt16s	–			17	0.57 ± 0.13	0.002 ± 0.001
			nucODH	–			17	0.85 ± 0.07	0.001 ± 0.001
			nucRhd				15	0.00 ± 0.00	0.003 ± 0.002
10	Nha Trang	53	mtCO1	41	0.59 ± 0.09	0.006 ± 0.004	14	0.84 ± 0.07	0.013 ± 0.007
	Vietnam		mt16s	40	0.23 ± 0.09	0.0005 ± 0.0006	14	0.60 ± 0.08	0.002 ± 0.002
			nucODH	36	0.96 ± 0.02	0.001 ± 0.001	12	0.85 ± 0.07	0.001 ± 0.001
			nucRhd	35	0.17 ± 0.08	0.000 ± 0.000	13	0.15 ± 0.13	0.000 ± 0.000
11	Pulau Seribu, Java	22	mtCO1	–			26	0.86 ± 0.07	0.003 ± 0.002
	Indonesia		mt16s	–			25	0.47 ± 0.11	0.001 ± 0.001
			nucODH	–			16	0.78 ± 0.07	0.001 ± 0.001
			nucRhd				16	0.13 ± 0.11	0.000 ± 0.000
12	Donggala, Sulawesi	41	mtCO1	1	*	*	25	0.90 ± 0.05	0.006 ± 0.003
	Indonesia		mt16s	1	*	*	25	0.53 ± 0.11	0.002 ± 0.001
			nucODH	1	*	*	17	0.76 ± 0.08	0.0001 ± 0.0002
			nucRhd	1	*	*	20	0.00 ± 0.00	0.000 ± 0.000
13	Manado, Sulawesi	44	mtCO1	23	0.73 ± 0.07	0.003 ± 0.003	19	0.82 ± 0.07	0.002 ± 0.001
	Indonesia		mt16s	25	0.48 ± 0.09	0.001 ± 0.001	19	0.37 ± 0.14	0.001 ± 0.001
			nucODH	15	0.48 ± 0.15	0.000 ± 0.000	14	0.57 ± 0.13	0.0001 ± 0.0002
			nucRhd	17	0.00 ± 0.00	0.000 ± 0.000	16	0.00 ± 0.00	0.000 ± 0.000

The total sample size (*n*), the population sample size for that lineage (n.p.), and standard deviations for diversity values are shown here. Insufficient sample sizes are indicated by asterisk and the absence of data by a "–"

shelter on the reef during the day and feed on it in the evenings. While they can make large vertical migrations (~100 m, Jereb & Roper, 2006), they are commonly observed in extremely shallow waters (1–3 m) in multiple areas of their range (pers. obs.). Furthermore, egg masses are commonly found deposited in shallow areas (majority 1–10 m) on reefs, debris, and mooring lines (Wada & Kobayashi, 1995,

Table 6 Genetic differentiation (AMOVA) results for populations of each lineage that had sequence data for $n > 10$

	mtco1					mt16s					nucODH				
	n	n.p.	%	ST	*P*	*n*	n.p.	%	ST	*P*	*n*	n.p.	%	ST	*P*
Lineage B	95	3				105	4				58	3			
Among populations			77.51	0.775	**0.000**			86.14	0.861	**0.000**			51.33	0.513	**0.000**
Within populations			22.49					13.86					48.67		
Lineage C	225	8				209	8				189	8			
Among populations			23.24	0.232	**0.000**			22.70	0.227	**0.000**			5.75		**0**
Within populations			76.76					77.30					94.25		

The number of individuals (*n*), number of populations (n.p.) percentage of variation explained by within and between population differences (%), ϕ_{ST} statistics and significance value (*P*) are shown for each lineage and gene region. Bold significance values indicate a *P* value <0.05

pers. obs.). Availability of suitable habitats may be a limiting factor for long-distance migration, as the short lifespan of the squid needs to be weighed against finding areas for feeding and spawning.

In the Coral Triangle, the Indonesian Throughflow transports water at 20 × 106 m^3/s from the Pacific to the Indian Ocean (Godfrey, 1996; Gordon & Fine, 1996), while surface currents can reach speeds of 1 m/s (Wyrtki, 1961), posing strong limits to migration for adult *S.* cf. *lessoniana*. While *S.* cf. *lessoniana* are strong swimmers, energetic limits restrict movement and adult squid actively seek out specific current regimes to conserve energy (O'Dor et al., 2002). The physical barriers of the Coral Triangle have been well-demonstrated to pose limits to gene flow and dispersal in many diverse marine organisms (e.g., DeBoer et al., 2008; Barber et al., 2011; Fauvelot & Borsa, 2011). While in the present study, all three lineages are co-distributed over the Coral Triangle, they demonstrate markedly different patterns of population differentiation. Particularly, AMOVA results do not indicate any barriers to gene flow in lineage C, while lineage B showed higher levels of genetic differentiation among localities (Table 5). Unfortunately, sample sizes were large enough for analyses in only three out of the nine localities for lineage B. However, in the three localities (Manado, Bali, and Nha Trang) examined, there are strong genetic breaks suggesting that gene flow and dispersal may be limited between geographic areas. Fast currents, deep water, landmasses, and long distances in particular separate these three localities. Overall, the biological characteristics of this squid—coastal spawning and feeding habitat, large hatchling size, and restricted adult movement and behavior—provide decreased opportunity and ability for widespread dispersal. In light of the pronounced genetic divergence and evidence for at least two to three delimited species in the present study and the life history of *S.* cf. *lessoniana*, the evolution of cryptic species is expected given the ample opportunities for genetically isolated populations.

While the biological characteristics of *S.* cf. *lessoniana* lend itself to scenarios of high rates of divergence and speciation, the co-occurrence of these cryptic species raises more complex questions regarding the evolution and ecology of this species complex. Previous studies detected genetically distinct cryptic species of *S.* cf. *lessoniana* with varying degrees of morphological variation co-occurring in two relatively small geographic locations (Izuka et al., 1994; Triantafillos & Adams, 2005). Triantafillos & Adams theorized that the co-occurrence in Shark Bay, Australia was a result of an occasional overlap between an Indian Ocean and Pacific Ocean species that otherwise have non-overlapping distributions (2005). However, this present study indicates that co-occurrence seems to be quite widespread (Fig. 6) from Indian Ocean to western Pacific locations. While it could be theorized that the co-occurrence in the Coral Triangle is overlap between Indian and Pacific Ocean sister taxa, more sampling at the edges of the range (e.g., Hawaii, Oceania nations, and eastern Africa) is needed to confirm.

The detection of co-occurring cryptic species raises interesting questions concerning the ecology and evolution of these species. However, more pressingly, it also raises serious questions about the validity of biological and ecological characteristics determined for *S.* cf. *lessoniana*. For example, the flexible

reproductive strategies demonstrated by Pecl (2001) and Jackson & Moltschaniwsky (2002) which identified marked spatial and temporal heterogeneity for growth and life history characteristics in *S.* cf. *lessoniana*, may actually represent different life histories between the cryptic species. Furthermore, these squids have been observed to spawn at different seasons, on different substrates, and with different egg morphologies throughout their range (Rao, 1954; Segawa, 1987; Ueta & Jo, 1989; Chotiyaputta, 1993; Segawa et al., 1993a, b; Izuka et al., 1994) raising the possibility that these variations may actually be fixed characteristics for each species.

The three lineages were present in markedly different abundances at all sites (Fig. 6). Lineage C was appears to be most abundant, while Lineages B and A were less abundant, but co-occurred with lineage C and occasionally each other (Fig. 6). Analyses were not conducted for lineage A due to small sample size. However, the low abundance of lineage A suggests that it either occurs in habitats not usually fished or it has a much more restricted distribution. While these differences may be due to differences in sampling effort or gear used, they may be rooted in different ecological, behavioral, or biological characteristics of the three cryptic species. Particularly, sympatric co-occurrence of closely related species in marine environments has been attributed to factors such as climatic gradients, asynchrony of maturation, habitat specificity, and ecological niche partitioning [e.g., *Aurelia aurita* (Schroth et al., 2002), marine microbes (rev. Fenchel, 2005), *Micromonas* microeukaryotic alga (Slapeta et al., 2006), *Amphipholis squamata* brittle stars complex (Sponer & Roy, 2002)]. *S.* cf. *lessoniana*'s large range encompasses a plethora of diverse physical and biological factors resulting in many different habitats, ecological communities, and oceanographic regimes that could facilitate co-occurrence. However, these questions require much more in-depth investigations.

Conclusions

These results expand on previous studies of *S.* cf. *lessoniana* in Shark Bay, Australia (Triantafillos & Adams, 2005) and Ishigaki, Japan (Segawa et al., 1993b; Izuka et al., 1994), which suggested the presence of cryptic co-occurring lineages. These studies in Australia and Japan only focused on small spatial areas and found evidence for sympatric cryptic species with limited morphological differences. Upon expanding the range of study to the Indo-Pacific and Indian Oceans, we found similar results of cryptic species occurring sympatrically (Fig. 6). Like these previous studies, recovered cryptic lineages from this study were strongly differentiated and separated by large genetic distances (Tables 3, 5). However, because these studies employed allozymes, it is impossible to determine whether these are the same cryptic lineages detected in this study or whether there may more than three cryptic species of *S.* cf. *lessoniana*. Our use of CO1 DNA sequence data, the universal barcode marker (Hebert et al., 2003; Allcock et al., 2011) will lay the groundwork for comparison of squid populations from other regions, allowing the determination of how many cryptic species exist in this taxon. However, this study provides a phylogenetic basis for species delimitation, but for a comprehensive taxonomic reassessment, these results need be combined with detailed morphological characterizations.

S. cf. *lessoniana* is of economic interest throughout its range, supporting commercial, and artisanal fisheries (Chikuni, 1983; Soselisa et al., 1986; Sudjoko, 1987; Chotiyaputta, 1993; Tokai & Ueta, 1999; Nateewathana et al., 2000; FAO, 2009). Maintenance of sustainable fisheries is dependent on accurate life history and growth information, particularly as successful recruitment and population abundance for squids are dependent on specific environmental conditions (Boyle & Boletzky, 1996; Forsythe et al., 2001; Agnew et al., 2005; Pecl & Jackson, 2008). Thus, flexible growth and reproductive strategies may represent a viable natural mechanism to cope with changing conditions, avoiding major crashes in population biomass. However, in light of the genetic evidence of widespread co-occurring cryptic species presented in this study, it is absolutely crucial that efforts are undertaken to accurately determine specific life histories for each species.

The GenBank accession numbers for all original sequences from this study are included in Table 7.

Table 7 GenBank accession numbers for sequences generated in this study

ID No.	Isolate	CO1	16s	ODH	Rhd
ACEH12MAY05	C	KF052359	KF052136		
ACEH12MAY02	B	KF052360	KF052137	KF052498	KF052275
ACEH12MAY03	B	KF052361	KF052138	KF052499	KF052276
ACEH12MAY04	B	KF052362	KF052139		
BAL00501	B	KF052363	KF052140		
BAL00604	C	KF052364	KF052141		
BAL00606	C	KF052365	KF052142		
BAL00608	C	KF052366	KF052143		
BAL00609	B	KF052367	KF052144		
BAL00610	B	KF052368	KF052145		
BAL00612	B	KF052369	KF052146		
BAL00613	C	KF052370	KF052147		
BAL00614	C	KF052371	KF052148		
BAL00618	B	KF052372	KF052149		
BAL00624	B	KF052373	KF052150		
BAL00628	B	KF052374	KF052151		
BAL00637	B	KF052375	KF052152		
BAL00639	B	KF052376	KF052153		
DON002	B	KF052377	KF052154	KF052500	KF052277
DON004	A	KF052378	KF052155	KF052501	KF052278
DON013	C	KF052379	KF052156		
DON014	C	KF052380	KF052157		
DON015	A	KF052381	KF052158	KF052502	KF052279
DON016	C	KF052382	KF052159	KF052503	KF052280
DON020	A	KF052383	KF052160	KF052504	KF052281
DON021	C	KF052384	KF052161		
DON023	C	KF052385	KF052162	KF052505	KF052282
DON024	C	KF052386	KF052163	KF052506	KF052283
DON025	C	KF052387	KF052164	KF052507	KF052284
DON027	C	KF052388	KF052165	KF052508	KF052285
DON029	A	KF052389	KF052166	KF052509	KF052286
DON030	A	KF052390	KF052167	KF052510	KF052287
DON031	C	KF052391	KF052168	KF052511	KF052288
DON032	A	KF052392	KF052169	KF052512	KF052289
DON033	A	KF052393	KF052170	KF052513	KF052290
DON034	C	KF052394	KF052171		
DON036	C	KF052395	KF052172	KF052514	KF052291
DON037	A	KF052396	KF052173	KF052515	KF052292
DON038	A	KF052397	KF052174		
DON039	A	KF052398	KF052175		
DON041	C	KF052399	KF052176		
DON044	C	KF052400	KF052177		
DUM035	C	KF052401	KF052178	KF052516	KF052293
DUM036	C	KF052402	KF052179	KF052517	KF052294

Table 7 continued

ID No.	Isolate	CO1	16s	ODH	Rhd
DUM04402	C	KF052403	KF052180	KF052518	KF052295
DUM04403	C	KF052404	KF052181		
DUM04404	C	KF052405	KF052182	KF052519	KF052296
DUM04410	C	KF052406	KF052183		
DUM04414	C	KF052407	KF052184	KF052520	KF052297
DUM04415	C	KF052408	KF052185		
DUM04416	C	KF052409	KF052186	KF052521	KF052298
DUM04418	C	KF052410	KF052187	KF052522	KF052299
GM1_AF	B	KF019365	KF019356		
GM1_IF	C	KF019366	KF019357		
GM2_AF	B	KF019367	KF019358		
GM2_IF	C	KF019368	KF019359		
GM3_IF	C	KF019369	KF019360		
MAN001	B	KF052411	KF052188		
MAN003	B	KF052412	KF052189	KF052523	KF052300
MAN007	B	KF052413	KF052190	KF052524	KF052301
MAN011	B	KF052414	KF052191		
MAN012	B	KF052415	KF052192	KF052525	KF052302
MAN014	B	KF052416	KF052193	KF052526	KF052303
MAN015	B	KF052417	KF052194	KF052527	KF052304
MAN018	B	KF052418	KF052195		
MAN023	B	KF052419	KF052196		
MAN024	B	KF052420	KF052197	KF052528	KF052305
MAN026	C	KF052421	KF052198		
MAN027	C	KF052422	KF052199		
MAN028	C	KF052423	KF052200	KF052529	KF052306
MAN029	C	KF052424	KF052201	KF052530	KF052307
MAN032	C	KF052425	KF052202	KF052531	KF052308
MAN033	A	KF052426	KF052203	KF052532	KF052309
MAN034	C	KF052427	KF052204	KF052533	KF052310
MAN040	C	KF052428	KF052205	KF052534	KF052311
MAN041	C	KF052429	KF052206	KF052535	KF052312
MAN042	A	KF052430	KF052207	KF052536	KF052313
MAN044	C	KF052431	KF052208	KF052537	KF052314
MAN046	C	KF052432	KF052209	KF052538	KF052315
MNG1	B	KF019370	KF019361		
MNG2	B	KF019371	KF019362		
PB1_AF	C	KF019372	KF019363		
PB1_IF	C	KF019373	KF019364		
PSE001	C	KF052433	KF052210		
PSE002	C	KF052434	KF052211	KF052539	KF052316
PSE003	C	KF052435	KF052212	KF052540	KF052317
PSE005	C	KF052436	KF052213		
PSE006	C	KF052437	KF052214		

Table 7 continued

ID No.	Isolate	CO1	16s	ODH	Rhd
PSE009	C	KF052438	KF052215	KF052541	KF052318
PSE011	C	KF052439	KF052216	KF052542	KF052319
PSE015	C	KF052440	KF052217		
PSE019	C	KF052441	KF052218	KF052543	KF052320
PSE020	C	KF052442	KF052219	KF052544	KF052321
PSE024	C	KF052443	KF052220		
PSE027	C	KF052444	KF052221	KF052545	KF052322
RAJ00401	C	KF052445	KF052222		
RAJ005	C	KF052446	KF052223		
RAJ006	C	KF052447	KF052224	KF052546	KF052323
RAJ007	C	KF052448	KF052225	KF052547	KF052324
RAJ008	C	KF052449	KF052226		
RAJ009	C	KF052450	KF052227	KF052548	KF052325
RAJ016	C	KF052451	KF052228	KF052549	KF052326
RAJ017	C	KF052452	KF052229	KF052550	KF052327
RAJ018	C	KF052453	KF052230	KF052551	KF052328
RAJ020	C	KF052454	KF052231	KF052552	KF052329
RAJ021	C	KF052455	KF052232	KF052553	KF052330
RAJ023	C	KF052456	KF052233		
RAJ024	B	KF052457	KF052234		
RAJ028	C	KF052458	KF052235	KF052554	KF052331
RAJ029	B	KF052459	KF052236		
RAJ030	A	KF052460	KF052237		
RAJ032	B	KF052461	KF052238	KF052555	KF052332
RAJ035	C	KF052462	KF052239		
RAJ036	C	KF052463	KF052240	KF052556	KF052333
RAJ038	B	KF052464	KF052241	KF052557	KF052334
SUM004	A	KF052465	KF052242	KF052558	KF052335
TIC00101	C	KF052466	KF052243	KF052559	KF052336
TIC00102	C	KF052467	KF052244		
TIC00103	C	KF052468	KF052245		
TIC00421	C	KF052469	KF052246	KF052560	KF052337
TIC00513	C	KF052470	KF052247	KF052561	KF052338
TIC00514	C	KF052471	KF052248		
TIC00519	C	KF052472	KF052249	KF052562	KF052339
TIC008	C	KF052473	KF052250	KF052563	KF052340
TIC016	C	KF052474	KF052251		
TIC022	C	KF052475	KF052252	KF052564	KF052341
TIC023	C	KF052476	KF052253		
TIC025	C	KF052477	KF052254	KF052565	KF052342
VN002	C	KF052478	KF052255	KF052566	KF052343
VN003	C	KF052479	KF052256	KF052567	KF052344
VN007	C	KF052480	KF052257	KF052568	KF052345
VN009	C	KF052481	KF052258	KF052569	KF052346

Table 7 continued

ID No.	Isolate	CO1	16s	ODH	Rhd
VN015	C	KF052482	KF052259		
VN016	C	KF052483	KF052260	KF052570	KF052347
VN068	B	KF052484	KF052261	KF052571	KF052348
VN070	C	KF052485	KF052262	KF052572	KF052349
VN079	B	KF052486	KF052263	KF052573	KF052350
VN080	B	KF052487	KF052264	KF052574	KF052351
VN082	B	KF052488	KF052265	KF052575	KF052352
VN086	C	KF052489	KF052266		
VN090	B	KF052490	KF052267		
VN095	B	KF052491	KF052268	KF052576	KF052353
VN097	B	KF052492	KF052269	KF052577	KF052354
VN098	B	KF052493	KF052270		
VN108	B	KF052494	KF052271	KF052578	KF052355
VN109	C	KF052495	KF052272	KF052579	KF052356
VN150	C	KF052496	KF052273	KF052580	KF052357
VN151	C	KF052497	KF052274	KF052581	KF052358

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CHAPTER 2

(Not) going the distance: contrasting patterns of geographical subdivision in sibling taxa of reef squid (Loliginidae: *Sepioteuthis* cf. *lessoniana*) in the Coral Triangle

Abstract

Diversification in the marine environment has long been assumed to be driven by primarily allopatric (i.e. physical) barriers to gene flow. Recent studies indicate that this explanation is too simplistic and that a multitude of both physical and ecological drivers exist and interact to generate and maintain diversity. However, insight into these drivers as limited as most previous studies focused on benthic organisms and they depended on primarily, single markers, lacking sufficient power to illuminate fine-scale patterns of population structure. In light of this, this study uses a comparative population genomic approach to examine barriers to gene flow in two co-occurring, cryptic species of the bigfin reef squid (*Sepioteuthis* cf. *lessoniana*), a neritic organism within the center of marine biodiversity (the Coral Triangle). We used mitochondrial COI and ~2,000 genome-wide single nucleotide polymorphisms generated using a restriction enzyme associated digest method (RADseq) (2b-RAD, Wang et al. 2012) to examine population structure across the Coral Triangle. We found that previously hypothesized barriers to gene flow in this region (Pleistocene glaciation cycles and prevailing physical oceanographic patterns) do not equally shape population structure in these reef squids. We observe drastically discordant patterns of connectivity between these sibling taxa, implying that lineage B has much more limited dispersal capacity than lineage C, highlighting the importance of dispersal life history for shaping individual species responses to physical drivers of gene flow. Genomic-scale examinations also allowed us to identify fine-scale patterns of very limited

connectivity in Lineage B. Use of these markers also revealed evidence of potential reinforcement of neutral divergence patterns through regional environmental differences, suggesting that selective processes play a role in driving population structure in bigfin reef squids. Overall, this study increases our understanding of the drivers of diversity in the Coral Triangle beyond inference from primarily benthic and strongly reef-associated taxa.

Introduction

Allopatric (physical) barriers have long been regarded as the primary starting point for restricting gene flow and driving population divergence in natural populations (Dobzhansky 1937; Mayr 1942; Avise 2000; Coyne and Orr 2004). However, this view stems from decades of studies heavily biased towards terrestrial and freshwater organisms (Miglietta et al. 2011), but is not a natural fit to marine ecosystems with their fluid nature and absence of conspicuous barriers to gene flow. The high dispersal capacity of adult and larval stages of many marine species (such as broadcast spawners and migratory species) was assumed to limit opportunities for allopatric divergence, resulting in widely distributed, panmictic species with little or no genetic differentiation (Palumbi 1994; Shanks et al. 2003). Clear examples of allopatry in the sea were limited to new terrestrial barriers like the rise of the Isthmus of Panama (Lessios 1981; Bermingham et al. 1997) or the inability of larvae to successfully disperse over vast stretches of open ocean regions like the East Pacific Barrier (Ekman 1953; Briggs 1973; Lessios and Robertson 2006).

The application of genetic tools over the past few decades has dramatically transformed our understanding of population divergence and speciation in the sea, revealing much more limited dispersal and higher levels of genetic structuring than expected. For example, genetic

studies revealed that many cosmopolitan species are, in fact, comprised of species complexes (Solé-Cava et al. 1991; Knowlton 1993; Schroth et al. 2002). Marine populations can by highly structured (Hellberg et al. 2002; Swearer et al. 2002) as larvae fail to reach their dispersal potential due to behavioral and environmental factors mediating effective dispersal (Kingsford et al. 2002; Pineda et al. 2007; Jones et al. 2009). Moreover, an increasing number of studies highlight the importance of selective processes in shaping marine population structure and divergence – such as host specificity, post-settlement survival, and ecological niche diversification (Johannesson 2001; Faucci et al. 2007; Vigliola et al. 2007). Combined, these and other studies indicate that the factors affecting marine larval dispersal and population diversity are much more complex than previously thought (Palumbi 1992; Shanks et al. 2003).

Spanning Indonesia, Malaysia, Papua New Guinea, the Philippines, and East Timor, the Coral Triangle is the world's most biodiverse marine ecosystem (Hoeksema 2007; Veron et al. 2009), spawning considerable interest in the processes generating this diversity (Bowen et al. 2013; Barber and Meyer 2015). Prevailing theories suggest that high levels of inter- and intra-specific diversity can be attributed to the dynamic geological history of the Coral Triangle and to its complex oceanographic patterns. Numerous phylogeographic studies in a wide diversity of marine taxa provide strong evidence for allopatric divergence over the Sunda Shelf, which was repeatedly exposed during Pleistocene glaciation cycles creating a land barrier between the Pacific and Indian Oceans (Barber et al. 2002; Barber et al. 2006; Crandall et al. 2008a;b; DeBoer et al. 2008; Drew and Barber 2009; Gaither et al. 2011; Carpenter et al. 2011). Similarly, phylogeographic studies (Barber et al. 2006; Barber et al. 2011; Ackiss et al. 2013; Timery S DeBoer et al. 2014) as well as coupled bio-physical dispersal models (Kool et al. 2011; Treml and Halpin 2012) suggest that physical oceanographic processes in the western boundary

currents can also promote isolation and divergence. Yet, large-scale comparative phylogeographic meta-analyses indicate an abundance of discordant phylogeographic patterns, even amongst closely related species, as well as potential cryptic species with substantial regions of sympatry (Meyer et al. 2005; Plaisance et al. 2009; Barber et al. 2011; Carpenter et al. 2011), despite the expectation that broad-scale physical processes should result in similar structure in co-distributed taxa (Avise 2000).

Mitochondrial DNA has been the steady workhorse in studies of marine diversification (Bowen et al. 2014), illuminating the complexity of processes shaping gene flow, isolation, and divergence in marine environments (Hedgecock 1994; Hellberg 2009), particularly in the Coral Triangle. While mitochondrial DNA and microsatellites have been (and still are) valuable tools for elucidating patterns of population differentiation (Bowen et al. 2014), they lack power to precisely estimate the relative effects of demographic factors (e.g. gene flow, drift) versus selection and adaptive variation (rev. Storz 2005; Narum et al. 2013). Moreover, while traditional genetic markers are extraordinarily useful for historical connectivity and divergence, their ability to understand connectivity on ecologically relevant timescales is limited (Hedgecock et al. 2007; Allendorf et al. 2010). This limits our understanding the physical and biological processes underlying contemporary patterns of gene flow.

The advent of next-generation sequencing (NGS) technology exponentially increases our ability to tease apart the relative contributions of neutral versus non-neutral processes governing gene flow and divergence at historical and contemporary time scales. NGS allows for simultaneous screening of thousands to tens of thousands of putative loci throughout the genome. This increased power has the potential to increase the reliability and accuracy of estimates of demographic and population parameters (Brumfield et al. 2003) as well as identify putative

adaptive loci to detect ongoing speciation and adaptive divergence (Feder and Nosil 2010), processes that could contribute to discordant patterns of population genetic structure in co-distributed taxa. The application of such genomic markers in marine species are few, but steadily growing (Willette et al. 2014). Specifically, RAD sequencing has been used to great success in detecting broad and fine-scale population structure in wild populations such as clownfish (Planes et al. 2009), herring (Lamichhaney et al. 2012), and anchovies (Zarraonaindia et al. 2012).

Another challenge to inferring barriers to gene flow and processes driving divergence in the Coral Triangle is that most studies to date have focused on reef-associated and inshore taxa, largely to the exclusion of mobile neritic marine taxa. Theoretically, the high fecundity, high levels of active migration and passive dispersal typical of pelagic and neritic marine organisms are predicted to result in dynamic, unstructured populations over large spatial areas (Waples 1998). However, the few studies to date of pelagic and neritic marine species in the Coral Triangle indicate fine-scale patterns of differentiation (Perrin and Borsa 2001; Borsa 2003; Jackson et al. 2014). In addition, these studies focus exclusively on teleost fishes. Even though cephalopods play key roles in marine trophic webs and ecosystems (Clarke 1996; Piatkowski et al. 2001), to date, there has been very little research conducted on genetic structure in cephalopods, much less squid, in the Indo-West Pacific (however, see Yeatman and Benzie 1994; Sin et al. 2009; Anderson et al. 2010; Tomano et al. 2015), and none within the Coral Triangle.

The bigfin reef squid (*Sepioteuthis* cf. *lessoniana*, Lesson 1830) is common in the tropical coastal waters of the Indo-West Pacific region. Recent genetic studies confirmed that this commercially valuable species is comprised of at least three reciprocally monophyletic, but co-distributed cryptic species (Cheng et al. 2014). Previous population-level studies in this

species complex demonstrated restricted gene flow over deep ocean basins and extremely large geographic distances (e.g., between Japan and Thailand, Aoki et al. 2008), although the scope and resolution of these single and multilocus studies was limited.

The goal of this study is to advance our understanding of evolution of marine biodiversity in the Coral Triangle by specifically investigating: 1) patterns of connectivity in neritic taxa and 2) the relative roles of neutral versus non-neutral processes in governing gene flow and divergence. We aim to address these using a comparative phylogeographic analysis of sympatrically distributed populations of sibling taxa of the neritic bigfin reef squid (S. cf. lessoniana). Using a multilocus approach using both traditional (mitochondrial) and next-generation sequencing methodologies (genome-wide SNPs from RAD-based sequencing), we compared two widely co-occurring sibling lineages of *S.* cf. *lessoniana* (B and C, Cheng et al. 2014) in the Coral Triangle and peripheral areas to test for divergence across multiple known phylogeographic barriers. We also searched for outlier loci that might indicate adaptive divergence. As large-scale physical processes should produce similar patterns of regional genetic structure in organisms with similar dispersal life histories and ecologies (Avise et al. 1987), comparing sibling taxa allows for a robust test of the relative influence of previously hypothesized physical barriers to gene flow. Furthermore, by utilizing genome-wide markers, we can begin to tease apart the relative roles of neutral versus selective processes in shaping gene flow in neritic organisms, allowing for a broader understanding of the processes underlying the center of marine biodiversity.

Methodology

Specimen collection

We collected a total of 706 embryos, hatchlings, juveniles and adults from local fish markets and by hand-jigging from small boats and shores (day and night) at 17 locations throughout the Indo-West Pacific range of *S. cf. lessoniana* (Figure 2.2.1) (Table 2.2.1). For juveniles and adults, a 1-2 cm piece of tissue from the ventral opening of the mantle was preserved in 95% ethanol. Embryos and/or hatchlings were excised from individual capsules and preserved in 95% ethanol. We used mitochondrial CO1 as a barcoding marker (as proposed in Cheng et al. 2014) to assign all individual samples to either lineage B or C. Because of variation in abundances of these lineages at each location, we excluded sampling sites with less than 10 individuals sampled.

Tissue preparation and mitochondrial DNA sequencing

We extracted high-quality genomic DNA from preserved mantle tissue using a modified phenol-chloroform method employed in the E.Z.N.A Mollusc DNA extraction kit (Omega). Building upon previous mtDNA work in *Sepioteuthis* (Cheng et al. 2014), we amplified a 686-bp fragment of mitochondrial (mtDNA) cytochrome oxidase subunit 1 (CO1) sequence following the methods described therein.

RAD library preparation and sequencing

We generated genome-wide single nucleotide polymorphism (SNP) data using a type of RAD (restriction site-associated DNA tag) sequencing method, called 2b-RAD (Wang et al. 2012). We chose RAD sequencing as this method allows for simultaneous single nucleotide polymorphism discovery and genotyping in large numbers of samples without established genomic resources (i.e. non-model organisms) (Baird et al. 2008). Furthermore, the simultaneous

discovery and genotyping characteristic of RAD significantly reduces issues caused by ascertainment bias arising from developing SNP panels from a subset of populations (Clark et al. 2007). The 2b-RAD method employed here features the use of IIB restriction enzymes, which generate a specific-sized fragment with the restriction site in the center, resulting in uniform fragment sizes (Wang et al. 2012). We chose this method as it is extremely flexible and simple in comparison to more typical RAD sequencing techniques, making it ideal for screening of large numbers of individuals in population-scale studies.

Library preparation followed the protocol developed by the Matz laboratory at the University of Texas-Austin (http://www.bio.utexas.edu/research/matz_lab/matzlab/Methods.html), with a few modifications detailed below. We chose to amplify between 10-15 individuals per sampling location for each lineage to maximize spatial coverage and sequencing efficiency. Preparation started with digestion of ~800-1000 ng genomic DNA using AlfI restriction enzyme. Library-specific adaptors designed for $1/16^{th}$ representation of digestion sites were then ligated to digested samples. Ligated products were then amplified with Illumina platform specific primers and unique 6-bp barcodes for 24 cycles in preparation for sequencing. Amplified products were individually run on a 2% agarose gel with SYBR SAFE fluorescent dye in 1X TBE buffer at 150 V for 90 minutes or until product bands were significantly separated. The ~176 bp target band was then excised from the gel and the PCR product extracted and purified using a commercially available gel extraction kit (Qiagen QIAquick). Purified PCR samples were then further cleaned and brought to appropriate volume and concentration using Ampure XP beads using a standard clean-up protocol (Beckman-Coulter).

The University of California-Berkeley Vincent J. Coates Genomic Sequencing Facility performed all sequencing on an Illumina Hi-Seq 2000 with single-end 50-bp reads with 40-55

samples per lane at approximately 20X coverage. This facility also performed initial quality control and demultiplexing. We used an informatics pipeline from the Meyer Lab at Oregon State University specifically designed for the 2b-RAD method (E. Meyer, pers. comm. http://people.oregonstate. edu/~meyere/tools.html) to conduct read processing and filtering. First, terminal tag positions associated with ligation sites were removed from each resultant 50-bp read (keeping positions 1-36). Reads were then stringently filtered for quality, removing any reads with ambiguous base calls and any low-quality positions (more than 1 position with quality less than 10); reads consisting only of adaptors were also removed.

We used a Stacks-like informatics pipeline specifically designed for analysis of 2b-RAD generated data to conduct *de novo* reference construction, mapping, and genotype calling (M. Matz, https://github.com/z0on/2bRAD_denovo). We also attempted mapping to an unpublished, unannotated draft genome (~40X coverage Illumina HiSeq) of another loliginid squid, *Doryteuthis* (formerly *Loligo*) *pealei* (C. Titus Brown, http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html) as the reference. AlfI sites were extracted from the *D. pealeii* genome, resulting in a reference assembly of 198,008 unique tags (7,128,288 total basepairs). A *de novo* reference was constructed using a custom perl script that finds unique tag sequences for each individual (analogous to creating "stacks" using Stacks) (M. Matz, *pers. comm*.). Because initial mapping to the *D. pealeii* reference was extremely poor (~50% reads matched), we proceeded with the *de novo* reference (~95% reads matched). Using *cd-hit-est* (Li and Godzik 2006; Fu et al. 2012), unique reads were clustered into pseudoalleles, and subsequently into pseudosites allowing for a maximum of 3 mismatches. Final *de novo* references were built from 393,032 clusters (from 993,505 unique tags) and 397,706 clusters (from 1,139,465 unique tages) for lineages B and C respectively. Reads per individual sample were then mapped onto the *de novo*

reference using a custom perl script (M. Matz), filtering by read depth (min 5) and strand bias (min 5).

SNP calling and validation

Genotypes appearing in at least 2 individuals were called with a maximum of 0.5 allowed heterozygocity and high total read depth (20X). They were then filtered to exclude the lowest quality 20% of data. We discarded loci with too many heterozygotes (>75%) as they likely indicated lumped paralogs. Lastly, from this resultant set, loci with more than 25% missing data were also excluded. We then thinned the SNP data set keeping the site with the highest minor allele frequency (MAF) for each tag, to control for any effects of linkage disequilibrium.

DATA ANALYSIS

Sample statistics and detection of outlier loci

Identifying the relative effects of genetic drift and natural selection is necessary to accurately estimate the demographic history of population. In theory, loci under directional or balancing selection should display either higher or lower genetic differentiation than that expected under neutral theory (termed "outlier loci") (Lewontin and Krakauer 1973). As inclusion of outlier loci can bias estimates of population parameters (Morin et al. 2004), data were analyzed in three sets: 1) including all loci, 2) excluding outlier loci, and 3) only including outlier loci. We employed two different outlier detection approaches. We first used Bayescan, which directly estimates the posterior probability of individual loci being under selection by explicitly testing two models, one with and one without selection, using a reversible-jump Monte Carlo Markov chain approach (Foll and Gaggiotti 2008). Bayescan was run with a panel of

putatively neutral loci with 20 pilot runs (5000 steps) and a final run for 100,000 generations sampling every 10 steps and a 50% burn-in. Outlier loci were called under a false discovery rate of 0.05. Second, we employed a more traditional summary statistic approach using *fdist* as applied in Lositan (Antao et al. 2008). To rigorously test for outlier loci from a panel that likely contains neutral and selected loci, we first ran a pilot run using all loci to estimate mean neutral F_{ST}. All loci that fell outside of a 99% confidence interval were removed as candidate selected loci, and a new mean neutral F_{ST} was recalculated with the remaining putative loci. We then optimized for deviations from model assumptions by comparing the average simulated F_{ST} from the first 10,000 simulations with the empirical average F_{ST} and iteratively slicing and sampling the range between the two values to reach optimal error margins. We ran Lositan for 50,000 simulations and called outlier loci as those outside the 99% confidence interval with a false discovery rate of 0.1.

Sample statistics for SNP and CO1 datasets were calculated in Arlequin 3.5 (Excoffier and Lischer 2010). Allele frequencies and observed and expected heterozygocities were estimated for neutral and outlier loci SNP panels as well as the CO1 datasets. Nucleotide and haplotype diversity were also calculated for the CO1 datasets. Population pairwise F-statistics (after Weir and Cockerham 1984) were calculated within an analysis of molecular variance (AMOVA) framework for all datasets.

Population assignment and clustering

For CO1 datasets, we inferred minimum spanning trees of unique haplotypes based on pairwise distances (p-distance) in Arlequin 3.5 (Excoffier and Lischer 2010) to estimate the likely number of clades within each lineage. Clades were defined as groups of haplotypes

separated by 10 or more mutational steps (~1.5% sequence differentiation). To visualize genetic structure in the two lineages over geography, we mapped the frequencies of each clade over all sampling locations.

For the SNP datasets, we did initial tests of population assignment using two methods to determine the most likely number and composition of populations without *a priori* assumptions. We first conducted an assignment test for the likely number of populations within the SNP sample sets using ADMIXTURE (Alexander et al. 2009). ADMIXTURE estimates ancestry of unrelated individuals and population allele frequencies using a maximum likelihood approach. We tested population sizes ranging from k=1 to k=8, with the maximum value for k equaling the total number of distinct samples collected over time and space. The k value with the lowest cross-validation error indicates the most likely number of populations. Second, we used discriminant analyses of principal components (DAPC) to look for likely genetic clustering. DAPC and other principal components based analyses have been shown to demonstrate similar power to detect population structure model-based clustering methods (e.g. Structure, Pritchard et al. 2000) (Patterson et al. 2006). We used the *ade4/adegenet* package in R (Jombart 2008; Jombart et al. 2010) to conduct DAPC analyses for all data sets (mtDNA, neutral and outlier SNPs). We employed a cross-validation method implemented in *adegenet* (*xValDapc)* to determine the optimal number of principal components to include in the analysis in order to maximize inference and minimize chances of overfitting.

Testing for population structure

We conducted analysis of molecular variance (AMOVA) to test both *a priori* and *ad hoc* hypotheses of population structure. Specifically, we first tested specific *a priori* hypotheses of

structure or limited gene flow over known spatial barriers in the Coral Triangle (summarized in Carpenter et al. 2011; Bowen et al. 2013) (Figure 2.2). These include: restricted gene flow **1)** over the Sunda shelf at the Thai peninsula/western Sumatra region (Williams and Benzie 1998; Barber and Bellwood 2005; Barber et al. 2006), **2)** east and west of the Halmahera Eddy (Barber et al. 2002; Barber et al. 2006; DeBoer et al. 2008; Barber et al. 2011; Ackiss et al. 2013; Timery S DeBoer et al. 2014), **3)** over deep ocean basins between Japan and the Ryuyku archipelago and the Coral Triangle (Aoki et al. 2008), **4)** over the Sulu Sea between the Philippines and Indonesia (Lourie et al. 2005), and finally, **5)** absence of genetic structure. Second, we then tested for differentiation between identified clusters from ADMIXTURE within the SNP datasets. Similarly, we tested for differentiation between identified mitochondrial clades identified in the minimum spanning trees. All AMOVA analyses were conducted in Arlequin with 10,000 permutations (Excoffier and Lischer 2010) using all successfully sequenced individuals with $n \geq 4$ per sampling location.

Testing for isolation by distance

The presence of isolation by distance can result in gradual clines of genetic differentiation that may result in false positives when testing for hierarchical structure (AMOVA) (Meirmans 2012). We tested for isolation by distance for the neutral and outlier datasets using Mantel tests controlling for ADMIXTURE cluster and geographic distance. Geographic distance was calculated as the shortest distance over water between sampling points, as straight-line Euclidean distances are not appropriate for marine organisms for which crossing land is not possible. Geographic distances were estimated using custom algorithms implemented within ArcGIS 9.3 (Etherington 2011). Pairwise non-Euclidean distance (Nei's distance) was estimated between

individuals, and isolation-by-distance calculations were carried out with 9999 permutations, using the package *adegenet* in R (Jombart 2008).

Results

Mitochondrial DNA

Building on Cheng et al. (2014), we successfully sequenced CO1 (~686 bp) from 72 and 273 additional individuals from lineages B (8 locations) and C (19 locations) respectively. In total, 165 and 498 individual sequences for lineages for B and C yielded 62 and 174 unique haplotypes, respectively.

SNP discovery and quality filtering

We sequenced 53 individuals from 7 locations for lineage B and 116 individuals from 9 locations for lineage C (Table 2.1). We obtained between 1 million to 5 million raw reads per individual, with ~3% sequences lost after removing adaptors and ambiguous reads, and stringent quality filtering. Combining all genotypes for Lineage B, out of 379,127 basepairs, 11.52% (43,703) passed stringent genotype calling filters, of which 16.78% (7335) were polymorphic (at least 2 genotypes). For lineage C, out of 563,830 putative SNPs, 6.77% (38,205) passed genotyping filters, of which, 24.96% (9537) were polymorphic. Filtering these polymorphic SNPs for coverage, excess heterozygotes, and missing data resulted in 2,108 and 2,920 loci at 20X coverage for lineages B and C, respectively. After thinning to one SNP per tag, 1,653 and 2,229 loci remained for analysis. Lastly, individuals with more than 25% missing genotypes were also removed – resulting in 48 individuals from 7 locations for lineage B and 105 individuals from 8 locations for lineage C.

Outlier detection and molecular diversity

Within lineage B, moderate levels of observed (average $H_o = 0.220$) and expected (average $H_e = 0.249$) heterozygocity were detected for all loci (Table 2.2). Percentage of polymorphic SNPs in each sampling location ranged from 45.9% to 77.9% (Table 2.2). Comparatively, within lineage C, we observed lower levels of observed (average $H_o = 0.168$) and expected (average $H_e = 0.171$) heterozygocity over all loci (Table 2.2). Percentage of polymorphic SNPs in each sampling location ranged from 24.0% to 77.6% (Table 2.2). Bayescan recovered 54 and 12 outlier loci (under diversifying selection) for B and C with a false discovery rate of 0.05 (Figure 2.3). Comparatively, Lositan recovered 40 and 152 outliers respectively for B and C (Figure 2.3). A total of 35 loci for lineage B and 10 loci for lineage C were detected as outliers in both analyses and only these "outlier sets" were used for downstream analysis.

Patterns of population assignment and clustering

Mitochondrial DNA

The minimum spanning tree (MST) of lineage B revealed two distinct clades separated by 12 mutational steps (~1.75% uncorrected sequence divergence). Clade 1 was comprised of eastern and central Indonesia sampling locations (Raja Ampat, Lembeh, Manado, and Timor Leste) and indicated slightly more partitioning between Timor Leste and the rest of the samples (4 mutational steps) (Figures 2.4 & 2.11). Clade 2 included samples from the Indian Ocean, the Philippines, central Indonesia, and the Southeast Asian peninsula (Aceh, Bali, Ticao, and Nha Trang) (Figures 2.4 & 2.11). The MST generated for lineage C has three star-like clusters each representing a distinct clade separated by 12 mutational steps each (~1.75% uncorrected sequence divergence) (Figures 2.5 & 2.12). The largest, Clade 1, consisted of samples from the

majority of our sampling locations with the exception of the three Ryukyu Archipelago locations and approximately half the samples from Raja Ampat. Samples from the Ryuykus (Okinawa, Ishigaki and Iriomote) comprised the majority of Clade 2 while Clade 3 only included 12 samples from Raja Ampat (Figures 2.5 & 2.12).

AMOVA analyses imposing regional structure based on these clade identities indicated high levels of differentiation within Lineage B (F_{CT} = 0.903, $p < 0.05$) (Table 2.3); differentiation between clades in Lineage C were not as pronounced nor was it statistically significant (F_{CT} = 0.345, $p = 0.07$) (Table 2.4). Rather, observed levels of genetic variation could be equally well explained by both between group variance as by within group variance.

Neutral and outlier SNPs

Similar population structure was detected in neutral and outlier loci for Lineage B employing both population assignment and clustering methods. A two-population scheme (k=2) was most well supported for lineage B using neutral loci (CV =0.28484), while three populations (k=3) was supported when examining only outlier loci (CV= 0.36900) (Figure 2.6a & b). With k=2, ADMIXTURE indicated very little mixing of inferred population groups between geographic sampling locations and the presence of two populations corresponding to the western Coral Triangle (Banda Aceh, Bali, Nha Trang, and Ticao) and central/eastern Indonesia (Lembeh, Manado and Raja Ampat) (Figure 2.6a), concordant with patterns from CO1 (Figures 2.4, 2.11). Using outlier loci and k=3, individuals from Aceh separated as a distinct third population with a significant amount of admixture from the western Coral Triangle population (Figure 2.6b). Support from neutral loci for k=2 vs. k=3 was equivocal (28.75% vs 24.35% respectively), while mtDNA indicated that three populations accounted for most of the genetic

variation (though the difference was not statistically significant) (Table 2.3). However, the level of differentiation was much higher when examining outlier loci ($F_{CT} = 0.855$, $p < 0.05$). Under this three-population scheme for the outlier loci, between-group variation accounted for 82.17% of the variation (Table 2.3).

DAPCs conducted for lineage B with neutral loci showed that DF1 accounted for the distinction between the two populations while DF2 distinguished between all sampling locations, except for Bali (Indonesia) and Bulan (Bicol, Philippines) which completely overlapped, signaling high rates of connectivity (Figure 2.7a). In contrast, while outlier loci similarly accounted for the distinction between the two populations in DF1, there was substantial overlap between sampling locations within populations. (e.g., Bali and Ticao; Aceh and Nha Trang). DF2 accounted for the separation of Lembeh from Manado and Raja Ampat in the central/eastern Indonesia population (Figure 2.7b).

In contrast to Lineage B, analysis of Lineage C revealed non-concordant patterns of population assignment between neutral and outlier loci. Neutral loci indicated k=3, with populations in 1) the Ryuykus, 2) Raja Ampat, and 3) the western Coral Triangle/central Indonesia (Bali, Aceh, Donggala, Andaman Sea and Gulf of Thailand) (Figure 2.8a). While this pattern is concordant with mtDNA, neutral SNPs recovered much lower levels of differentiation ($F_{CT} = 0.060$, $p < 0.01$ for SNPs vs. $F_{CT} = 0.345$, $p < 0.05$ for mtDNA) (Table 2.4). Moreover, DAPC with neutral loci not only distinguished the three groups above, but also was able to distinguish among all three Ryukyu islands (Okinawa, Ishigaki, Iriomote) (Figure 2.8a).

ADMIXTURE analysis of Lineage C outlier loci (n=10), in contrast, recovered 8 populations (k=8) with no clear geographic patterns. ADMIXTURE is designed to rapidly assess ancestry in a large number of markers, making it ideal for next-generation sequencing

applications. However, the outlier dataset for lineage C only contained 10 loci, thus making it highly unlikely for this low number of loci to distinguish any signals from noise. Running this dataset through Structure revealed that k=2 was most well-supported (using the Delta K method, Evanno et al. 2005). DAPC of outlier loci also indicated two groups with some degree of overlap (Figure 2.9b). Populations from the western Coral Triangle/central Indonesia populations tightly clustered together and individuals from Raja Ampat formed a cluster along DF2; however, a number of individuals fell within the western Coral Triangle/central Indonesia group. Along DF1, the Ryuykus emerged as another group albeit with more variation among individuals. Examination of the ADMIXTURE plot for outliers (k=3) indicates a significant amount of mixed ancestry between the three populations (Figure 2.8b). Among these outlier loci, however, we observed higher levels of differentiation between the three populations than either neutral loci or CO1 indicated ($F_{CT} = 0.444$, $p < 0.01$).

Given the different patterns of population assignment, we assessed the ability of neutral versus outlier loci to assign individuals back to their original sampling location using DAPCs. Overall, neutral loci performed much better than outlier sets at assigning individuals back to original sampling locations (Table 2.5). Neutral loci for lineage B assigned nearly 100% of individuals back to their original sampling locations, while assignment proportions were much lower for lineage C. In particular, neutral loci were able to successfully assign Ryuyku Arichipelago and Raja Ampat individuals; however, individuals from the more widely distributed western Coral Triangle/central Indonesia population were not often successfully reassigned.

Testing hypotheses of spatial population structure

AMOVA showed significant genetic structure within both lineages of *Sepioteuthis* cf. *lessoniana* (Table 2.3 & 4). However, none supported differentiation across the Halmahera Eddy, Java Sea, or Sulu Sea. Within Lineage B, strong differentiation was only supported between the three groups inferred by ADMIXTURE. While neutral SNPs revealed strong structure (F_{CT} = 0.2875, $p<0.001$), mtDNA and outlier loci indicate near fixation between these groups (F_{CT} = 0.9025, F_{CT}=0.8554, $p<0.0001$, respectively). In lineage C, AMOVA only supported a genetic break between the Ryuyku Archipelago and the Coral Triangle with all datasets, although mtDNA showed significantly low levels of connectivity across the Halmahera Eddy (Table 2.4).

Lineage C had much lower levels of overall differentiation (F_{ST} =0.0479, $p<0.0001$) than lineage B (F_{ST} =0.2024, $p<0.0001$) when comparing differentiation inferred from neutral loci. Comparatively, global levels of differentiation were much higher for both lineages when examined with outlier loci (B: F_{ST}=0.7821, $p<0.0001$, C: F_{ST}=0.3720, $p<0.0001$). This high level of differentiation in outlier loci is reflected as well in the very strong differentiation observed in CO1 data (B: F_{ST}=0.8782, $p<0.0001$, C: F_{ST}=0.6238, $p<0.05$).

We did not detect any signals of IBD in lineage B (r^2 = 0.0001-0.02914, $p>0.05$) in any of the datasets. After accounting for population structure, no signal of IBD was detected in either the western Coral Triangle population or in the central/eastern Indonesia population (Figure 2.10a & b). Comparatively, there was a significant pattern of isolation by distance (IBD) in lineage C for all populations at all loci (r^2=0.0347-0.2313, $p<0.05$) (Figure 2.10c & d). However, underlying population structure can confound estimations of IBD, thus when we tested for IBD just within the Coral Triangle (omitting the divergent Japan population), we did not recover any patterns of isolation by distance.

Discussion

Numerous studies of nearshore and reef-associated taxa using mtDNA data have shown strong differentiation among populations spanning the Sunda Shelf and Halmahera Eddy (rev. Carpenter et al. 2011; Ludt and Rocha 2014). However, these putative filters to gene flow do not appear to equally impact lineages of *Sepioteuthis* c.f. *lessoniana*, suggesting that highly mobile neritic taxa may not be influenced by sea level changes and physical oceanography the same way as reef-associated species that only disperse as larvae. This study does reveal strong, contrasting patterns of genetic structure between these sibling taxa highlighting that even slight differences in life history can result in drastically different patterns of connectivity. Moreover, the use of genome-wide SNPs adds substantial resolution for identifying fine-scale patterns of connectivity and for detecting potential selective processes at play. Specifically, the use of genomic markers in this study reveals that limited gene flow can in fact, occur in neritic taxa and that regional environmental differences may play a much larger role than expected in shaping divergence patterns.

Lineage B exhibits overall broad-scale structure distinguishing Sunda Shelf populations from oceanic island populations to the north and east (Figure 2.11). This pattern is similar to patterns from snails (Reid et al. 2006), seahorses (Lourie and Vincent 2004), and mackerel (Rohfritsch and Borsa 2005). Genome-wide SNPs add substantial resolution to recovered patterns of population structure, revealing strong distinctions not only between inferred populations, but between sampling localities as well, indicating that lineage B has very limited dispersal (Figure 2.7a). Moreover, divergent patterns detected with outlier loci indicate that selective processes stemming from regional environmental differences likely plays a role in population structure (Figure 2.6b, 2.7b). In contrast, we do not detect strong signals of structure

in lineage C, lending support to it having wide dispersal capacity (Table 2.4, Figures 2.8, 2.9, 2.12). However, the separation of the Ryuku Islands from the remainder of the Coral Triangle suggests that distance may limit gene flow among these regions. Combined insight from mitochondrial and genomic markers reveal that both neutral and non-neutral processes both play a significant role in shaping patterns of population divergence in neritic species in the Coral Triangle.

Consolidation of cryptic species identities over Pacific and Indian Oceans

While numerous efforts have been made to determine the identity, number and characteristics of cryptic species within *S. cf. lessoniana*, insight from these studies has been limited because these studies used different sets of information and were spatially limited, making it extremely difficult to compare between studies and regions (e.g. Segawa et al. 1993a;b; Izuka et al. 1994; Izuka et al. 1996; Triantafillos and Adams 2005). CO1 barcoding of samples in the Pacific and Indian Oceans conducted by Cheng et al. (2014) and expanded upon in this study confirm that lineage C corresponds to the *shiro-ika* type (*sensu* Segawa et al. 1993) also known as species 2 (*sensu* Izuka et al. 1996; Imai and Aoki 2009). Lineage A, which was not examined on the population level in this study, corresponds with *kua-ika* (*sensu* Segawa et al. 1993) also known as species 3 (*sensu* Izuka et al. 1996; Imai and Aoki 2009). While lineage B was not detected in individuals sampled in the Ryuykus in this study, barcoding of existing sequences from GenBank indicate that lineage B corresponds to *aka-ika* (*sensu* Segawa et al. 1993) also known as species 1 (*sensu* Izuka et al. 1996) (S. Tomano, *pers. comm.*).

Population structure in neritic taxa in the Coral Triangle

Phylogeographic structure inferred with mtDNA and genome-wide SNPs of these two lineages of reef squid do not precisely correspond to the two most commonly cited phylogeographic barriers in the Coral Triangle, the Sunda Shelf and Halmahera Eddy (Barber et al. 2011; Carpenter et al. 2011; Ludt and Rocha 2014). For lineage B, our results indicate a distinct Sunda Shelf population and an oceanic island populations roughly corresponding to divisions between western and central/eastern Indonesia (Figures 2.6, 2.11). As lineage B is not as common as lineage C, sampling for this lineage was much coarser (Cheng et al. 2014), thus the precise location of this break is unclear. Traditionally, the Sunda Shelf break stemming from Pleistocene glaciation is situated along the southern margins of the Sunda Shelf (corresponding to modern day Java and Sumatra). While ample evidence supports Indian/Pacific Ocean vicariance during low sea level stands, the contact point between these two regions is highly variable across taxa (rev. Ludt and Rocha 2014, Barber et al. 2000, 2006, DeBoer et al. 2008, 2014, Ackiss et al. 2013, Jackson et al. 2013, Crandall et al. 2008a, 2008b, 2011). For lineage B, it is possible that the Sunda Shelf population represents the edge of an Indian Ocean population that has recolonized more rapidly than the Pacific Ocean population following sea level rises. Extremely high levels of differentiation in mitochondrial CO1 along with high differentiation genome-wide support this theory of allopatric divergence and subsequent recolonization (Table 2.3).

On the other hand, the strong break observed in Lineage B also suggests significant influence of prevailing oceanography in limiting gene flow in this species. Western and central/eastern Indonesia are separated by the Makassar Strait between the islands of Sulawesi and Kalimantan (Figure 2.11). The Indonesian Throughflow moves nearly 20 million m^3/s of water from the Pacific Ocean to the Indian Ocean (Godfrey 1996) through this narrow strait, at

velocities topping 1m/sec (Wyrtki 1961). Coupled biophysical larval dispersal models suggest that this fast current is a significant physical barrier for east/west dispersal of larvae and adults within the Coral Triangle (Kool et al. 2011), and as a neritic species, it is unlikely that *S.* c.f. *lessoniana* would purposely navigate this span of deep and fast-moving water. Data supporting genetic structure on either side of the Makassar Strait has been found in a number of diverse marine organisms including giant clams (*Tridacna maxima,* Nuryanto and Kochzius 2009, DeBoer et al. 2014), snails (Reid et al. 2006), and scad mackerel (*Decapterus russelli*, Rohfritsch and Borsa 2005). High levels of admixture and clustering highlighted by genome-wide SNPs between Philippines and Bali samples is likely the result of high connectivity facilitated by the Indonesian Throughflow, lending further support to the influence of oceanography to shaping patterns of gene flow in lineage B. Coupled biophysical larval dispersal models predict that the Indonesian Throughflow facilitates high rates of gene flow and dispersal from the Philippines to central and southern Indonesia (Kool et al. 2011). Similarly, the moderate levels of admixture between the Philippines and Vietnam are likely the result of westerly currents in the South China Sea (SCS) (Figures 2.6a, 2.7a).

Despite the well-known filters to gene flow in marine species in the Coral Triangle (see above) we do not observe significant limits to dispersal and gene flow in Lineage C across most of this region (Table 2.4 & 6, Figures 2.8, 2.9 & 2.12). Instead, genetic differentiation over all locations can be explained by isolation by distance (Figure 2.10c & d), although this pattern is driven by divergence between the Coral Triangle and the Ryuyku archipelago (mtDNA: F_{CT}=0.9386, p<0.001; N: F_{CT} =0.1363, p<0.001; O: F_{CT} =0.4406, p<0.001); within the Coral Triangle, there is no significant signal of IBD. Intuitively, divergence between the Ryuykus and the Coral Triangle is expected given the considerable distance of deep water (~3000 km from the

center) separating the two regions, which poses a significant dispersal barrier for a neritic species. For the majority of Indo-West Pacific marine organisms, the Ryukyus represent the northern limits of ranges (Briggs 1999). For many of these species, strong divergence is often observed between populations in this peripheral portion of the range and those in the center of the range (Nakajima et al. 2014; He et al. 2015). Divergence between Japan and areas from the South China Sea and further afield have been detected before in the *shiro-ika* form of *S.* cf. *lessoniana* (lineage C) (Aoki et al. 2008).

Results also support differentiation between Raja Ampat and the rest of the Coral Triangle, a pattern that likely results from the isolating effects of the Halmahera Eddy. This isolation is predicted by coupled biophysical models (Kool et al. 2011; Treml and Halpin 2012) and is seen in a diversity of phylogeographic studies, including in fish (Timm and Kochzius 2008; Jackson et al. 2014), giant clams (DeBoer et al. 2008; 2014a; 2014b; Kochzius and Nuryanto 2008), stomatopods (Barber et al 2006, 2011), nautilus (Wray et al. 1995), and other invertebrates (Crandall et al. 2008; 2012). However, the position of this break is unclear, as we do not have samples across the Maluku Sea, but rather from either end of it at Lembeh and Raja Ampat. Overall however, historical sea level changes and physical oceanographic patterns do not seem to strongly influence patterns of connectivity in lineage C.

Non-concordance of population structure in sibling taxa

Comparison of population structure over the Coral Triangle and peripheral regions reveals a stark contrast in divergence patterns between two sibling taxa. This result suggests that differences in traits such as life history, dispersal capacity and behavior, habitat preference and recruitment can result in massively differential responses to regional-scale physical processes

(Kingsford et al. 2002; Pineda et al. 2007). The low levels of differentiation in lineage C are more similar to highly dispersive pelagic organisms such as mackerel (Perrin and Borsa 2001) and tuna (Jackson et al. 2014) while the strong differentiation in lineage B is more similar to the patterns observed in less mobile, benthic and reef-associated taxa (see references above).

Limited disperser (Lineage B)

Although *Sepioteuthis* sp. are members of the neritic squid family Loliginidae, they display morphological and life history traits that more closely resemble benthic cuttlefish (family Sepiidae) (Segawa 1987; Segawa et al. 1993; Anderson 2000; Jereb and Roper 2010). Their main feeding areas and spawning areas are found in shallow coastal and reef habitats, rarely extending out over the continental shelf (Wada and Kobayashi 1995; Jereb and Roper 2010), and adults shy away from strong currents (pers. obs, LEK from Indonesian fisherman). Tagging studies of *S. australis* in Australia (Pecl et al. 2006) and *S*. cf. *lessoniana* in Japan (Ueta and Jo 1990) suggest that spawning adults may only travel between 10-40 km along the coast. Given these similarities to cuttlefish life history (Boycott 1965), perhaps it is not surprising that patterns of population structure detected in lineage B resemble limited regional scale connectivity in pharaoh cuttlefish (*Sepia pharoanis*) in this area (Anderson et al. 2010), suggesting similar responses to prevailing physical processes in the region.

Strong distinctions between sampling locations could also stem from specific requirements defining recruitment instead of, if not in addition to, limited dispersal capacity. Both myopsid and oegopsid squid are known to display spawning site fidelity, which may drive a significant degree of self-recruitment (e.g. Buresch et al. 2006; Thorpe et al. 1986, Brierley et al. 1993). Like other Loliginidae, *Sepioteuthis* species spawn in aggregations of varying sizes

(Jackson and Pecl 2003; Moltschaniwskyj and Pecl 2007; Venkatesan and Rajagopal 2012) at different reef habitats. Studies suggest that differences in spawning site are fixed between the three cryptic lineages of *S.* cf. *lessoniana* (Imai and Aoki 2009). On the same reef, *aka-ika* (Lineage B) is observed to spawn at much deeper depths than *shiro-ika* (Lineage C) (Tomano et al. unpubl.). If the availability of deeper reef habitat may be more limited compared to shallow habitats, this would limit the degree of connectivity between more far-flung sampling locations. Thus, even within a population, there may be limited exchange of individuals between spawning sites driving the signals of low connectivity observed with genome-wide data. However, very few studies have examined movement and migration in specific lineages within the species complex. Thus, further research is needed to investigate the specifics of dispersal behavior and capacity in lineage B.

Broad disperser (Lineage C).

Conversely, low overall levels of differentiation suggest that lineage C is a broad-scale disperser (Figures 2.8, 2.9, 2.12), more similar to *Loligo*/*Doryteuthis* (family Loliginidae). While lineage C shows strong evidence for wide dispersal capacity, we do observe a significant break between the Coral Triangle and the Ryuyku Islands (Figure 2.12), reflecting results from previous studies suggesting limited dispersal over the fast-moving Kuroshio Current (Aoki et al. 2008). Past genetic studies detect evidence of founder effects in species 2 (lineage C, *shiro-ika*) around mainland Japan (Aoki et al. 2008, Tomano et al. 2015), suggesting colonization by a few long-distance dispersers lending additional support to limited dispersal over this region. Within the Ryuykus, neutral SNPs indicated moderate levels of structure between individual islands (Figure 2.9) (Table 2.7) that were not detected with mitochondrial DNA (Figure 2.5). Ishigaki,

Iriomote and Okinawa are relatively close together, ranging from ~50 km to 500 km apart, but these distances span areas of deep ocean. Decreased dispersal between islands may be attributed to lack of contiguous coastline as has been observed between mainland Japan and the Ryukyus, but this hypothesis has had mixed support in studies of *S.* cf. *lessoniana* (Aoki et al. 2008, Imai and Aoki 2009, Tomano et al. 2015, Izuka et al. 1996, Yokogawa and Ueta 2000). Samples from the Ryukyu Islands in this study were collected from individual embryos from egg masses laid at approximately the same time, suggesting that simultaneously spawning individuals for each island are significantly differentiated. Overall, these results lend strong support for the hypothesis that deep water and non-contiguous coastlines are important barriers to dispersal for lineage C.

Role of selective processes

One of the major criticisms of relying solely on mitochondrial DNA is that it cannot indicate whether observed divergence stems from selective or neutral processes, hindering our understanding of the mechanisms underlying diversification (Edwards and Beerli 2000; Edwards et al. 2005 but see Bowen et al. 2014 for counter-argument). Using a genome-wide approach, we were able to identify putative outlier loci, which represent portions of the genome that may be under divergent selection. This novel approach allowed for us to infer when selective processes may be important in shaping historical and ongoing patterns of gene flow. For these sibling species of reef squid, outlier loci revealed that regional environmental differences likely play a significant role in reinforcing divergence from neutral processes.

Using outlier loci, Lineage B distinguishes Aceh as a third distinct region (Figure 2.6b). Although most studies have attributed this pattern to Pleistocene vicariance (Williams and

Benzie 1998; Duda Jr and Palumbi 1999; Barber and Bellwood 2005; Ackiss et al. 2013) the coupled biophysical models of Kool et al. suggest that currents can limit flow between the Indian Ocean and the Java Sea (2011). Given that the low estimates of genetic differentiation between Aceh and the rest of the western Coral Triangle population for Lineage B, this divergence is likely recent and may argue for a physical oceanographic origin, rather than Pleistocene sea level fluctuations. Alternatively, strong divergence in outlier loci but not in neutral loci in Aceh may reflect ongoing divergence with gene flow in the region. The mechanisms of ecological speciation in marine environments are not well understood and at times, controversial, however, there is increasing genetic evidence hinting that it is more common than previous thought (e.g. Rocha et al. 2005; Faucci et al. 2007, Fritts-Penniman et al. unpubl, Simmonds et al. unpub.). Unique abiotic characteristics of the waters around Banda Aceh may drive divergence. Furthermore, a significant portion of reef biomass and available reef habitat was extirpated in the region following the devastating tsunami in 2004 (Paris et al. 2007; Rao et al. 2007). Selective pressure may stem from any of these forces; however, further investigation is required to determine this.

Complete concordance in population structure between neutral, outlier and mtDNA loci in Lineage C may suggest a complementary role of selective and neutral processes. The breaks observed in lineage C with all loci correspond to major breaks between biogeographic provinces in the Indo-West Pacific (Spalding et al. 2007). The biogeographic provinces are defined by the presence of distinct biotas, which have arisen due to both historical isolation and distinctive abiotic features including geomorphological, hydrographic, and geochemical features (Spalding et al. 2007). The Ryuyku Archipelago and the Coral Triangle are in different marine provinces, thus selective pressures imposed differences in abiotic features likely plays a role in reinforcing

patterns driven by neutral divergence. While Raja Ampat and rest of the Coral Triangle are within the same marine province, they are in different ecoregions, suggesting that specific differences in biogeographic shaping factors between these regions may also serve to reinforce neutral divergence. However, this study only represents the first step towards disentangling the effect of selective processes from neutral processes in diversification of marine organisms. Further research on the specific life histories and ecologies of these reef squid are required before we can make solid inferences about the specific role of selection.

Population structure inferred from mitochondrial DNA versus SNPs

The majority of phylogeographic studies in marine systems use mtDNA because of its ability to readily discern phylogeographic patterns and the relative ease of obtaining sequence data (rev. Avise 2000). The growing ability and the decreasing cost of generating and examining hundreds to thousands of loci across the genome has terrific potential to revolutionize our understanding of the evolutionary history of species and processes shaping population patterns (Brumfield et al. 2003; Helyar et al. 2011). However, comparatively, conducting a population-scale study with sufficient spatial coverage using NGS markers is still prohibitively expensive, particularly for researchers in developing countries, such as those within the Coral Triangle (Willette et al. 2014).

Within lineage B, mtDNA and ~1,650 neutral and outlier SNPs support divergence among a western Indonesia population and a central/eastern Indonesia population. Given the strong signal of divergence in neutral SNPs, there is no reason to implicate selective sweeps in shaping mtDNA patterns. Similarly, although lineage C has lower levels of differentiation, both mtDNA and SNPs distinguish the Ryuyku Archipelago from the remainder of the Coral Triangle.

Thus, SNPs provided a minimal advantage in discerning broad-scale patterns of differentiation. Similar conclusions come from mtDNA and microsatellite analyses of giant clams in this region (Timery S DeBoer et al. 2014). However, neutral and outlier SNP panels detected fine-scale structure not detected by CO1 alone, such as distinguishing among the three Ryukus sampling sites in Lineage C, echoing results from other studies that detected population boundaries in marine organisms that were difficult to discern using traditional markers (Freamo et al. 2011; Karlsson et al. 2011). Furthermore, outlier loci in Lineage B show more structure than neutral loci, suggesting that selection may be reinforcing other physical or behavioral isolating forces. In Lineage C, lower levels of clustering in outlier loci than neutral loci suggests that neutral processes are the main drivers of divergence in this lineage and that all locations have similar responses to shared selective pressures. Despite several caveats regarding detecting loci under selection (Foll and Gaggiotti 2008), if care is taken to reduce false positives by using multiple outlier tests (Luikart et al. 2003; Bonin et al. 2006), these and other results show the utility of genome-wide SNP assays (Eckert et al. 2010; Hohenlohe et al. 2010; Freamo et al. 2011; Bradbury et al. 2013; Nadeau et al. 2013).

Implications for conservation and fisheries management

Marine ecosystems provide a significant portion of global food supplies as well as support national economies and livelihoods (Pauly et al. 2005). Effective management and sustainability of these critical ecological and economic resources requires accurate data to delineate management units and assess harvesting pressure. Conservation and fisheries biologists are increasingly emphasizing the utility of genetic data (Frankham et al. 2002; Frankham 2005), both as an inherent biological element to be conserved (Lande 1988; Crozier 1997; Amos and

Balmford 2001) and as a vital tool to inform conservation planning and management, particularly for harvested natural resources (Frankham 2010). In the marine realm, genetic information is particularly useful for informing 1) correct species identification and 2) spatial and temporal structure of populations (Hilborn and Waters 1992; Palumbi 1994), elements key to delineating management units and conservation areas.

The results from this study have two major implications for management of this fishery. First, the strong differentiation among populations revealed by SNPs indicates strong limits to connectivity among populations. Even within the Ryuku Islands, gene flow is insufficient to limit differentiation among reefs. This fine-scale genetic differentiation suggests strong limits to demographic exchange, and that management efforts may need to focus on the local, rather than regional, scale. Second, Lineages B and C have very different patterns of genetic structure and connectivity, yet they are caught in the same nets on the same reefs. Given that these cryptic taxa can only be distinguished genetically at present, the most judicious approach to management of this mixed fishery would be to manage for lineage with the more restricted dispersal, Lineage B.

Rapid development of genetic tools and methods of analyses have propelled the study of conservation genetics into the new era of conservation genomics (Kohn et al. 2006; Allendorf et al. 2010; Avise 2010). However, the use of the most technologically advanced or most recent tools may not generate directly relevant information needed for rapid decision making, as is needed by practitioners. Our study demonstrates that SNP data can detect fine-scale patterns of genetic with significant management implications for this fishery. However, in developing countries, such as those in the Coral Triangle, access to facilities and equipment required to collect cutting-edge genetic information is extremely limited (Barber et al. 2014) and cost-prohibitive (Willette et al. 2014). Thus, while SNP data refines our understanding of the

dynamics of the bigfin reef squid fisheries, financial limitations in developing countries may preclude the application of these techniques. As such, mitochondrial DNA may remain an important tool for management as it can provide important insights to population structure at a fraction of the cost.

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FIGURES AND TABLES

Table 2.1. Sampling localities and sample sizes for mitochondrial and SNP datasets. Note, sample sizes for SNP data reflect how many individuals were retained following stringent filtering for quality and coverage (denoted by *)

				Lineage B		**Lineage C**	
	Sampling locality	***Province***	***Country***	***CO1 data***	***SNP data****	***CO1 data***	***SNP data****
1	Kota Banda Aceh	Western Sumatra	Indonesia	50	8	84	8
2	Ban Pak Bara	Satun	Thailand	--	--	38	15
3	Songkhla	Songkhla	Thailand	--	--	27	13
4	Jimbaran	Bali	Indonesia	31	5	9	3
5	Donggala	Central Sulawesi	Indonesia	--	--	25	24
6	Dumaguete	Negros Oriental	Philippines	--	--	33	--
7	Midara	Iriomote	Japan	--	--	17	7
8	Nosoko Bay	Ishigaki	Japan	--	--	34	7
9	Lembeh	North Sulawesi	Indonesia	6	5	29	--
10	Manado	North Sulawesi	Indonesia	22	10	19	--
11	Muncar	East Java	Indonesia	--	--	4	--
12	Seragaki Diamond Beach/Futyaku	Okinawa	Japan	--	--	26	12
13	Pulau Panggang	Pulau Seribu	Indonesia	--	--	26	--
14	Dili		Timor Leste	4	--		
15	Waiwo/Sorong	Raja Ampat	Indonesia	9	5	26	17
16	Baladingan	Ticao	Philippines	--	--	41	--
17	Bulan	Bicol	Philippines	4	4	8	--
18	San Jacinto	Ticao	Philippines	--	--	21	--
19	Phu Quoc		Vietnam	--	--	17	--
20	Nha Trang		Vietnam	39	10	14	--
			Total	***165***	***47***	***498***	***106***

Table 2.2 . Summary statistics for mitochondrial and genome-wide single nucleotide polymorphisms employed in this study

			Lineage B					Lineage C				
			mtCO1		SNPs			mtCO1		SNPs		
Country	Region	Locality	Hap. div.	Nuc. div.	H_o	H_e	% poly	Hap. div.	Nuc. div.	H_o	H_e	% poly
Indonesia	Western Sumatra	Kota Banda Aceh	1.000 ± 0.004	0.001 ± 0.001	0.295 ± 0.186	0.293 ± 0.144	62.6%	1.000 ± 0.002	0.003 ± 0.002	0.277 ± 0.179	0.275 ± 0.142	57.5%
Thailand	Satun	Ban Pak Bara						1.000 ± 0.006	0.002 ± 0.002	0.230 ± 0.167	0.229 ± 0.149	66.2%
Thailand	Songkhla	Songkhla						1.000 ± 0.010	0.009 ± 0.005	0.251 ± 0.179	0.238 ± 0.147	70.0%
Indonesia	Bali	Jimbaran	1.000 ± 0.008	0.001 ± 0.001	0.417 ± 0.243	0.384 ± 0.153	45.9%			0.522 ± 0.269	0.495 ± 0.179	24.0%
Indonesia	Central Sulawesi	Donggala						1.000 ± 0.011	0.006 ± 0.003	0.210 ± 0.160	0.210 ± 0.147	71.9%
Philippines	Negros Oriental	Dumaguete						1.000 ± 0.007	0.003 ± 0.002			
Japan	Iriomote	Midara						1.000 ± 0.020	0.001 ± 0.001	0.323 ± 0.201	0.311 ± 0.149	37.6%
Japan	Ishigaki	Nosoko Bay						1.000 ± 0.007	0.001 ± 0.001	0.331 ± 0.216	0.318 ± 0.149	36.2%
Indonesia	North Sulawesi	Lembeh	1.000 ± 0.096	0.003 ± 0.003	0.423 ± 0.229	0.374 ± 0.136	54.4%	1.000 ± 0.001	0.003 ± 0.002			
Indonesia	North Sulawesi	Manado	1.000 ± 0.013	0.003 ± 0.002	0.280 ± 0.187	0.262 ± 0.143	62.5%	1.000 ± 0.016	0.014 ± 0.008			
Indonesia	East Java	Muncar						1.000 ± 0.177	0.000 ± 0.000			
Japan	Okinawa	Seragaki						1.000 ± 0.011	0.001 ± 0.001	0.273 ± 0.185	0.266 ± 0.154	51.2%
Indonesia	Pulau Seribu	Pulau Panggang						1.000 ± 0.011	0.003 ± 0.002			
Timor Leste		Dili	1.000 ± 0.177	0.001 ± 0.001								
Indonesia	Raja Ampat	Waiwo/ Sorong	1.000 ± 0.052	0.003 ± 0.002	0.433 ± 0.249	0.373 ± 0.139	47.4%	1.000 ± 0.011	0.014 ± 0.008	0.276 ± 0.185	0.260 ± 0.149	80.4%
Philippines	Ticao	Baladingan						1.000 ± 0.005	0.003 ± 0.002			
Philippines	Bicol	Bulan	1.000 ± 0.177	0.002 ± 0.002	0.494 ± 0.244	0.410 ± 0.121	55.8%	1.000 ± 0.063	0.003 ± 0.002			
Philippines	Ticao	San Jacinto						1.000 ± 0.015	0.003 ± 0.002			
Vietnam		Phu Quoc						1.000 ± 0.020	0.004 ± 0.003			
Vietnam		Nha Trang	1.000 ± 0.006	0.001 ± 0.001	0.308 ± 0.204	0.274 ± 0.147	77.9%	1.000 ± 0.024	0.026 ± 0.014			

Table 2.3. Genetic differentiation over hypothesized barriers to dispersal for Lineage B using SNP and mtDNA CO1 datasets. Significance indicated by + = p < 0.1,* = p < 0.05, ** = p < 0.01

Hypothesized barrier	Data type	Among groups	Among populations within groups	Within populations
None (global differentiation)	Neutral		20.24 % **	79.76 %
	Outlier		78.21 % **	21.79 %
	CO1		87.82%**	12.18 %
Pleistocene glaciation (Sunda shelf)	Neutral	1.69 %	-0.14% **	98.45 % +
	Outlier	6.42 %	-3.21 %	96.79 %
	CO1	15.15 %	8.02 % **	76.82 % **
Halmahera Eddy	Neutral	1.17 %	1.82 % **	97.01 % **
	Outlier	-8.60 %	10.96 % **	97.64 % **
	CO1	-41.58 %	79.68 %**	61.90 %**
Sulu Sea	Neutral	4.71 %	-0.83 % **	96.12 %
	Outlier	6.54 %	-1.79 %	95.25 %
	CO1	8.38 %	17.41 %	74.21 %
ADMIXTURE inferred populations (k=2)	Neutral	28.75 % *	1.06 % **	70.19 % **
	Outlier	85.54 % *	0.46 % **	14.01 % **
	CO1	90.25 % *	3.38 % **	6.36 % **
ADMIXTURE inferred populations (k=3)	Neutral	24.35 % *	0.81 % **	74.84 % **
	Outlier	82.17 % *	0.58 % *	17.25 % **
	CO1	82.56 % +	7.14 % **	10.30% **

Table 2.4. Genetic differentiation over hypothesized barriers to dispersal for Lineage C using SNP and mtDNA CO1 datasets. Significance indicated by + = p < 0.1,* = p < 0.05, ** = p < 0.01

Hypothesized barrier	Data type	Among groups	Among populations within groups	Within populations
None (overall FST)	Neutral		4.79 % **	95.21 %
	Outlier		37.20 % **	62.80 %
	CO1		62.38 % **	37.62 %
Pleistocene glaciation (Sunda shelf)	Neutral	0.88 % +	-1.46 %	100.58 %
	Outlier	0.15 %	-0.16 %	100.01 %
	CO1	-1.97 %	8.82 % **	93.15 % **
Halmahera Eddy	Neutral	6.63 %	-1.08 %	94.45 % **
	Outlier	24.27 %	-0.44 %	76.17 % **
	CO1	47.75 % *	3.26 % **	48.99 % **
Deep water	Neutral	5.03 % *	1.11 % **	93.86 % **
	Outlier	53.40 % *	2.54 % **	44.06 % **
	CO1	85.12 % **	1.25 % **	13.63 % **
Sulu Sea	Neutral			
	Outlier			
	CO1	-0.68 %	8.12 % **	92.56 % **
ADMIXTURE populations	Neutral	6.03 % **	0.75 % **	93.22 % **
	Outlier	44.35 % **	2.51 % **	53.15 % **
	CO1	34.50% +	35.93 % **	29.57 % **

Table 2.5. Back-assignment proportions of individuals to original sampling locations using discriminant analysis of principal components

Lineage B	Aceh	Bali	Lembeh	Manado	Nha Trang	Raja Ampat	Bulan
Neutral	1.0	0.80	1.00	1.00	1.00	1.00	0.75
Outlier	0.50	1.00	1.00	1.00	0.80	1.00	0.75

Lineage C	Aceh	Satun	Bali	Donggala	Songkhla	Iriomote	Ishigaki	Okinawa	Raja Ampat
Neutral	0.50	0.33	0.33	0.86	0.36	1.0	1.0	1.0	1.0
Outlier	0.00	0.40	0.00	0.77	0.21	0.29	0.57	0.75	0.94

Table 2.6. Pairwise F_{ST} between sampling locations for Lineage B. Values above the diagonals represent analysis with neutral loci and values below represent outlier loci. Values in bold are statistically significant ($p<0.0001$)

	Banda Aceh	Nha Trang	Bali	Bulan	Manado	Lembeh	Raja Ampat
Banda Aceh		**0.00516**	**0.00189**	**0.06734**	**0.36677**	**0.24937**	**0.35158**
Nha Trang	**0.04457**		-0.03534	**0.04498**	**0.33289**	**0.20887**	**0.30376**
Bali	-0.11014	-0.07971		-0.04793	**0.3087**	**0.17406**	**0.28494**
Bulan	**0.0868**	**0.05933**	0		**0.3184**	**0.16306**	**0.28453**
Manado	**0.85777**	**0.87808**	-0.16525	0		**0.01805**	**0.03603**
Lembeh	**0.78811**	**0.8246**	**0.82019**	**0.84718**	**0.08397**		**0.01937**
Raja Ampat	**0.88639**	**0.90765**	**0.7268**	**0.75589**	-0.02807	**0.12121**	

Table 2.7. Pairwise F_{ST} between sampling locations for Lineage C. Values above the diagonals represent analysis with neutral loci and values below represent outlier loci. Values in bold are statistically significant ($p<0.0001$)

	Satun	Banda Aceh	Bali	Songkhla	Donggala	Ishigaki	Raja Ampat	Okinawa	Iriomote
Satun		***0.00612***	*-0.07564*	*-0.00012*	***0.00126***	***0.08082***	***0.06182***	***0.07363***	***0.07392***
Banda Aceh	0.02785		*-0.10444*	***0.00343***	***0.00587***	***0.08718***	***0.05174***	***0.08092***	***0.08549***
Bali	-0.11522	0.05819		*-0.11088*	*-0.08577*	*0.03586*	*-0.07553*	*0.00595*	*0.01738*
Songkhla	-0.00308	0.01251	-0.16474		*-0.00292*	***0.06114***	***0.05671***	***0.05977***	***0.05882***
Donggala	0.0161	-0.00455	-0.00003	0.00101		***0.08411***	***0.04497***	***0.07785***	***0.0761***
Ishigaki	**0.42461**	**0.37223**	**0.20087**	**0.31355**	**0.40784**		***0.10077***	***0.07952***	***0.05823***
Raja Ampat	**0.2692**	**0.19288**	**0.15872**	**0.18541**	**0.18394**	**0.30817**		***0.11459***	***0.10303***
Okinawa	**0.67076**	**0.69001**	**0.59731**	**0.59834**	**0.65926**	**0.25421**	**0.58037**		***0.08064***
Iriomote	**0.60132**	**0.62291**	**0.4145**	**0.50253**	**0.60537**	**0.18273**	**0.49629**	**0.11702**	

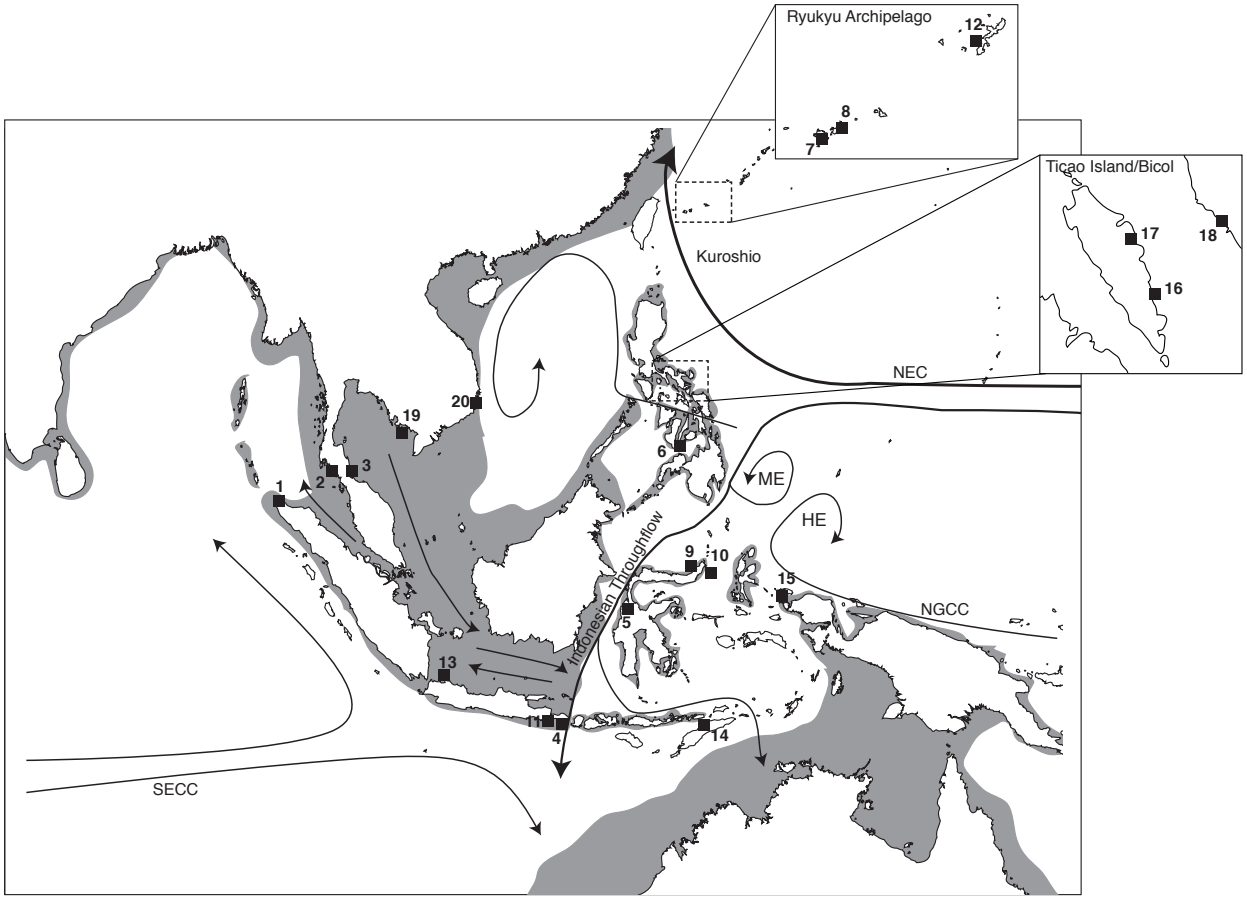


Figure 2.1. Sampling locations through the Coral Triangle and adjacent regions. Gray shading indicates exposed continental shelf during low sea level stands during the Pleistocene (after Voris 2000). Primary oceanographic features are illustrated as well (after Wykrti 1971): NEC = North Equatorial Current, NGCC = New Guinea Coastal Current, SECC = Southeast Counter Current, ME = Makassar Eddy, HE = Halmahera Eddy.

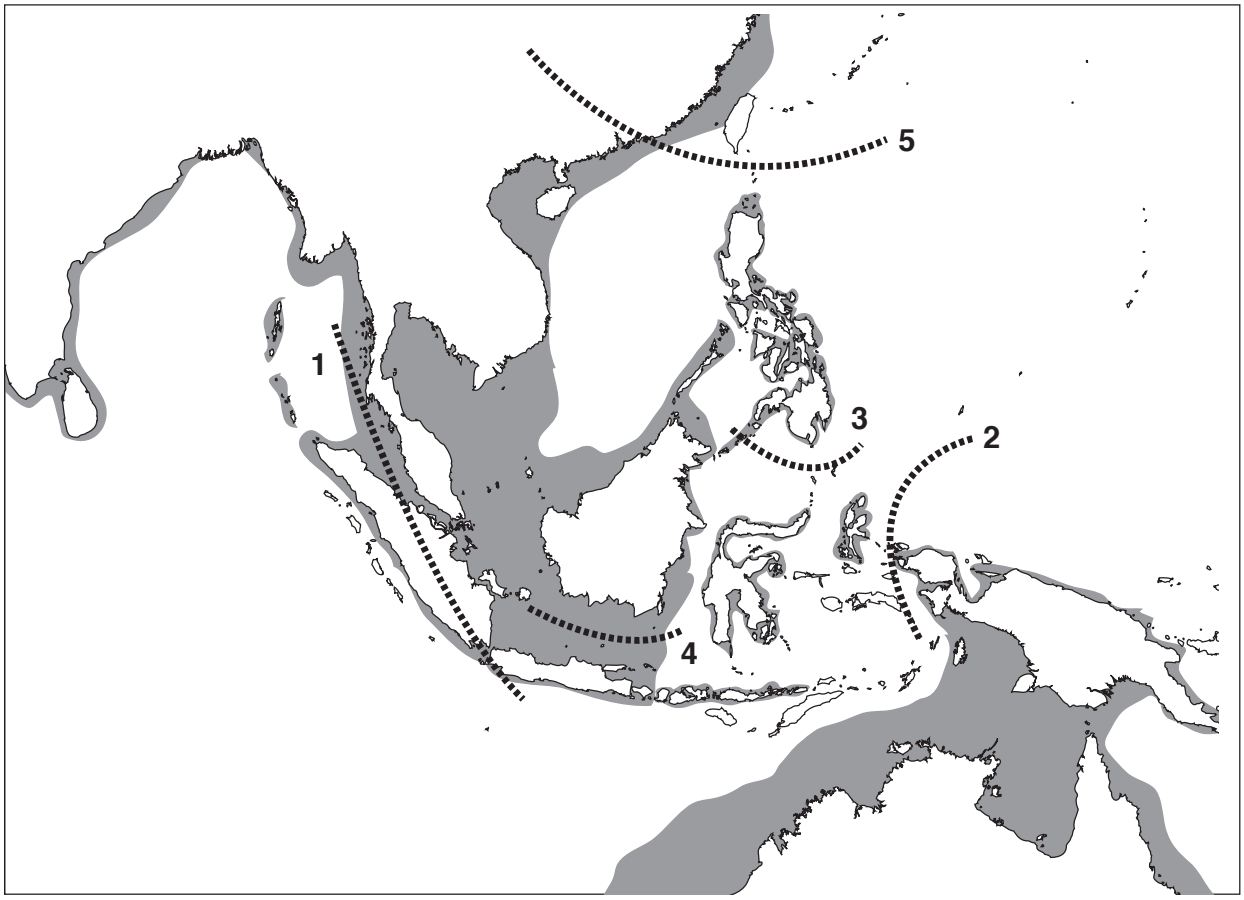


Figure 2.2. Commonly hypothesized phylogeographic breaks in the region (after Carpenter et al. 2010)

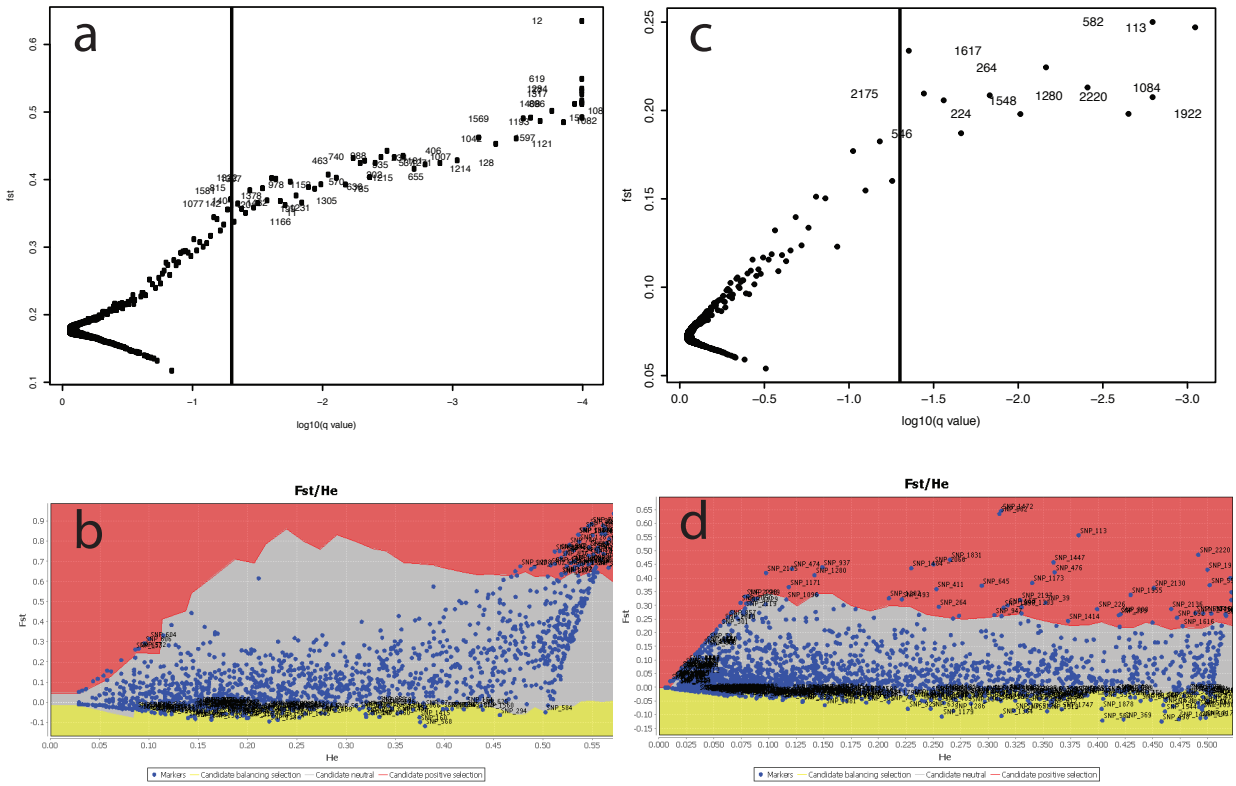


Figure 2.3. Putative outlier loci within each lineage were detected using a probabilistic method (Bayescan, Foll and Gaggiotti 2008) and a summary statistic method (Lositan, Antao et al. 2008). Outliers were called at 0.05 false discovery rate for Bayescan (a, c) and at 0.1 false discovery rate and 0.99 confidence interval for Lositan (b, d).

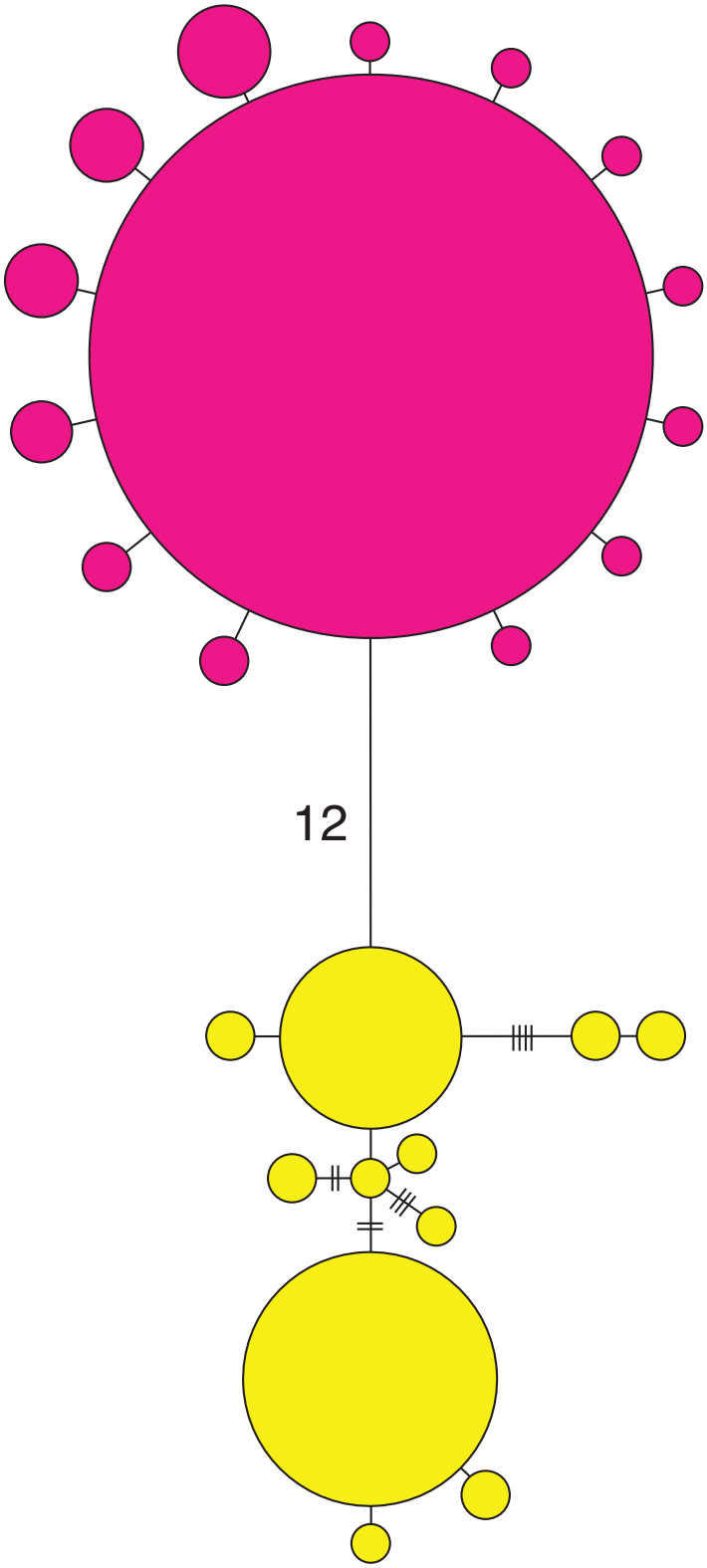


Figure 2.4. Minimum spanning tree for lineage B derived from mitochondrial CO1 data. Colors indicate clades separated by at least 10 mutational steps. Hash marks indicate how many mutational steps separate haplotypes. No hash marks indicate 1 mutational step

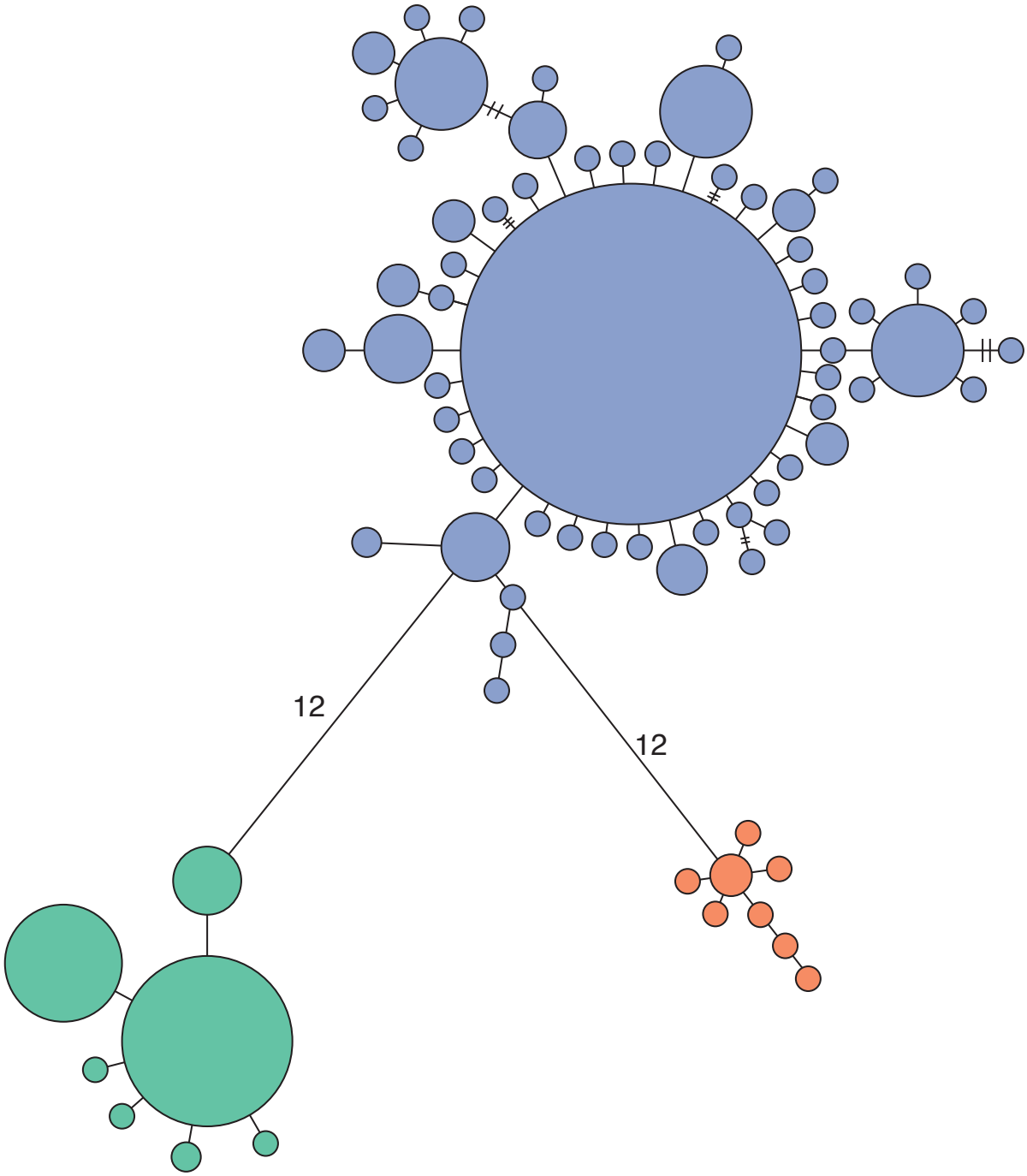


Figure 2.5. Minimum spanning tree for lineage C from derived from mitochondrial CO1 data. Colors indicate clades separated by at least 10 mutational steps. Hash marks indicate how many mutational steps separate haplotypes. No hash marks indicate 1 mutational step

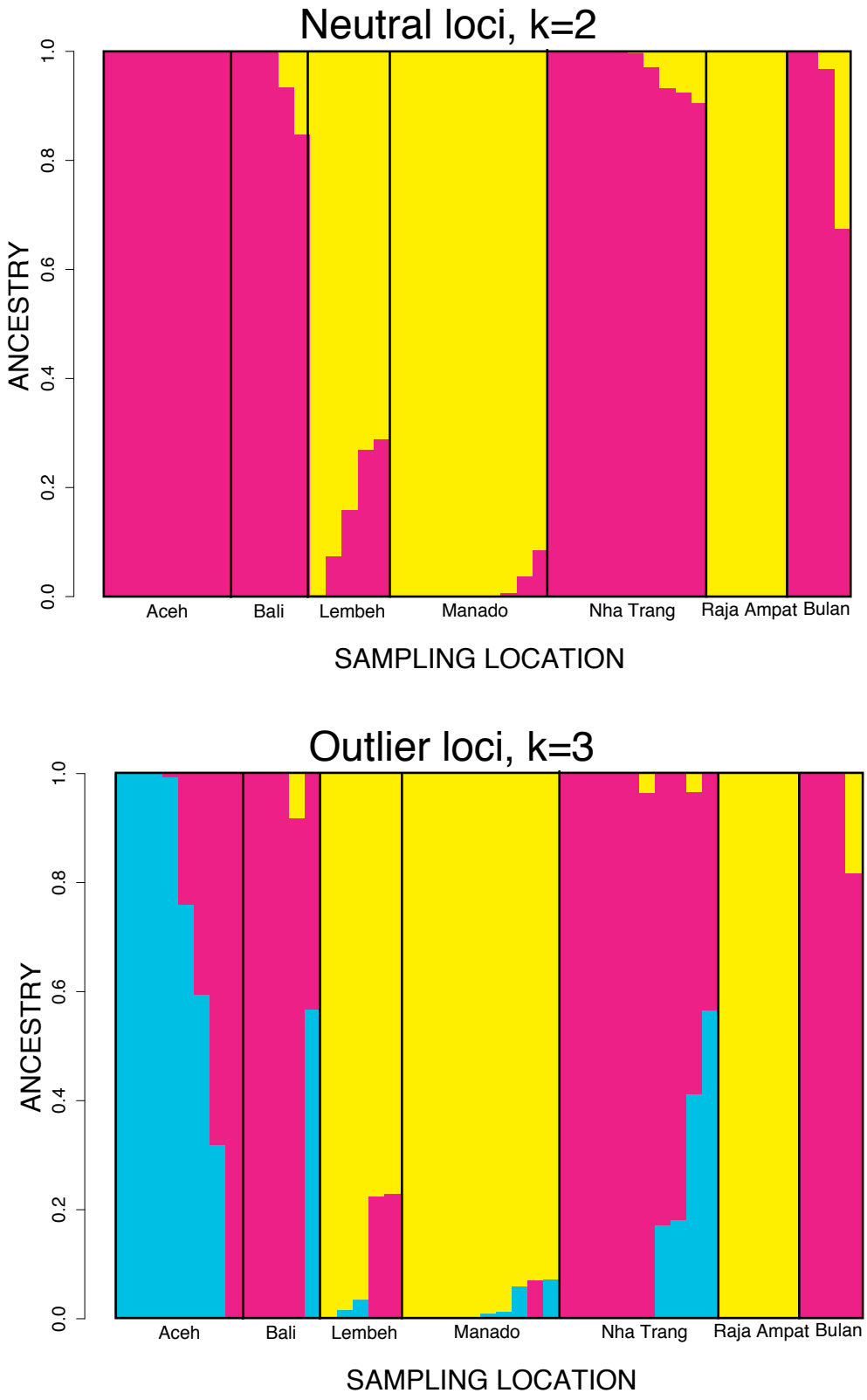


Figure 2.6a & b. Individual ancestry plots for lineage B inferred from neutral and outlier SNPs using ADMIXTURE.

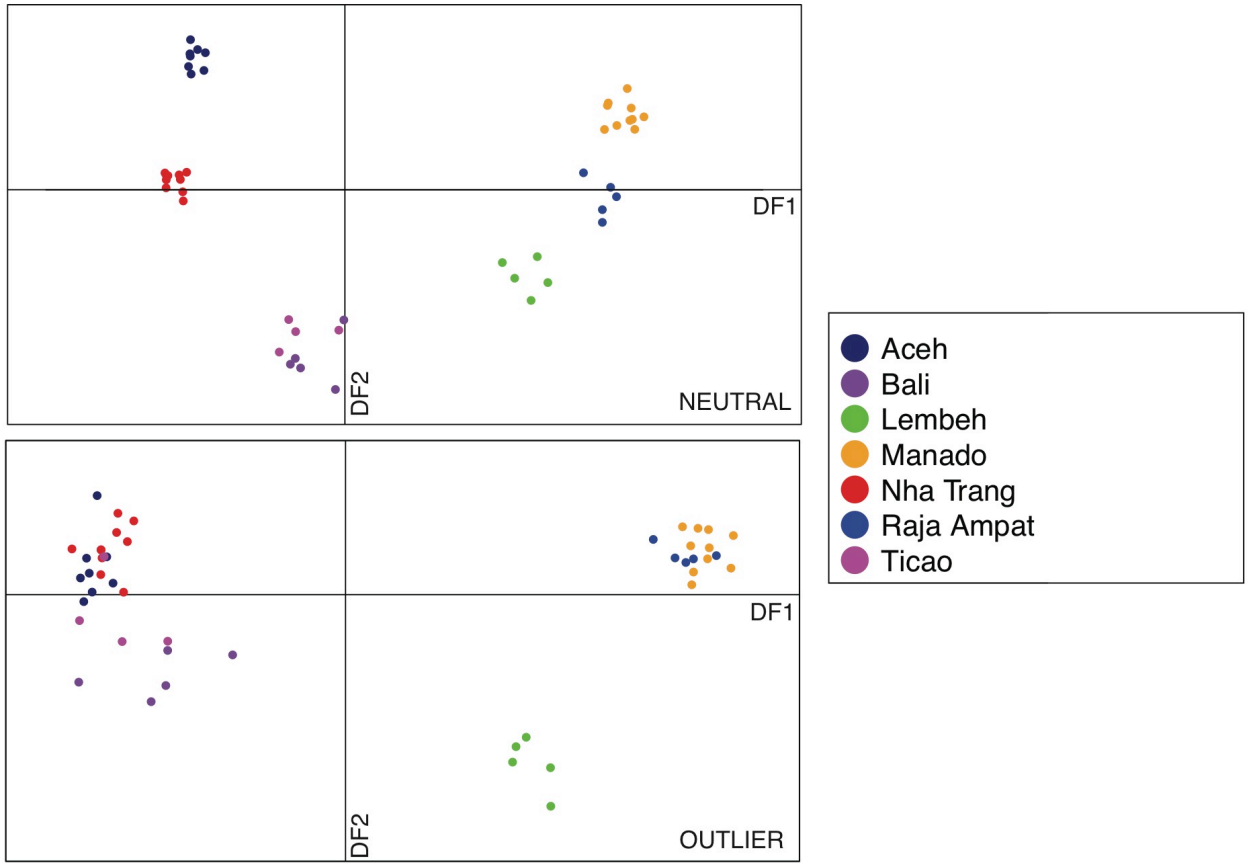


Figure 2.7a & b. Discriminant analysis of principal components indicate varying patterns of clustering with different data types for lineage B

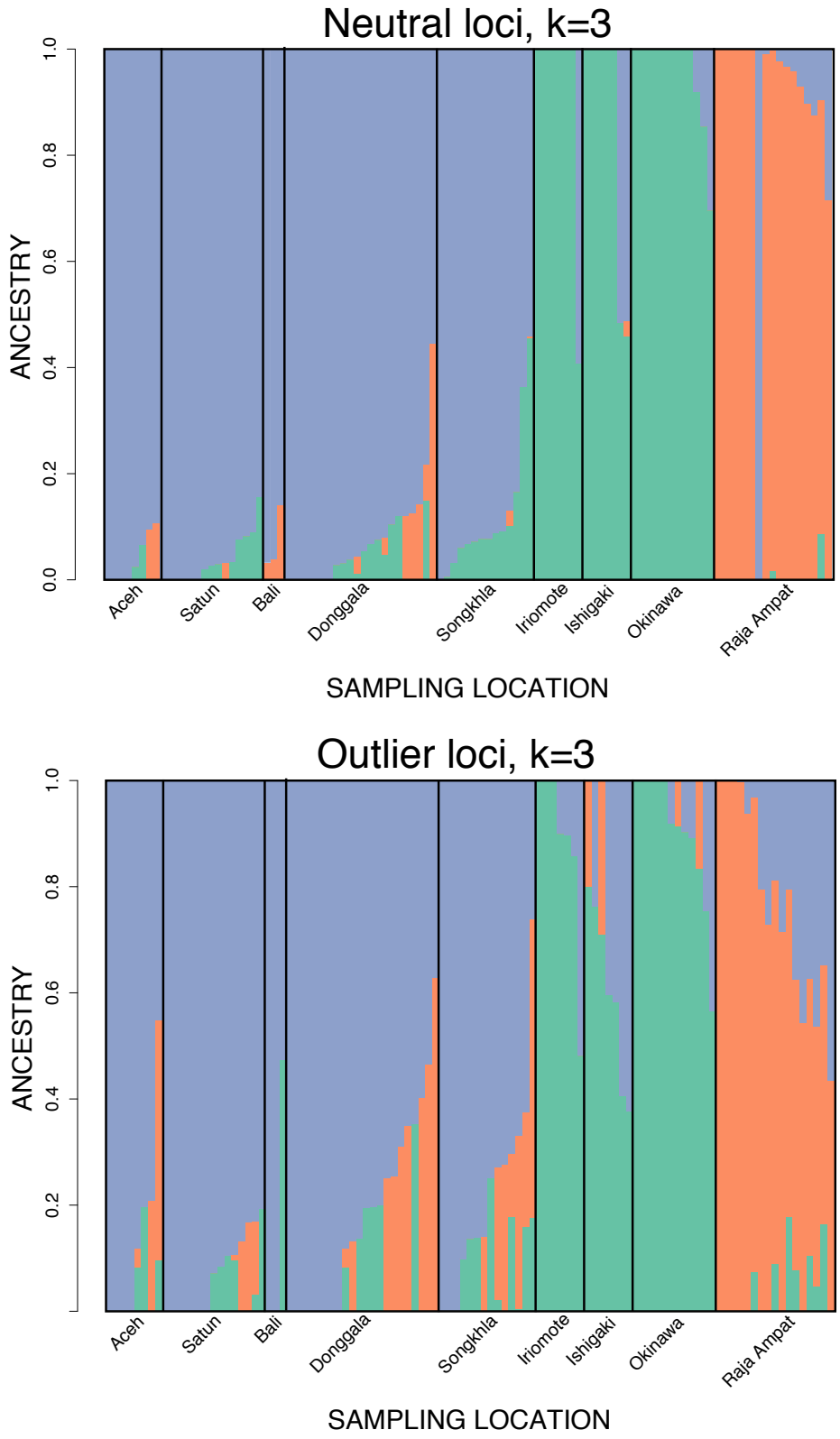


Figure 2.8a & b. Individual ancestry plots for lineage C inferred from neutral and outlier SNPs using ADMIXTURE

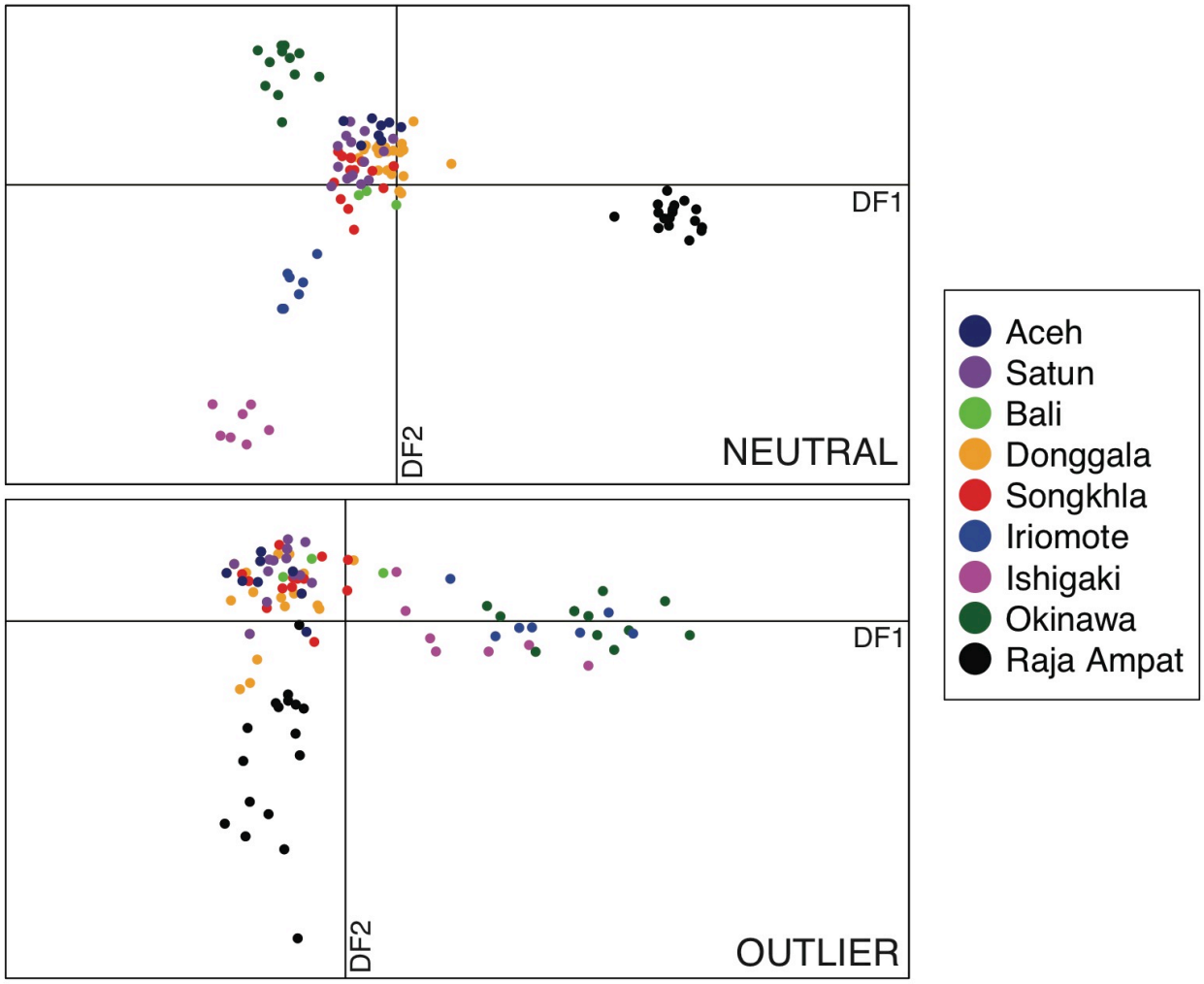
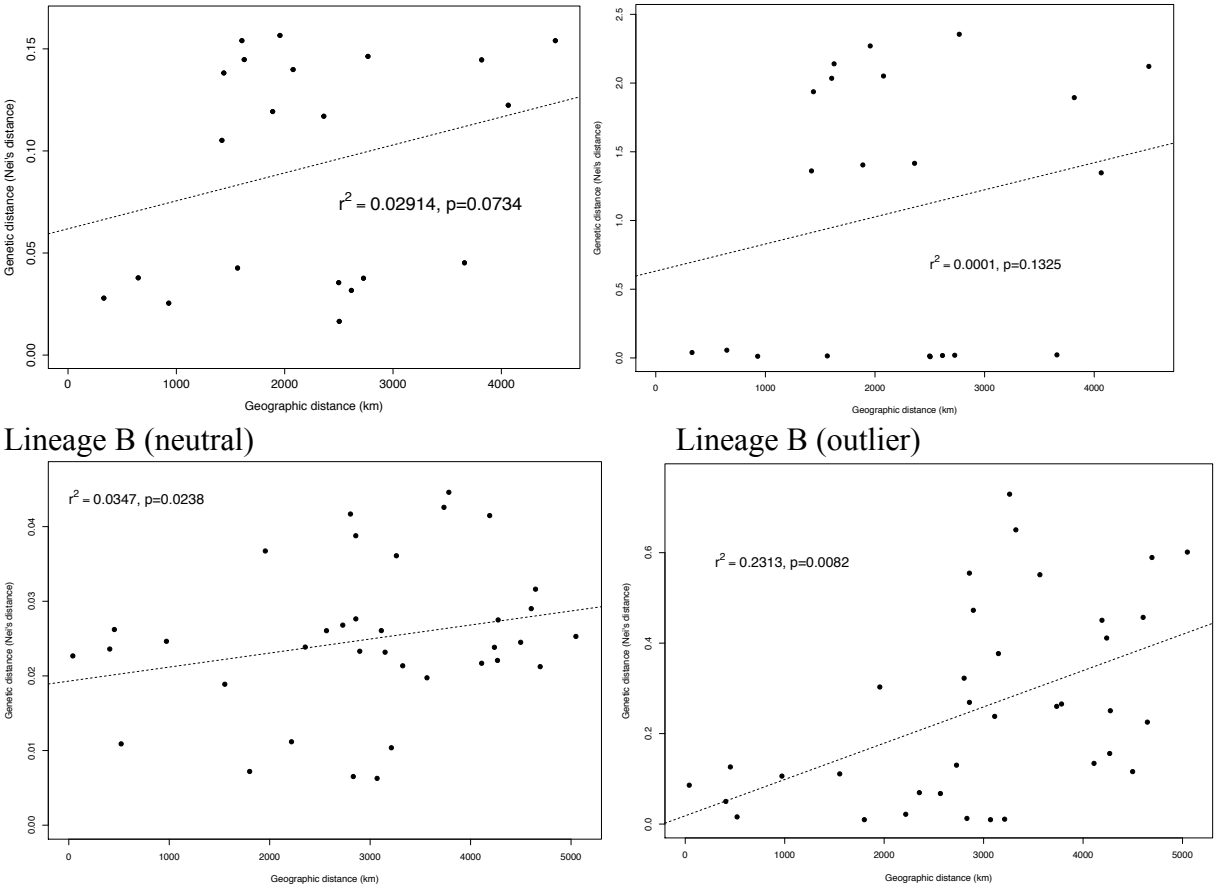


Figure 2.9a & b. Discriminant analysis of principal components indicate varying patterns of clustering with different data types for lineage C



Lineage B (neutral) Lineage B (outlier)

Lineage C – Neutral Lineage C - outlier

Figure 2.10. Isolation by distance was detected in Lineage C but not in Lineage B.

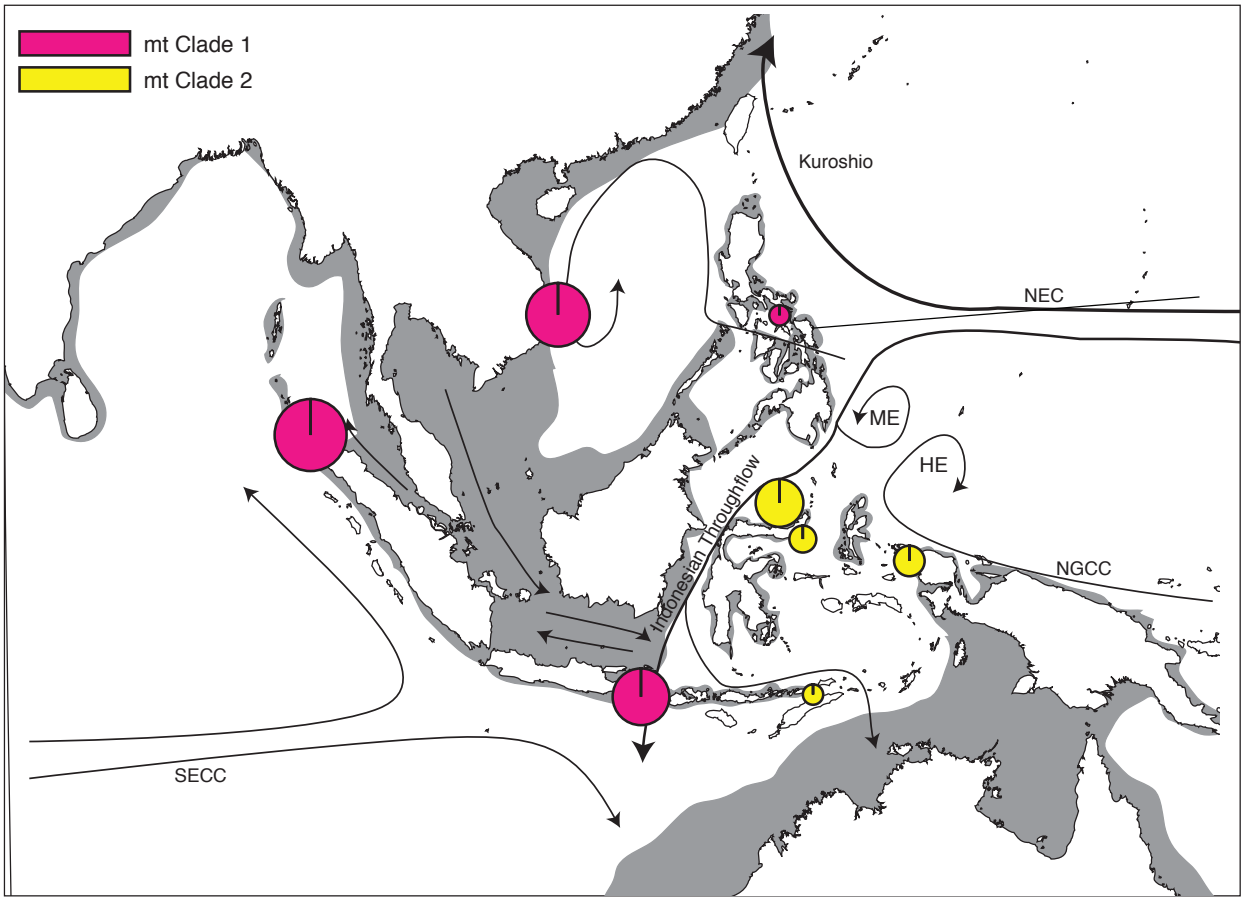


Figure 2.11. Distribution of mitochondrial clades over the sampling regions in the Coral Triangle and peripheral areas for lineage B. Gray shading indicates exposed continental shelf during low sea level stands during the Pleistocene (after Voris 2000). Primary oceanographic features are illustrated as well (after Wykrti 1971): NEC = North Equatorial Current, NGCC = New Guinea Coastal Current, SECC = Southeast Counter Current, ME = Makassar Eddy, HE = Halmahera Eddy.

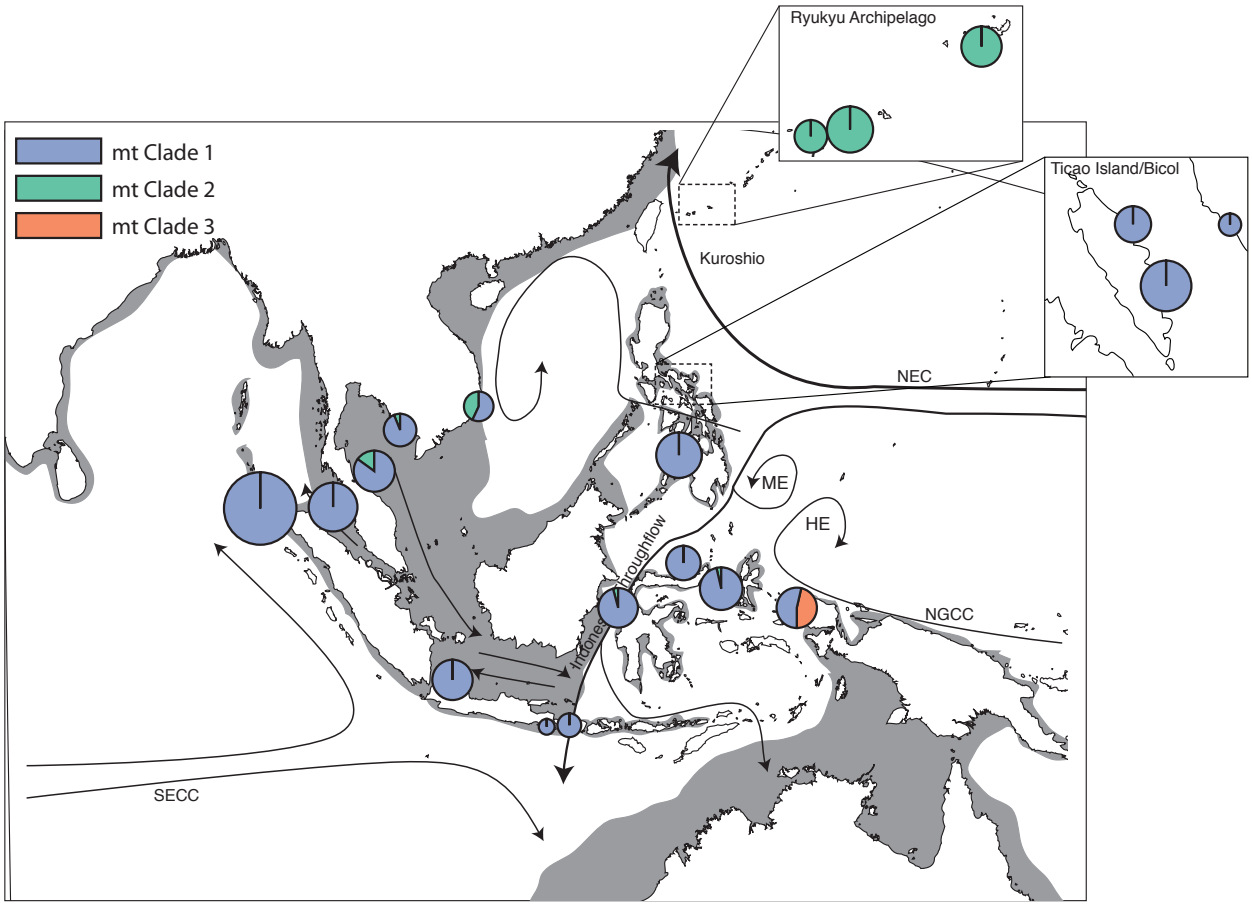


Figure 2.12. Distribution of mitochondrial clades over the sampling regions in the Coral Triangle and peripheral areas for lineage C. Gray shading indicates exposed continental shelf during low sea level stands during the Pleistocene (after Voris 2000). Primary oceanographic features are illustrated as well (after Wykrti 1971): NEC = North Equatorial Current, NGCC = New Guinea Coastal Current, SECC = Southeast Counter Current, ME = Makassar Eddy, HE = Halmahera Eddy.

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CHAPTER 3

Genome-wide SNPs reveal complex fine-scale population structure in the California market squid fishery (*Doryteuthis opalescens*)

Abstract

Marine fisheries are critical for supporting local economies and sustaining local and global food security. In California, market squid (*Doryteuthis opalescens*) is one of the largest and most valuable fisheries. Understanding the extent and distribution of genetic structure is crucial for informing effective conservation and management for this commercially important species. We generated 662 single nucleotide polymorphism markers from across the genome and along with corresponding morphometric data, aimed to determine whether multiple breeding stocks of *D. opalescens* occur within the California fishery. A total of 156 individuals were amplified from five monthly sampling groups (May-September 2014) in the northern (Monterey) and southern (Southern California) regions of the fishery (n = 8). Overall, low levels of genetic differentiation were detected between all sampling groups (F_{ST}=0.008, p>0.05) with no significant differentiation between all northern and southern samples (F_{CT}=-0.006, p > 0.05). Closer inspection of sampling groups using discriminant analysis of principal components and pairwise comparisons of genetic differentiation reveal unexpected complex patterns of fine-scale population structure. Specifically, we observe that some spawning groups recruiting to spawning grounds at different times are genetically distinct. These results lend preliminary support to the existence of smaller genetically distinct cohorts that continually spawn in California (Jackson and Domeier 2003), as opposed to the prevailing notion that spawning occurs in two asynchronous peaks in the northern and southern regions of the fishery (Hixon 1983, Spratt

1979). This study represents the first in-depth genomic examination of temporal-spatial population structure in *D. opalescens* and demonstrates the applicability of genome-wide SNPs for detecting fine-scale structure in squid fisheries. However, this study only covered part of one harvest season and thus, to accurately inform changes in fisheries management, a systematic analysis using fisheries independent data over multiple spawning seasons is required.

Introduction

Management of marine resources depends heavily on information regarding spatial and temporal structure of populations (Hilborn and Walters 1992, Palumbi 1994). Generally, management of harvested mobile marine organisms centers around catch limits, spatial and temporal delimitations for harvest, and size restrictions. Therefore, it is vital to have accurate information about the timing and location of breeding populations, and the connectivity among these populations (Sale 2004). Physically observing movement of individuals and connectivity among marine populations is extremely difficult given the fluid and expansive nature of the ocean. Inference of these parameters is, therefore, limited to other sources of data (e.g. tag-recapture, modeling, etc…) (Semmens et al. 2007). Advances in genetic resources have made it possible to detect both historical and contemporary patterns of population structure and assess demographic parameters, revolutionizing our understanding of wild populations and how to manage them. For example, the use of genetic methodologies in marine management has been extremely successful in distinguishing breeding populations of harvested oceanic salmon and assigning them back to their natal streams (Waples et al. 1990; Larson et al. 2014).Genetic methods using markers ranging from allozymes to mitochondrial DNA and microsatellites have been increasingly applied over the past few decades to numerous commercially important species

for identifying stocks and populations (Carvalho and Hauser 1994; Morin et al. 2010).

Population genetic approaches can help delineate populations, indicate patterns of gene flow (Slatkin 1987; Waples and Gaggiotti 2006), identify adaptive variation (Beaumont and Balding 2004), detect inbreeding (Charlesworth and Willis 2009) and tease apart parentage (Chakraborty et al. 1988). However, traditional population genetic methods (e.g. mtDNA, microsatellites, etc) typically employ a relatively small number of loci, limiting inferences of demographic and population patterns due to low power and high error rates (Knowles and Maddison 2002) and thus, can only provide limited insight for fisheries management and conservation (rev. Allendorf et al. 2010). In contrast, modern genomic approaches can generate millions of reads from across the entire genome, allowing the detection of hundreds to thousands of putative genetic variants, including single nucleotide polymorphisms (SNPs). This advance is exponentially increasing the reliability and accuracy of estimates of demographic and population parameters (Brumfield et al. 2003). While the field of population genomics is still in its infancy, numerous studies have demonstrated its potential to lend deep insight into the dynamics of natural populations (Hohenlohe et al. 2010; Bourret et al. 2013; Larson et al. 2014).

California market squid (*Doryteuthis opalescens*, formerly *Loligo opalescens* Berry; Anderson 2000; Vecchione et al. 2005) is the most valuable fishery resource in California, currently valued at $73.3 million a year in 2010 (Pacific Fishery Management Council 2011). This neritic loliginid squid ranges widely along the west coast of North America, from southeastern Alaska (Wing and Mercer 1990) to Baja California (Fields 1965). While the fishery for market squid in California has been active since 1863 (Fields 1965), it was historically perceived as an underutilized resource until as late as 1978 (Vojkovich 1998). The fishery rapidly increased in the 1980s and 1990s due to rising international demand for squid and the

precipitous decline of the regional Pacific anchovy fishery. Today, market squid comprises the largest biomass of any harvested species in the coastal California fisheries (Rogers-Bennett and Pearse 2001). Not only are market squid ranked as the highest value of any California fishery, but they are a crucial link in the coastal food web. Market squid are a primary prey/forage species for at least 38 species of fish, birds and mammals (Morejohn et al. 1978). As such, a major loss in abundance of this important prey resource could have significant impacts on the California coastal marine ecosystem. Effective and appropriate management of market squid must encompass this species' extremely high economic and ecological importance.

Despite the tremendous importance of the market squid fishery, it is still unclear whether the fishery is composed of one or multiple breeding stocks. Despite its wide latitudinal range, major spawning areas and concentrated abundance are only known from central and southern California (Fields 1965). Market squid have sub-annual recruitment in two major events from April-August in Monterey Bay and from November-March in southern California (Vojkovich 1998; Zeidberg et al. 2006). Active harvest of market squid typically runs from mid spring until the established tonnage quota has been landed, typically in late summer/early fall depending on the season (Zeidberg 2013); thus, the majority of the harvest spans both reported spawning peaks. Spatial and temporal separation of spawning events indicates the likelihood of either separate populations or sub-populations, and consequentially, the potential need for distinct regional management plans.

Morphometric examinations conducted on samples both from within (Fields 1965; Evans 1976) California and throughout the species range (Kashiwada and Recksiek 1978) suggested possible geographically distinct populations and temporally separated spawning stocks, but results were inconclusive. Early genetic studies using protein assays and isozymes were similarly

ambiguous, hinting at the potential of two populations (Ally and Keck 1978; Christofferson et al. 1978). Most recently, Reichow and Smith (2001) used microsatellites to determine the level of gene flow between different parts of the range, concluding there was high genetic connectivity (exchange of genes) from Alaska to Baja California. However, the inference from this study is limited as it pooled samples from multiple years and used relatively low numbers of loci. The absence of genetic differentiation should not necessarily be interpreted as support for one panmictic population (Lowe and Allendorf 2010). For species with very large effective population sizes, like pelagic and neritic fish and invertebrates, low migration rates ($m < 0.001$) can still result in differentiation (FST) values near zero (Waples 1998). This low number of migrants, while sufficient to achieve measurements of high gene flow, is likely not sufficient to maintain demographic connectivity which is a critical element for population and species persistence (Hedgecock et al. 2007; Waples and Gaggiotti 2006). Understanding both of these connectivity patterns is vital to ensure the sustainability of this commercial valuable fishery.

Here we employ genome-wide single nucleotide polymorphism loci (SNPs) in conjunction with morphometric data to test for the possibility of spatial or temporal connectivity between the northern and southern fishing grounds during the 2014 harvest season for *D. opalescens*.

Methodology

Specimen collection

A total of 400 whole adult samples were collected during regular monitoring activities by California Fish and Wildlife staff in both northern (Monterey landings) and southern California (San Pedro, Catalina landings) ports during the active fishing seasons in 2014 (Figure 3.1).

Temporally paired samples (n=50 per location per month) were collected from June through August. Only northern samples were collected in May, and only southern samples in September due to no or low numbers of landings (Table 3.1). Adults were frozen and transported to the University of California, Los Angeles for processing. Squid were subsampled for genetic analyses by excising a 1-2 cm piece of mantle tissue from the ventral side of the mantle opening and preserving in 95% ethanol.

RAD library preparation and sequencing

We generated genome-wide single nucleotide polymorphism (SNP) data using a type of RAD (restriction site-associated DNA tag) sequencing method, called 2b-RAD (Wang et al. 2012). We chose RAD sequencing as this method allows for simultaneous single nucleotide polymorphism disδ^{15}covery and genotyping in large numbers of samples without established genomic resources (i.e. non-model organisms) (Baird et al. 2008). Furthermore, the simultaneous discovery and genotyping characteristic of RAD significantly reduces issues caused by ascertainment bias arising from developing SNP panels from a subset of populations (Clark et al. 2007). The 2b-RAD method employed here features the use of IIB restriction enzymes, which generate a specific-sized fragment with the restriction site in the center, resulting in uniform fragment sizes (Wang et al. 2012). We chose this method because it is extremely flexible and simple in comparison to more typical RAD sequencing techniques, making it ideal for screening of large numbers of individuals in population-scale studies.

We extracted high-quality genomic DNA from preserved mantle tissue using a modified phenol-chloroform method employed in the E.Z.N.A Mollusc DNA extraction kit (Omega). Library preparation followed the most recent protocol publically available on the Matz

laboratory website at the University of Texas-Austin, with a few modifications (http://www.bio.utexas.edu/research/matz_lab/matzlab/Methods.html). Preparation started with digestion of ~800-1000 ng genomic DNA using AlfI restriction enzyme. In the interest of increasing the number of individuals sequenced per lane, we reduced total marker density by $1/16^{th}$ (of all digestion sites) using library-specific adaptors. Ligated products were then amplified with Illumina platform-specific primers and unique 6-bp barcodes for 24 cycles in preparation for sequencing. Amplified products were individually run on a 2% agarose gel with SYBR SAFE fluorescent dye in 1X TBE buffer at 150 V for 90 minutes or until product bands were significantly separated. The ~176 bp target band was excised from the gel and the PCR product extracted and purified using a commercially available gel extraction kit (Qiagen QIAquick). Purified PCR samples were further cleaned and brought to appropriate volume and concentration using Ampure XP beads using standard clean-up protocol (Beckman-Coulter).

Sequencing was performed at the University of California-Berkeley Vincent J. Coates Genomic Sequencing Facility on an Illumina Hi-Seq 2000 with single-end 50-bp reads with 40-55 samples per lane. Initial quality control and demultiplexing were performed by the UC Berkeley facility. Read processing, filtering, mapping and genotype calling were conducted using a pipeline from the Meyer Lab at Oregon State University, specifically designed for the 2b-RAD method (E. Meyer, pers. comm. http://people.oregonstate.edu /~meyere/tools.html). First, terminal tag positions were removed from each 50-bp read (keeping positions 1-36) to remove sections associated with ligation sites. Reads were then stringently filtered for quality; removing any reads with ambiguous base calls and any low-quality positions (more than 1 position with quality less than 10). From these high-quality reads, any reads consisting only of adaptors were also removed.

We constructed a reference assembly using an unpublished, unannotated draft genome of a closely related species of squid to *D. opalescens* – *Doryteuthis* (formerly *Loligo*) *pealei*. This draft genome is publicly available from C. Titus Brown (University of California, Davis) (http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html) and was generated from ~40X coverage Illumina HiSeq reads. AlfI sites were extracted from the *D. pealei* genome, resulting in a reference assembly of 198,008 unique tags (7,128,288 total basepairs). Reads per individual sample were then mapped onto the reference assembly using *gmapper* employed in SHRiMP (Rumble et al. 2009), with stringent settings to minimize mismatches (maximum 2 mismatches per read). Mapped reads were filtered to remove weak, partial, and ambiguous mappings in SHRiMP, which tests the likelihood that strong matches would be found by chance given the characteristics of the reads and the reference (Rumble et al. 2009). Mapped reads were then converted to a readable format for SNP calling using *prettyprint* as implemented in SHRiMP. Nucleotide frequencies at sites with at least 5x coverage excluding the AlfI recognition site were then counted for genotype calling. Genotypes were called with maximum 0.01 minor allele frequency (homozygous calls), minimum 0.25 minor allele frequency (heterozygous calls) and minimum 10x coverage.

SNP calling and validation

We combined genotypes from all mapped samples to detect putative single nucleotide polymorphisms (SNPs). SNPs were called optimizing for polymorphism rate (maximum 2 per tag), low coverage (individuals must have at least 5000 loci genotyped) and missing data (loci must be genotyped in at least 75% of individuals). The dataset was then filtered to remove repetitive tags by detecting highly variable tags containing too many SNPs. This was set to a

maximum of 2 SNPs per tag as there is fairly low likelihood of having more than 2 variable sites in the 29 total bases around a 6-bp recognition site (E. Meyer, *pers. comm*.).

Morphometric measurements

To complement genetic assessment of population structure, we measured macro-morphological characters on all successfully sequenced individuals. Eight individual measurements were made (Table 3.2a, Figure 3.2). DML and weight are standard measures of size commonly used in squid, particularly as DML reflects the size of the rigid structure in squid, the pen. Anecdotal evidence suggests that fin width and fin length could be useful in differentiating populations (Evans 1976). Mantle thickness and gonad condition were chosen to estimate stages of maturity. As squids mature, their mantle grows thicker; however, post-spawning as they progress into senescence, the mantle tissue deteriorates. Given that there is ample evidence for distinct spawning peaks between northern and southern fishing areas (Zeidberg et al. 2006), estimating maturity allowed us to compare genetic information with temporal changes in spawning. All measurements and features were determined as follows in Table 3.2a.

DATA ANALYSIS

Sample statistics and population assignment

Allele frequencies, observed and expected heterozygocities, and departures from Hardy-Weinberg equilibrium (HWE) were estimated using Arlequin 3.5 (Excoffier and Lischer 2010). Departure from HWE was calculated for each locus in each population using an analogous method to Fisher's exact test as implemented in Arlequin (1,000,000 steps in the Markov chain

and 100,000 dememorization steps). As multiple tests were conducted over populations, we applied a sequential Bonferroni correction procedure (Holm 1979) in order to minimize the number of significant departures due to chance (Waples 2015). While we already controlled for linkage disequilibrium by picking one SNP per 2bRAD tag, we also conducted tests of linkage disequilibrium between remaining pairs of SNP loci in each population in Arlequin. Any additional SNPs demonstrating high levels of linkage disequilibrium were removed from the final panel of neutral loci. Pairwise F-statistics (after Weir and Cockerham 1984)were calculated between each sampling group (temporal-spatial groups) within an analysis of molecular variance (AMOVA) framework also in Arlequin. Mean, standard deviation, median, and mode of morphometric measurements were calculated for each sampling group and each region (north and south) and compared between sexes, sampling groups and regions using analysis of variance (ANOVA), Tukey HSD tests, and unpaired Student's t-tests as implemented in R (R Core Team 2013).

In order to avoid biased estimates of population parameters, outlier loci that may reflect areas of the genome under selection were separately analysed (Luikart et al. 2003). We employed a reversible-jump Monte Carlo Markov chain method to explore models with and without selection to identify candidate non-neutral loci from a dataset using Bayescan (Foll and Gaggiotti 2008). Bayescan was run with 20 pilot runs (5000 steps) and a final run for 100,000 generations sampling every 10 steps and a 50% burn-in. Outlier loci discovered with a false discovery rate of 0.05 were then separated from the dataset to obtain a neutral panel of SNPs.

We conducted tests of population assignment to determine the most likely number and composition of populations without *a priori* assumptions using ADMIXTURE (Alexander et al. 2009). ADMIXTURE estimates ancestry of unrelated individuals and population allele

frequencies using a maximum likelihood approach. We tested population sizes ranging from k=1 to k=8, representing hypotheses of increasing population structure from one panmictic population (k=1), two spatially distinct populations (k=2) all the way to testing the likelihood that each sampling group was a separate population (k=8). The k value with the lowest cross-validation error indicates the most likely number of populations.

Testing for population structure

Using an analysis of molecular variance (AMOVA), we tested a specific hypothesis of structure or limited gene flow between northern and southern fishing areas. All AMOVA analyses were conducted in Arlequin with 10,000 permutations (Excoffier and Lischer 2010).

We also tested for population structure with morphometric data combined with genetic data. Prior to the proliferation of genetic methodologies to examine population structure, differences in morphometric variables were used to distinguish between different stocks or populations, thus we tested for differentiation of morphometric characteristics between northern and southern samples (rev. Cadrin 2000) using linear regressions combined with analysis of covariance (ANCOVA) and analysis of variance (ANOVA) implemented in R (R Core Team 2013). First, we conducted ANCOVAs comparing regression slopes with sex as the covariate within northern and southern sites as well as for all sites combined to determine if any sexual dimorphism exists in our samples. In order to thoroughly test the different measures of growth, we compared regression slopes of DML vs. weight, FW, and FL respectively, between sexes. We used DML as the independent variable as it reflects the most accurate measurement of size (Fields 1965). Two models were examined within the ANCOVA – one with an interaction between sex and the dependent variable and one without the interaction. The fit of these models

was then tested using an Analysis of Variance (ANOVA) with significance level set to $p < 0.05$. These tests were then repeated using sampling site (northern or southern) as the covariate to explicitly test for differences in growth pattern between harvest regions.

In addition, we employed multivariate analyses using 1) just the morphometric dataset and 2) the SNP dataset and the morphometric dataset combined, to assess biological or geographic determinants of genetic clustering of individuals. To do this we, used a discriminant analysis of principal components (DAPC) conducted in the *adegenet* package in R (Jombart 2008; Jombart et al. 2010) in order to describe observed clustering of genetic and morphometric data. First, we tested whether variation in morphometric variables could capture differences in sampling location and region. Retaining too few principal components (PC) sacrifices power for discrimination between groups, while on the other hand, retaining too many PCs increases the chance of overfitting. Thus, we used a cross-validation method implemented in *adegenet* (*xvalDapc*) to find the optimal number of PCs for each comparison. Using a DAPC on the log-transformed morphometric data (DML, weight, FL, FW), we mapped sampling group (time and location) and region divided by sex on to the first two discriminant functions and described resultant clusters. Second, we used DAPC to examine the clusters described by the SNP data using the same mapping scheme with region by sex and sampling group.

Estimating migration rates and direction

Beyond understanding if population structure exists, determining the direction and rate of migration is also crucial for informing fisheries management as it helps illustrate rates of connectivity over more relevant ecological timescales (Allendorf et al. 2010). We therefore estimated population pairwise migration rates and direction using a Bayesian approach as

employed in BAYESASS+ (Wilson and Rannala 2003). Unlike other estimators of migration rate which estimate long-term gene flow based on the F_{ST}/*Nm* model (rev. (Pearse and Crandall 2004), BAYESASS+ uses multi-locus genotypes to probabilistically identify source populations of known individuals and estimate inbreeding coefficients, population allele frequencies, and population migration rates (Wilson and Rannala 2003). Notably, BAYEASS+ also has few assumptions, making it ideal for examining migration in wild populations, which may not be in equilibrium. As BAYESASS+ can only run a certain number of loci due to memory and CPU limits, we ran 3 randomized subsets of 400 loci each from the dataset. Each subset was run 3 times and migration rates averaged from all three runs and subsets. In order to optimize mixing, parameters were adjusted for each run for each subset to keep the acceptance rate between 20% and 60%. Final runs for each subset and replicate were run for 1,000,000 generations sampling every 1,000 steps with a 10% burn-in and random start seed, resulting in 900 sampling points per run. Final estimates of population pairwise migration rates and inbreeding coefficients (F_{IS}) were averaged over all runs and subsets.

Results

Temporal and spatial variation in life history data

The majority of individuals sampled were either in maturity stage 3 or 4 (gravid or spent) (Figure 3.3A). On average, males were larger than females in terms of all measures of growth (Figure 3.3A). Males were also generally significantly larger than females in weight (Student's t-test, t=3.30, df=126.97, p=0.001) but not significantly different in any other measure. In contrast, all measures of growth (dorsal mantle length, weight, fin length, fin width) were significantly different between males and females in the north and south fishing regions (ANOVA, DML:

F=9.51, p>0.0001, weight: F=24.07, p > 0.0001, FL: F=6.51, p=0.0004, FW: F=3.60, P=0.015) (Figure 3.3A). Upon closer examination of these pairwise comparisons, males and females caught in northern fishing areas were larger than those caught in the south; however, not all of these differences were significant (Table 3.3). Within the northern region, we observed that individuals were generally larger earlier in the season than at the end, while in the southern region, differences in size between months were not as pronounced (Figure 3.3B & C, Table 3.3).

Comparing regression slopes for growth between sexes did not yield a significant interaction (ANCOVA, F=3.049, p=0.083), indicating that males and females generally grow at the same rate in general, but that males are generally larger at maturity than females (Figure 3.4). However, when we split males and females between regions, there was a significant interaction between region/sex in the relationship between DML and weight (ANCOVA, F=5.23, p=0.0019). This significant covariance stems from the males (ANCOVA, F=5.80, p=0.019) and not the females (ANCOVA, F=3.32, p=0.073) (Figure 3.5). Discriminant analysis of principal components indicated subtle clustering of individuals from northern and southern regions with the majority of variation defined by discriminant function 1 (DF1) with weight contributing the largest proportion of loadings (Figure 3.6A). However, the proportion of successful reassignments of individuals to regions by sex was only moderate (31.4%-69.2%) indicating likely admixture, or incomplete differentiation based on these morphological characters. On a finer scale, morphometric data was able to slightly discriminate between temporal replicates within the northern and southern regions, as well as between them (without separating out sexes) (Figure 3.6B). Temporal replicates in the south were discriminated by DF1, with weight contributing 81.2% of loadings. DF2 (FW and FL) indicated slight differentiation between

northern samples as well as between northern and southern samples. However, successful reassignments to sampling groups were fairly low for all groups (10.0%-50%) except for July and August samples from both regions (69.6%-88.9%). Combined, while these morphometric data indicate some difference in size between northern and southern regions and sampling groups, reassignment probabilities were too low to reliably use this data as an indicator for separate populations.

Sequencing, SNP discovery and quality filtering

Out of 174 individuals submitted for sequencing, we discarded 18 individuals due to low number and quality of reads and 4% of sequences after stringent quality filtering. This resulted in 156 total samples with an average of 3 million to 11 million raw reads per individual. An average of 76% of quality-filtered reads mapped to the *D. pealeii* reference. Combining all genotypes, we had 384,469 loci over all individuals, of which 5.83% (22422) were polymorphic (at least 2 genotypes). We optimized for the highest number of loci genotyped in the most individuals, keeping 5,000 loci genotyped in 149 out of 156 samples. We then discarded loci that had more than 25% missing data over individuals (3982 putative SNP loci remaining) and subsequently, tags with more than 2 SNPs sites (925 loci remaining). After thinning for 1 SNP per tag, a total of 662 loci representing 149 individuals from 8 sampling points (4 time points each from northern and southern California fishing grounds) were included in the final dataset.

Molecular diversity and outlier detection

Varying levels of observed (H_o = 0.07098-0.15245) and expected (H_e = 0.07044-0.14576) heterozygocity were detected across sampled locations (Table 3.4). Levels were similar between

samples collected in northern California in May, July and August and in southern California in August. In comparison, southern samples from June and July had slightly higher H_e and H_o and samples from Ventura had higher H_e and H_o than all other samples. Samples collected in June from northern California stood out as having much higher H_o and H_e than all other sampling locations. The mean percentage of polymorphic SNPs in each sampling location ranged from 14.59% to 27.85% (Table 3.4). After testing for HWE and sequential Bonferroni correction (Holm 1979), one locus showed significant departures from HWE at more than half of the locations sampled and was excluded from the final dataset. Over all 661 loci in all sampling locations, Bayescan did not detect any outlier loci. Furthermore, results from a less conservative ($\log_{10}$PO>1.5) and more stringent criteria ($\log_{10}$PO>2) also revealed that no outliers were detected (Figure 3.7). Thus, we proceeded on the assumption that the panel of SNPs generated were neutral, however, it could also be the case that outliers were not detected because we did not have sufficient power.

Patterns of population assignment and structure

AMOVA results indicate that there is no significant genetic differentiation between northern and southern California (F_{CT}=-0.006, $p > 0.05$) with overall high rates of gene flow (F_{ST} =0.008, $p>0.05$). ADMIXTURE indicated that a one-population scheme was most well-supported (CV=0.10265) (Figure 3.8). When we examine patterns of individual ancestry assuming k=2, there was no clear spatial patterns between northern and southern regions (Figure 3.8). While this suggests a single spawning stock, pairwise comparisons from different sampling locations and times throughout the season indicate that patterns of genetic structure throughout the season is not so straightforward. We found varying patterns of differentiation between

temporal replicates from each region (Tables 3.5A-C). Examining paired temporal replicates, we observe significant spatial differentiation in July and August (Table 3.5C). However, looking at all samples, pairwise comparisons suggest temporal differentiation between samples collected in May/June and those collected in July-September (Table 3.5C).

Within each region, pairwise genetic differentiation is higher as time between temporal replicates increases. For example, in northern region samples, all monthly samples were not significantly different from each other except for increased differentiation between samples collected in May with those in August (F_{ST} =0.01041, $p<0.05$) (Table 3.5A). In southern samples, differentiation is more pronounced between monthly samples (Table 3.5B). Specifically, we observe that samples collected at least two months apart tend to have low levels of differentiation. Interestingly, while overall we did not recover any significant indicators of structure between northern and southern California during the fishing season as a whole, we do observe significant levels of differentiation between northern and southern California during July and August (F_{ST} =0.014 and 0.0072 respectively, $p>0.05$), as well as recover signals of temporal differentiation independent of region (Tables 3.5A-C). These complex patterns of subtle structuring between temporal replicates at each region indicate that patterns of gene flow are not symmetrical over time and space.

Given the complex differences in morphometric measurements and SNP data, we used discriminant analysis on principal components to tease apart spatial and temporal determinants of differentiation. Initial scatterplots of individuals over the first two discriminant functions (representing the first 40 PCs) indicates slight partitioning between northern and southern regions, but stronger distinctions between males and females in the north (Figure 3.6C). However, the SNP dataset only had a moderate ability to reassign individuals back to northern or

southern region by sex (68.57%-79.49%). Discriminant analysis of the first 40 PCs indicated stronger partitioning between temporal replicates, particularly between samples collected early in the season (May and June) and those collected later (July-September) (Figure 3.6D). Discriminant function analysis had variable success in assigning individuals back to their original sampling groups (~69.57%-100.00%) lending some support for subtle differentiation between certain locations and times. Overall, the DAPC analyses indicate that despite the likelihood of one population sampled during the fishing season, substantial fine-scale structuring does exist between specific time periods in the season and between fishing regions.

Direction and magnitude of migration

The means of immigration rates (averaged over posterior probabilities of subsets and replicate runs) between sampling locations indicate a high degree of self-recruitment. All spatial-temporal sampling groups (e.g. North-June, North-July, etc..) indicated a self-recruitment rate of 0.65 to 0.69, except for Southern California samples in August that had much higher self-recruitment (0.92) (Table 3.6). Migration rates in both directions between all sampling times and locations were fairly consistent at $m \sim 0.010$-0.018. While migration rates were symmetrical for all sampling locations, migration rates were higher (m=0.20-0.26) coming from samples collected in southern California in August and all other sites. However, it is worth noting that for seven out of eight sampling locations, the rate of self-recruitment closely approached the threshold for the immigration rate prior (0.70) defined by the program (Wilson and Rannala 2003). Thus, it is distinctly possible that these results do not reflect the realistic migration rates and that we may be getting these values due to the chain getting trapped in local optima (see Discussion).

Discussion

Distinct size groups combined with the stark discordance in spawning peaks between northern and southern California has long suggested the presence of more than one population of *D. opalescens* in California. Previous studies using morphometric (Evans 1976; Kashiwada and Recksiek 1978), electrophoretic (Ally and Keck 1978), allozyme (Christofferson et al. 1978), and microsatellite data (Reichow and Smith 2001) have been unable to definitely resolve population structure of *D. opalescens* in California. Similarly, in this study, when compared with genomic data, morphometric data was unable to resolve similar patterns of population structure. However, results from this study using genomic data reveal a subtle but distinct signal of temporally mediated spatial structure in California during the 2014 summer harvest season. This stands in contrast to previous studies (above) and current management policy that assumes a temporally stable single stock with high gene flow between northern and southern California. Rather, evidence from this study suggests that 1) genetically distinct spawning groups are recruiting to spawning grounds at different times and 2) inconsistent patterns of spatial distinctions, indicating variations in recruitment patterns and preferences. This insight into the complex nature of population structure in market squid necessitates a change in current fisheries management plans.

Temporally mediated population structure

Examination between temporal replicates reveals complex population patterns in market squid harvested in 2014. While morphometric data was unable to clearly resolve any temporal or spatial patterns (Figure 3.6C), we recovered a subtle but distinct signal of differentiation between northern and southern California in July and August (F_{ST} = 0.014 and 0.007 respectively,

$p<0.05$), however not in June (F_{ST} =-0.05, $p>0.05$) This indicates that population structure is not as straightforward as previously thought (Figure 3.6). As the season progressed, we observed more differentiation between northern and southern regions. Pairwise comparisons of individuals present at each temporal replication within each region indicate that individuals spawning in the southern fishing area in August-September were significantly different from those present in June and July (Table 3.5B). We also observed that individuals present in August in the north were significantly different from those present earlier (Table 3.5A). These patterns observed with genome-wide SNP data indicates that 1) monthly regional catches may be dominated by distinct spawning groups that change over time and 2) spatial distinction between northern and southern regions does exist, but is not temporally consistent.

Growth in squid is strongly tied to environmental conditions at both development and maturation (Jackson and Forsythe 1997; Jackson and Domeier 2003; Forsythe 2004), thus morphometric and morphological characters may be used to distinguish between populations experiencing different environments or displaying different responses to shared environments. While in this study, northern samples were generally larger at maturity than southern samples (Figure 3.3B) (ANOVA, $p<0.05$) we did not consistently observe this difference. In fact, there was a significant amount of overlap in morphometric indicators between sampling groups (Figure 3.3B). Similarly, studies employing growth (Evans 1976) and other morphological measurements, such as beak morphology (Kashiwada and Recksiek 1979), arm length (Kashiwada 1981) and tentacle suckers (Kashiwada and Recksiek 1978) were also not able to distinctly resolve distinct geographical differences. The size of recruiting adult *D. opalescens* is highly variable depending on specific hatching conditions (Reiss et al. 2004). Thus, the

variability detected in this study suggests that different sized individuals stem from different cohorts hatched and matured under different environmental conditions.

Strong spawning site fidelity has been observed to drive divergence in other marine species such as salmon (Banks et al. 2000), artic charr (Adams et al. 2006), cod (Skjæraasen et al. 2011; Zemeckis et al. 2014), and pike (Miller et al. 2001). Numerous physical tagging studies as well as genetic studies using a variety of molecular markers, including microsatellites and genome-wide SNPs, have found distinct spawning stocks that correspond to spawning sites in specific river basins and streams that mix offshore (Seeb et al. 2011; Larson et al. 2014). Similarly, consistent temporal lags in recruitment have also been observed to drive divergence as a form of temporal reproductive isolation. For example, gel electrophoresis was able to detect different populations of salmon between fall and spring spawning in the Gulf of St. Lawrence (Kornfheld and Gagnon 1982).

The prevalence of spawning aggregations at specific shallow-water sites within loliginid species (Boyle and Boletzky 1996) suggests that within these species, population structure could derive from spawning site fidelity as well. Examinations with allozyme data resolved at least three separate populations of *D. pealeii* in the NW Atlantic (Garthwaite et al. 1989). More in-depth investigation using microsatellite loci indicated that populations corresponded to distinct inshore spawning aggregations with populations occurring together in offshore feeding grounds (Buresch et al. 2006). Pelagic (oegopsid) squids, which have much higher dispersal capacity and range than neritic (myopsid) squids (including loliginids) were also found to have genetically distinct spawning populations recruiting into the same spawning site at different times throughout the year (Thorpe et al. 1986; Brierley et al. 1993). Similarly, *D. opalescens* are observed to utilize the same spawning sites along the California coastline (Fields 1965;

Vojkovich 1998; Zeidberg et al. 2012), leading many to hypothesize that some degree of self-recruitment may exist. Paralarvae of *D. opalescens* also tend to become entrained in specific water masses close to spawning grounds (Zeidberg and Hamner 2002). Additionally, complex schooling behavior develops very early in other loliginid paralarvae (Boal and Gonzalez 1998). Thus it is feasible that *D. opalescens* forms schools shortly after hatching and may recruit together to a specific spawning site, in a similar fashion as *D. pealeii* (Buresch et al. 2006). However, this has not been explicitly examined in this species.

Previous studies report that *D. opalescens* spawning has two distinct peaks – one in the spring/summer in northern California (Spratt 1979) and one in the winter in southern California (Hixon 1983) and are thought to represent different spawning populations (Ally and Keck 1978; Christofferson et al. 1978; Jackson and Domeier 2003). However, observations of overlapping size cohorts and year-round spawning in *D. opalescens* (Jackson and Domeier 2003; Navarro 2014) and *L. pealeii* (Macy III and Brodziak 2001) suggest that large spawning aggregations are rare and continual spawning by smaller cohorts is more common than expected. This study also does not support two distinct populations defined by these spawning peaks, rather indicating that genetic identity of spawning cohorts can fluctuate from month to month in the same region supporting temporal lags in recruitment between cohorts. However, while we sampled between the two reported major peaks, our sampling reflects the most active time of the fishery in 2014 when theoretically, spawning abundance should be highest (Figure 3.9). Despite a clear shift in harvest concentration from the northern to the southern region during our sampling period, we do not observe signals of a markedly different population shift from north to south. Rather, these results lend more support to continual spawning throughout California comprised of temporally distinct cohorts.

Low levels of genetic differentiation

Recovery of overall low levels of genetic differentiation is not unexpected in this highly mobile marine species. In fact, the majority of research on population structure of neritic loliginids indicates either very little or no population structure (e.g., *Sepioteuthis lessoniana* (Aoki et al. 2008), *D. opalescens* (Reichow and Smith 2001), *Loligo forbesi* (Shaw et al. 1999), *L. forbesi* (Brierley et al. 1995). Previous studies of *D. opalescens* also found high levels of gene flow over wide spatial scales (Ally and Keck 1978; Christofferson et al. 1978; Reichow and Smith 2001). Small-scale tagging studies on *D. opalescens* indicate that these squid have the capability to travel long distances, potentially up to 400-500 km in their lifetime (Zeidberg 2004; Payne et al. 2006). However, with large population sizes, even low migration rates ($m < 0.001$) can result in very low values of genetic differentiation (F_{ST}) (Waples and Gaggiotti 2006; Lowe and Allendorf 2010). While there are no reliable estimates of total biomass for *D. opalescens*, models based on egg-escapement place the number of spawning females at a few million during seasons of low abundance (Dorval et al. 2013). Thus, under the assumption of large population sizes, a small number of individuals migrating between spawning cohorts or between regions could result in the low levels of genetic differentiation observed in this study.

The hundreds of genome-wide markers utilized in this study significantly increased power to make more robust estimates of population parameters, such as migration rate and number of migrants (rev. Brumfield et al. 2003). Specifically, we wanted to determine if there was evidence for long-distance migration between spawning grounds *within* a season. Using Bayesian methods employed in BAYESASS indicated ~⅔ self-recruitment at all sampling points, with wildly varying rates of migration between sampling points. While some migration rates may have been physically feasible, others indicate significant migration backwards in time

(Table 3.6). Critical examinations of BAYESASS have revealed major issues with convergence when used to on datasets where overall F_{ST} is very low (<0.05) and migration rates are generally high (priors limited to maximum ⅓ migrant composition) (Faubet et al. 2007), as in the case here. Thus, as purely a heuristic measure, we estimated *Nm* from the overall F_{ST} for all sampling points using the assumptions put forward by Sewall Wright (1931) and find that as few as 31 individuals migrating would be sufficient to maintain this low level of genetic differentiation. Previous estimates of the likely number of migrants between northern and southern California using microsatellite data indicate anywhere between ~20-80 individuals migrating per generation may be enough to maintain observed levels of genetic homogeneity (Reichow and Smith 2001). Although these methods of estimation are fraught with assumptions and the subject of substantial debate (see Whitlock and McCauley 1999), they do support a theory of low levels of migration is possible between spawning grounds and spawning groups.

Applicability of SNP and RAD-seq methodology for market squid fishery examination

Investigating population structure and dynamics in cephalopods, as for most marine organisms, has historically been limited due to the difficult of physically observing or tracking migration and movement during very dispersive life stages and for very large population sizes (Semmens et al. 2007). Specifically, developments in next-generation sequencing methods allow for the detection and development of hundreds to thousands of molecular markers that can be used to assign individuals to source populations (Bernatchez and Duchesne 2000; Luikart et al. 2003) and make more accurate estimates of demographic parameters (Brumfield et al. 2003). To date, studies utilizing genome-wide markers to estimate population parameters and migration patterns in marine species are steadily growing. Specifically, RAD sequencing and other

approaches have been used to great success in detecting broad and fine-scale population structure in non-model organisms and in wild marine populations such as clownfish (Planes et al. 2009), herring (Lamichhaney et al. 2012), and anchovies (Zarraonaindia et al. 2012). However, genome-wide markers have not yet been applied to cephalopod populations, despite their tremendous potential to inform spatial and temporal management of extremely dynamic and valuable fisheries species. Understanding population structure and population dynamics in cephalopod fisheries is particularly pressing as the elusive nature of cephalopod species, squid in particular, result in management plans founded on very limited data. Moreover, as world finfish stocks are in rapid decline, we have also seen a rapid increase in exploitation of cephalopod fishery stocks. Without clear understanding of what is being harvested and how populations are genetically and demographically connected, we cannot sustainably manage the fisheries.

To date, this study and the one documented in the previous chapter are the first to use genome-wide single nucleotide polymorphisms to examine population differentiation in cephalopod species. The SNPs in this study show tremendous potential for teasing apart fine-scale variation that was previously undetected using traditional morphometric techniques and allozyme and microsatellite loci. However, inferences on spatial and temporal structure of the fishery cannot be based on one pilot study alone. Based on the patterns observed in this study, we were not able to confidently make inferences about why this pattern of temporally mediated spatial structure exists. This was not because the genetic data were insufficient,, but rather that there is insufficient information concerning life history and reproductive behavior over both the species range and multiple generations. In order for genomic-scale data to fulfill its potential for highlighting population dynamics, it needs to be implemented within a rigorous and well-structured sampling scheme and as a complement to other types of data so as to provide a full

picture of the biological and physical processes governing population structure.

Current situation of fishery and potential threats

Current management is comprised of annual landings limits, weekend closures, limited vessel entry and spatial closures in spawning habitats located in California Marine Protected Areas (California Fish and Wildlife). Policies are based on landings and egg escapement modeling (Macewicz et al. 2004; Dorval et al. 2013). However, the short life cycle and sub-annual recruitment schedule means that population abundance is completely dependent on the previous season's spawning success. Genetic evidence from this study reveals that the fishery in California is very likely not composed of one population, and the spatial and temporal patterns of population structure are more complex than previously thought. This raises serious questions regarding the origin of recruiting adults and the dynamics of population connectivity between these major spawning and fishing grounds. For example, our study suggests that there is substantial monthly variation in the composition of catch in both northern and southern regions. Within the five months sampled in 2014, we detect signals of genetically distinct groups present at different times. During months where one population dominates both northern and southern California, if spawning is severely impacted in one site, overall abundance and spawning success may not be severely impacted. However, when genetically different populations dominate northern and southern regions, and there is heavy harvest pressure on one site or another, this could severely impact future abundance and recruitment of one or both populations. Moreover, major population crashes in the past from strong El Nino events highlight the extreme sensitivity of *D. opalescens* populations to environmental changes. Ongoing research indicates that the availability of suitable habitat for spawning drastically decreases during strong El Nino events

(Zeidberg et al. 2012; Navarro 2014). It is then critical to understand whether genetically distinct populations are defined by a degree of spawning site fidelity in order to understand how populations may be affected by strong climatic events. Thus, understanding the nuances and complexities of the spatio-temporal composition and dynamics of spawning populations is absolutely vital for informing distribution of fishing effort.

While SNPs add tremendous resolution into the complexities of market squid population structure, recommendations and insight for informing management is still limited as the samples collected in this study are fisheries dependent. This reflects a growing issue in fisheries studies, in that it is often difficult to obtain sufficient sample sizes and sampling range for highly mobile fisheries species. While the insight we've gained in this study may be helpful, they still only reflect what is happening with the bounds of the fishery and does not necessarily reflect the biological processes underlying this complex temporal population structure. Therefore, in order to corroborate and expand upon these findings, we need to conduct further research combining both fisheries dependent and independent data as well as expand sampling over multiple generations to get an idea of 1) the stability of these population patterns and 2) identify where these populations come from and 3) when they recruit in order to accurately inform long-term sustainable management of this economically critical species. Finding the answers to these questions is absolutely crucial to sustainably managing market squid as not only do they support California fishing economy and local livelihoods, but also for understanding the dynamics of the populations in the context of climate change.

Conclusions

Despite examining a very small time window and just a portion of the total range of the

species, single nucleotide polymorphisms were still able to detect subtle differentiation between sampling locations and sampling times. The findings from this study lend support to the idea that more than one population of *D. opalescens* likely exists within California and that effective dispersal is more restricted than expected, despite overall high levels of gene flow. Given that we did not find consistent patterns of spatial separation at all sampling times, it is likely that these populations are not present year-round and varies in abundance over time. However, further research is required to determine where they come from and where they go (e.g. offshore, areas outside of the target range of the fishery, etc…). Furthermore, complex patterns of differentiation suggest that gene flow varies with season and that the occurrence of different spawning populations fluctuates over time. While this study demonstrates the remarkable utility of RAD-sequencing for examining population structure in a highly dynamic fishery species, it also highlights limitations of using genomic data in wild populations. Despite the capability for delineating population structure with high resolution and assigning individuals, our inference into the biological implications of our findings is limited by the use of fisheries dependent data and the lack of comprehensive information on the life history and migration patterns of *D. opalescens*. While the location of some large and small spawning beds are known along the range, very little is known about where the squid are before they come inshore to spawn. Thus, in order to more fully understand the population structure of these vital commercial species, an integrated approach is absolutely necessary.

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FIGURES AND TABLES

Table 3.1. Sample collection of *D. opalescens* from northern and southern California landings during the 2014 harvest season.

Month	Region	Port	Fishing block	Sample ID	No. collected	No. successfully genotyped
May	North	Monterey (550)	Lover's Point (526)	LP01	50	19
June	North	Monterey (550)	Lover's Point (526)	LP02	50	10
	South	San Pedro (770)	Long Beach Breakwall (718)	SP01	50	19
July	North	Moss Landing (592)	Monterey (525)	MT01	50	24
	South	Terminal Island (745)	Catalina Harbor (762)	CT01	50	19
August	North	Monterey (550)	El Dorado (526)	MT02	39	13
	North	Monterey (550)	Lover's Point (526)	LP03	52	8
	South	San Pedro (770)	Catalina- back-side (807)	CT02	50	23
September	South	Ventura	Ventura County Line	CL01	50	14
				Total	441	149

Table 3.2a. List of morphometric measurements made for each individual squid

Dorsal mantle length (DML)	From tip of the rostrum to the posterior end of the mantle (+/- 0.1 cm) (Figure 3.3).
Weight	Measured after defrosting animals and draining of excess liquid but not allowing bodily fluids to escape (+/- 0.01 g).
Fin length (FL)	Dorsal side up, from the posterior end of the mantle to the insertion point of the fin into the mantle (+/- 0.1 cm) (Figure 3.3).
Fin width (FW)	Ventral side up, perpendicular to the longitudinal axis from the side of the mantle to the most distal part of the fin (+/- 0.1 cm) (Figure 3.3).
Mantle thickness	Measured after the ventral side of the mantle had been split anterior to posterior. Thickness was measured anterior to the fin (+/- 0.01 cm).
Sex	Determined by examining internal morphology of each individual (Figure 3.3).
Gonad condition	Estimated using a four-stage maturity scale (Table 3.2b).

Table 3.2b. Description of maturity scale employed for *D. opalescens* (after Evans 1976)

	Female stages	Male stages
1	Ovary small without visible eggs	Testis small; spermatophoric sac without visible spermatophores
2	Ovary medium with some eggs; mantle moderately thick and firm	Testis medium sized; spermatophoric sac with some spermatophores; mantle somewhat thick and firm
3	Oviduct and ovary full of eggs, large and completely filling posterior of mantle cavity; nidamental and oviducal glands large, firm and white; accessory nidamental glands orange red; mantle thick and firm	Testis large; spermatophoric organ large and firm, spermatophoric sac full of spermatophores and extending to end of posterior end of mantle cavity; mantle thick and firm
4	Oviduct and ovary has few eggs; nidamental and oviducal gland small and flaccid, accessory nidamental glands light pink; mantle thin and limp	Testis small; spermatophoric sac small with few spermatophores; mantle thin and limp

Table 3.3. Average differences in morphometric measurements between individuals grouped by sex by region **(A)** and location by time **(B)**. Bold values indicate average measurements between comparisons that are significantly different as indicated by Tukey HSD tests ($p>0.05$). Groups for sex by region (A) are north males (NM), north females (NF), south males (SM) and south females (SF). Groups for location by time (B) are as follows: North - May (N5), June (N6), July (N7), August (N8); South – June (S6), July (S7), August (S8), September (S9). Morphometric measurements abbreviated as DML (dorsal mantle length), FW (fin width), and FL (fin length). Negative differences signify that the second group is larger than the first group.

A

	DML (cm)	Weight (g)	FW (cm)	FL (cm)
NM – NF	0.4895	9.4412	0.0680	0.2830
SF – NF	**-0.9360**	**-11.0489**	-0.2901	-0.3363
SM – NF	-0.7079	-6.5624	-0.0371	-0.3708
SF – NM	**-1.4255**	**-20.4901**	**-0.3581**	**-0.6193**
SM – NM	**-1.1974**	**-16.0036**	-0.1051	**-0.6538**
SM – SF	0.2281	4.4865	0.2529	-0.0345

B

		DML (cm)	Weight (g)	FW (cm)	FL (cm)
N5	N6	0.1353	-0.7022	0.0858	-0.2600
	N7	0.4719	**10.2025**	0.0825	0.2333
	N8	**-1.5185**	**-12.5323**	**-0.5271**	**-0.7952**
	S6	**-1.5558**	**-12.1148**	0.2825	**-1.0000**
	S7	-1.0725	**-12.5764**	-0.3231	**-0.7222**
	S8	**-1.3121**	**-15.7068**	**-0.6842**	-0.4739
	S9	**-1.4090**	**-15.2949**	-0.3985	-0.6000
N6	N7	0.3367	10.9047	-0.0033	0.4933
	N8	**-1.6538**	**-11.8301**	**-0.6129**	-0.5352
	S6	**-1.6911**	-11.4126	0.1967	-0.7400
	S7	-1.2078	**-11.8742**	-0.4089	-0.4622
	S8	**-1.4474**	**-15.0046**	**-0.7700**	-0.2139
	S9	**-1.5443**	**-14.5927**	-0.4843	-0.3400
N7	N8	**-1.9905**	**-22.7348**	**-0.6095**	**-1.0286**
	S6	**-2.0278**	**-22.3172**	**0.2000**	**-1.2333**
	S7	**-1.5444**	**-22.7789**	**-0.4056**	**-0.9556**
	S8	**-1.7841**	**-25.9093**	-0.7667	**-0.7072**
	S9	**-1.8810**	**-25.4974**	-0.4810	**-0.8333**
N8	S6	-0.0373	0.4175	0.8095	-0.2048
	S7	0.4460	-0.0441	0.2040	0.0730
	S8	0.2064	-3.1745	-0.1571	0.3213
	S9	0.1095	-2.7626	0.1286	0.1952
S6	S7	0.4833	-0.4617	**-0.6056**	0.2778
	S8	0.2437	-3.5921	**-0.9667**	0.5261
	S9	0.1468	-3.1802	**-0.6810**	0.4000
S7	S8	-0.2396	-3.1304	-0.3611	0.2483
	S9	-0.3365	-2.7185	-0.0754	0.1222
S8	S9	-0.0969	0.4119	0.2857	-0.1261

Table 3.4. Summary statistics: Number of successfully sequenced individuals (n) per sampling location, expected heterozygocity (H_e), observed heterozygocity (H_o), percent polymorphic loci (%)

Sampling period	Region	Port	Fishing block	Sample ID	No. successfully genotyped	H_e	H_o	% polymorphic
May	North	Monterey (550)	Lover's Point (526)	LP01	19	0.0804	0.0797	26.01%
June	North	Monterey (550)	Lover's Point (526)	LP02	10	0.1457	0.1524	14.59%
	South	San Pedro (770)	Long Beach Breakwall (718)	SP01	19	0.0836	0.0797	27.85%
July	North	Moss Landing (592)	Monterey (525)	MT01	24	0.0703	0.0731	23.21%
	South	Terminal Island (745)	Catalina Harbor (762)	CT01	19	0.0875	0.0921	18.95%
August	North	Monterey (550)	El Dorado (526)	MT02	13	0.0769	0.0782	22.70%
	North	Monterey (550)	Lover's Point (526)	LP03	8			
	South	San Pedro (770)	Catalina- back-side (807)	CT02	23	0.0704	0.0710	24.05%
September	South	Ventura	Ventura County Line	CL01	14	0.1066	0.1106	15.90%

Table 3.5A. Pairwise F_{ST} between temporal replicates in northern California. F_{ST} with p>0.05 indicated in bold.

	North-May	*North-June*	*North-July*
North-May			
North-June	-0.00837		
North-July	0.00469	0.00132	
North-August	**0.01041**	-0.02505	-0.01909

Table 3.5B. Pairwise F_{ST} between temporal replicates in southern California. F_{ST} with p>0.05 indicated in bold.

	South-June	*South-July*	*South-August*
South-June			
South-July	-0.0036		
South-August	**0.00971**	0.00691	
South-September	**0.01167**	**0.02361**	**0.01709**

Table 3.5C. Pairwise F_{ST} between temporal replicates from northern vs. southern California. F_{ST} with p>0.05 indicated in bold.

	North-May	*North-June*	*North-July*	*North-August*
South-June	-0.00104	-0.04993	-0.02017	**0.01166**
South-July	**0.01249**	0.00978	**0.01381**	0.00458
South-August	**0.02386**	-0.01068	0.00287	**0.0072**
South-September	**0.01167**	0.00939	**0.0112**	0.00983

Table 3.6. Estimated migration rates between temporal replicates in northern and southern California fishing regions for *D. opalescens*. Columns indicate sampling locations where migrants are coming from and rows indicate where they are migrating to. Values in italics indicate level of self-recruitment.

		MIGRATING FROM							
		North-May	North-June	South-June	North-July	South-July	North-August	South-August	South-September
MIGRATING TO	North-May	*0.679*	0.012	0.012	0.012	0.012	0.030	0.231	0.012
	North-June	0.018	*0.686*	0.019	0.018	0.018	0.018	0.204	0.018
	South-June	0.012	0.012	*0.679*	0.012	0.012	0.026	0.236	0.012
	North-July	0.010	0.010	0.010	*0.677*	0.010	0.024	0.248	0.010
	South-July	0.012	0.012	0.012	0.012	*0.679*	0.024	0.237	0.012
	North-August	0.011	0.011	0.011	0.011	0.011	*0.678*	0.254	0.012
	South-August	0.011	0.011	0.011	0.011	0.011	0.011	*0.925*	0.011
	South-September	0.014	0.015	0.015	0.015	0.015	0.016	0.226	*0.682*

Figure 3.1. Sampling sites during 2014 fishing season in northern and southern California.

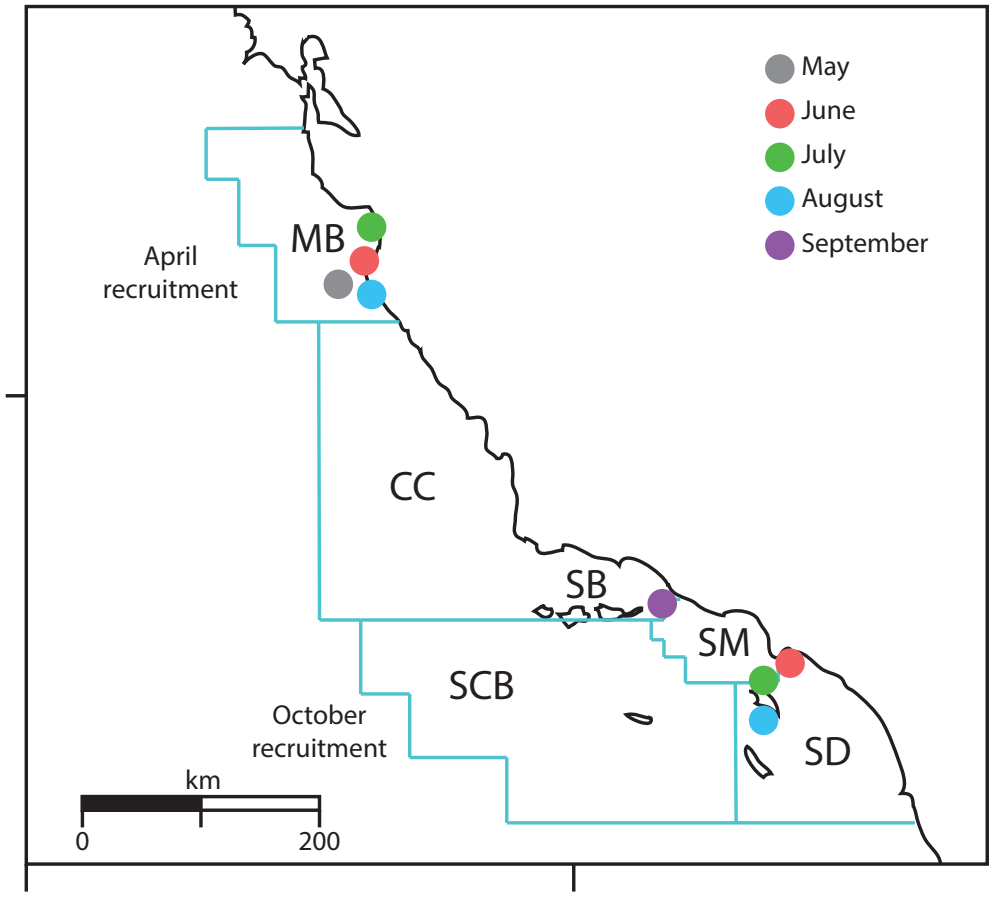
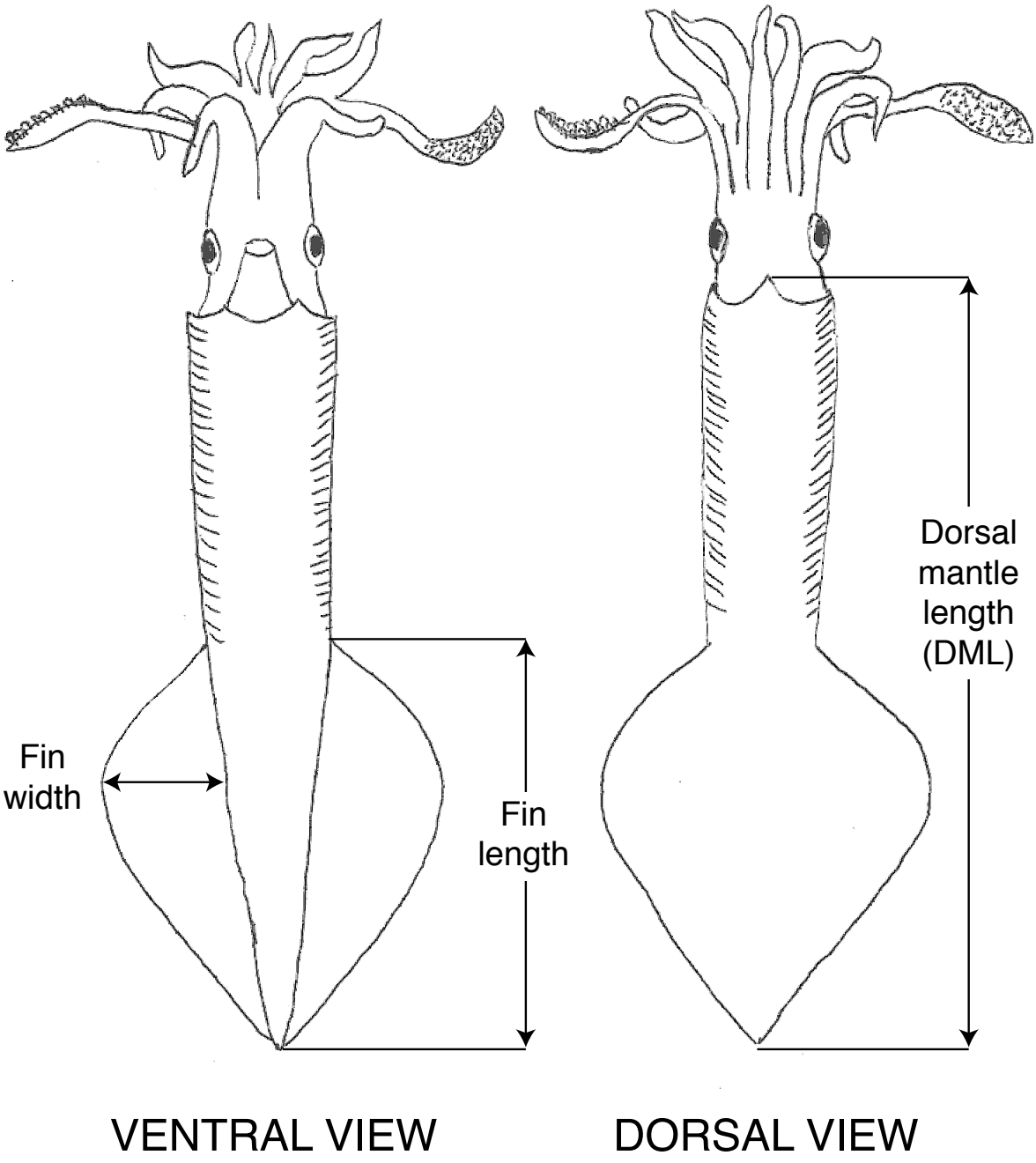


Figure 3.2. General external morphology of *Doryteuthis opalescens* with morphometric measurements indicated



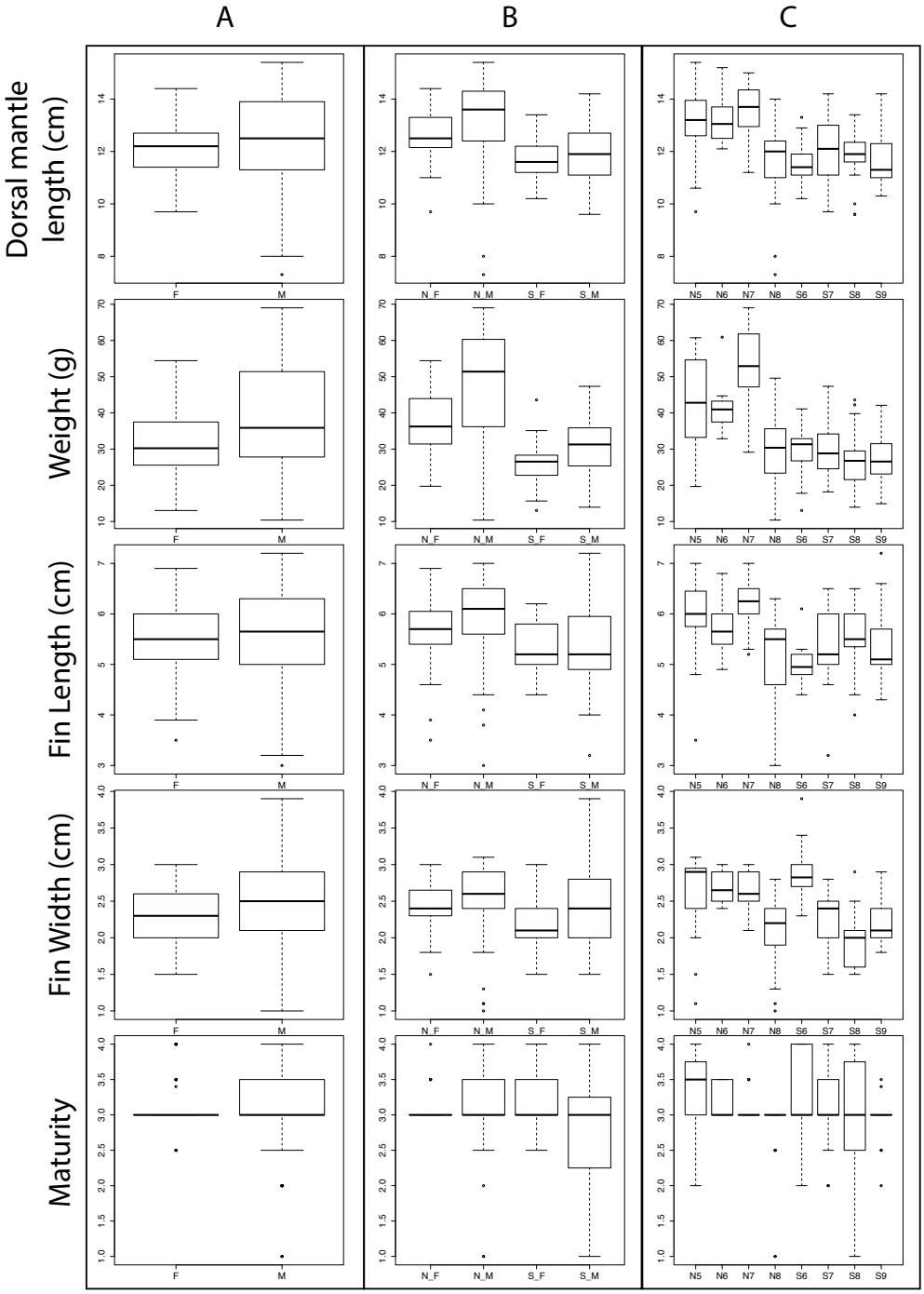


Figure 3.3. Morphometric measurements of growth and gonad condition. Ranges, means and standard deviations indicated in boxplots and compared between sexes (A), regions divided by sex (B), and sampling locations (C). Abbreviations are as follows: F – all females, M - all males, N_F – all females collected in the northern region, N_M - all males collected in the northern region, S_F – all females collected in the southern region, S_M – all males collected in the southern region, N5 – samples collected in May in the northern region, N6 – north-June, N7 – north-July, N8 – north-August, S6 – south-June, S7 – south-July, S8 – south-August, S9 – south-September.

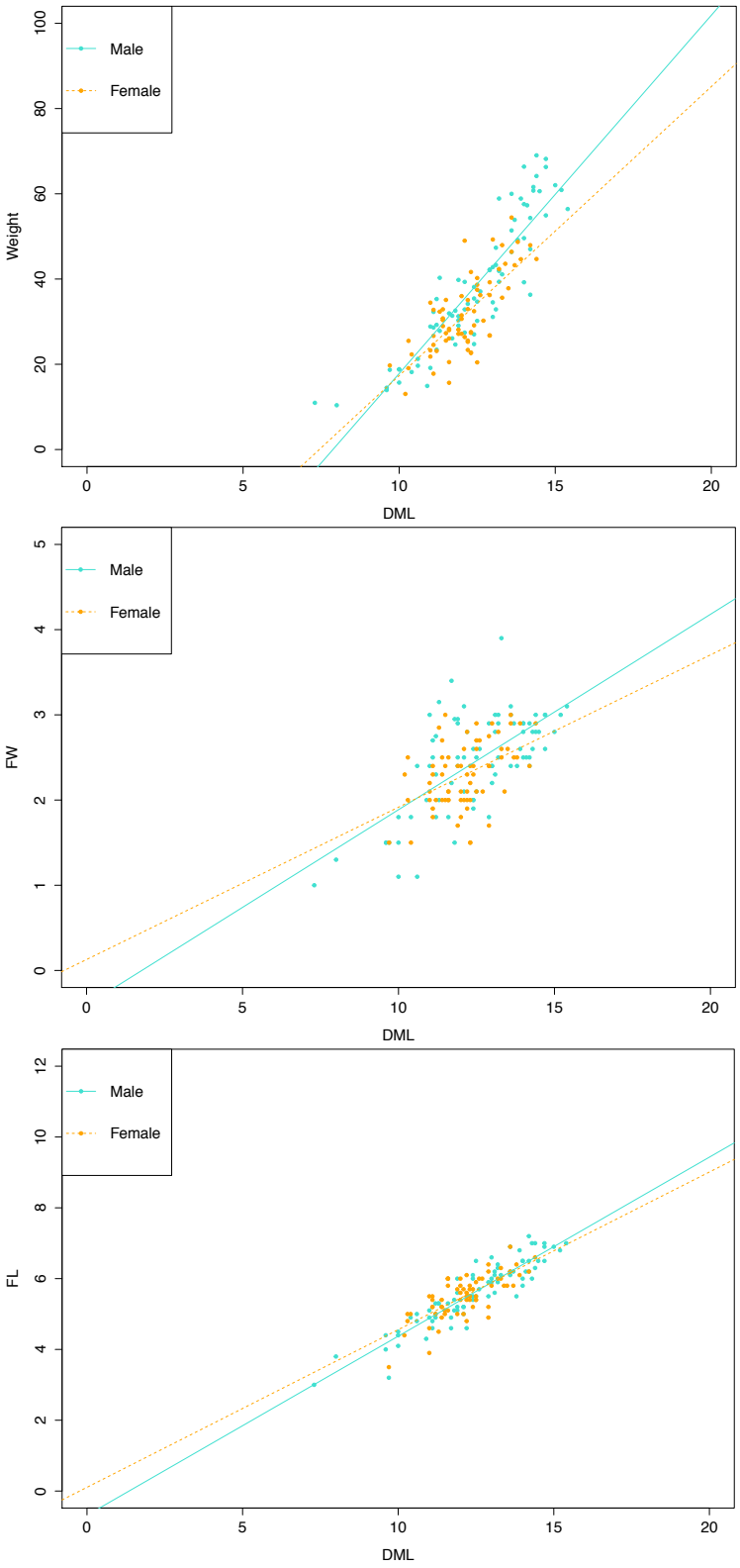


Figure 3.4. Analysis of covariance of regression indicates no significant interaction between sex and weight, fin width (FW) and fin length (FL) with DML as the independent variable

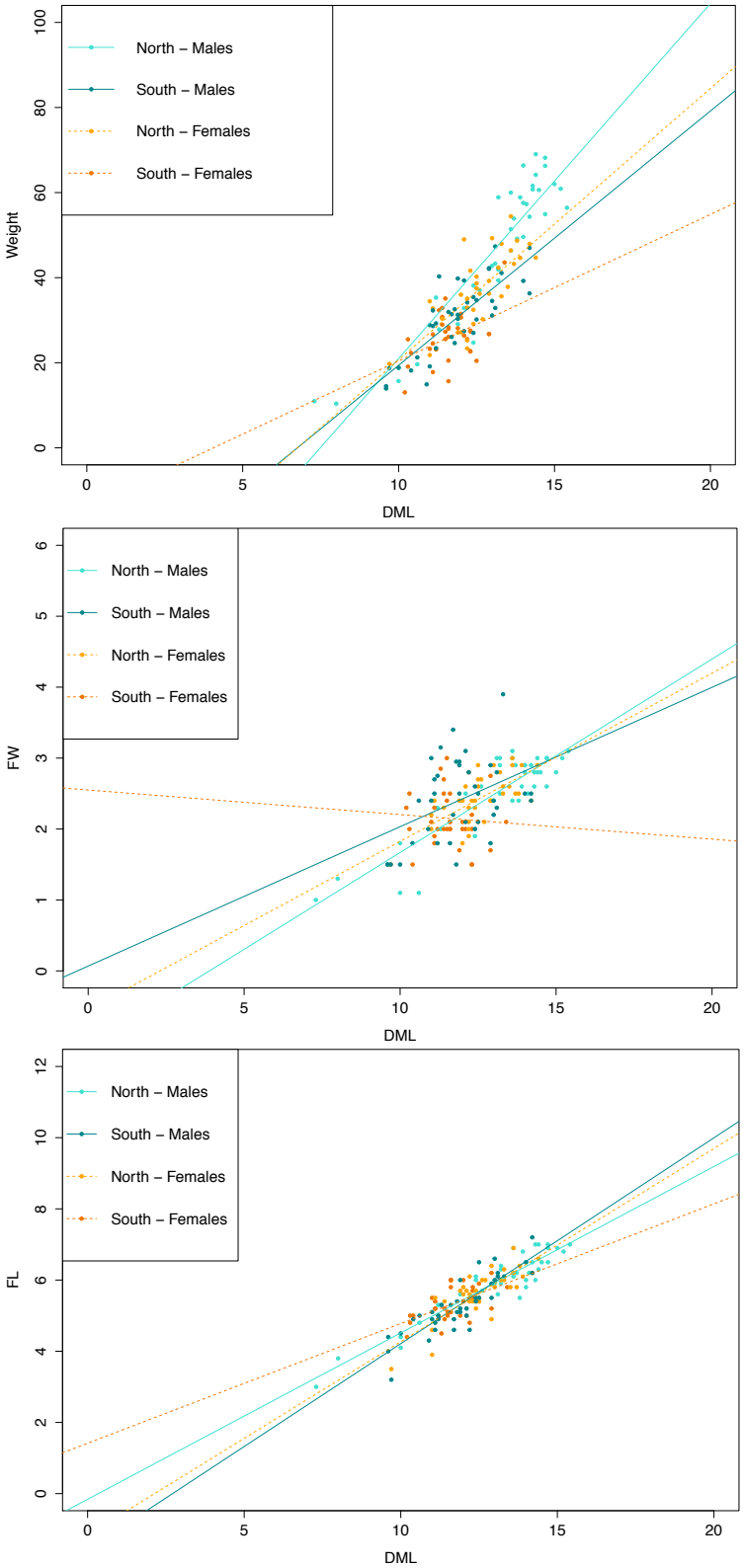


Figure 3.5. Comparison of regression lines indicates significant interaction between regions (by sex) and weight, fin width (FW) and fin length (FL) with DML as the independent variable

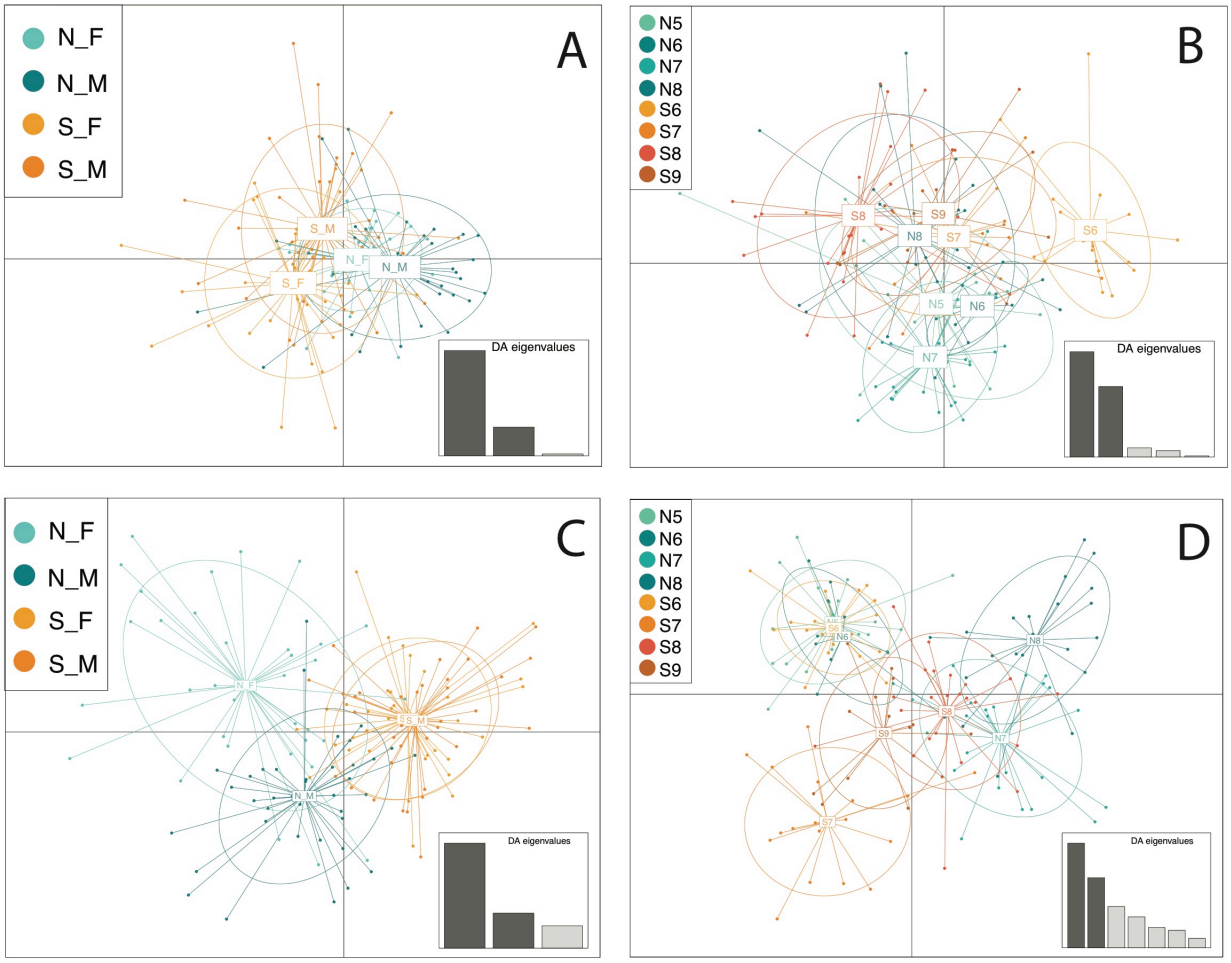


Figure 3.6. Discriminant analysis of principal components (DAPC) of morphometric data and region divided by sex (A) and by sampling location (B). DAPC of SNP data and region divided by sex (C) and by sampling location (D)

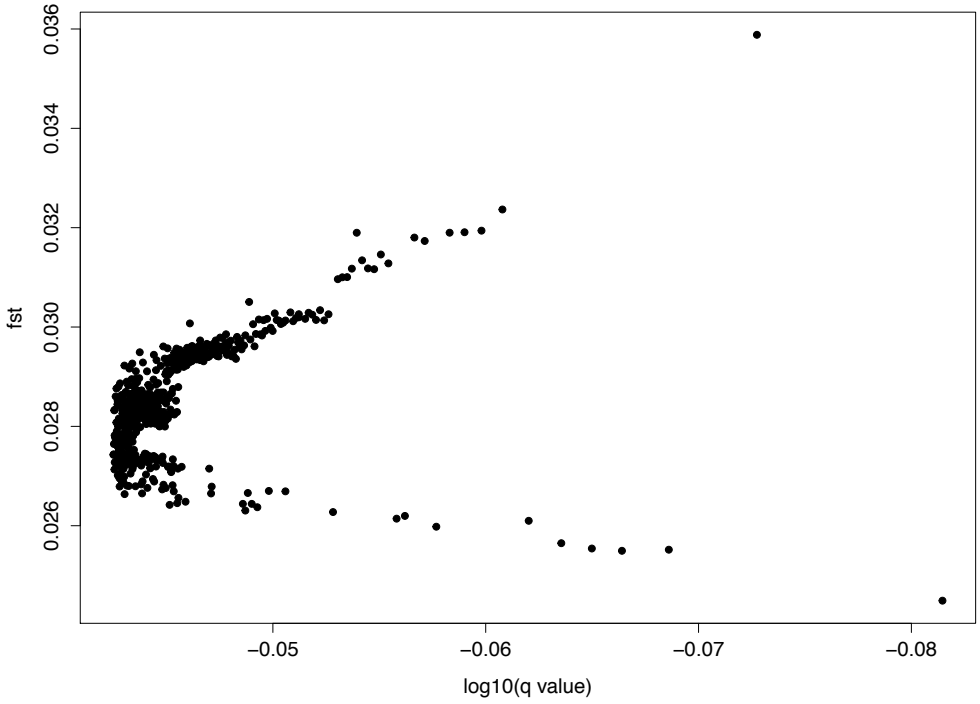


Figure 3.7. Distribution of FSTs and the probability (log10Q) of each SNP being classified as an "outlier" loci as determined by the probabilistic method used in Bayescan (Foll and Gaggiotti 2008)

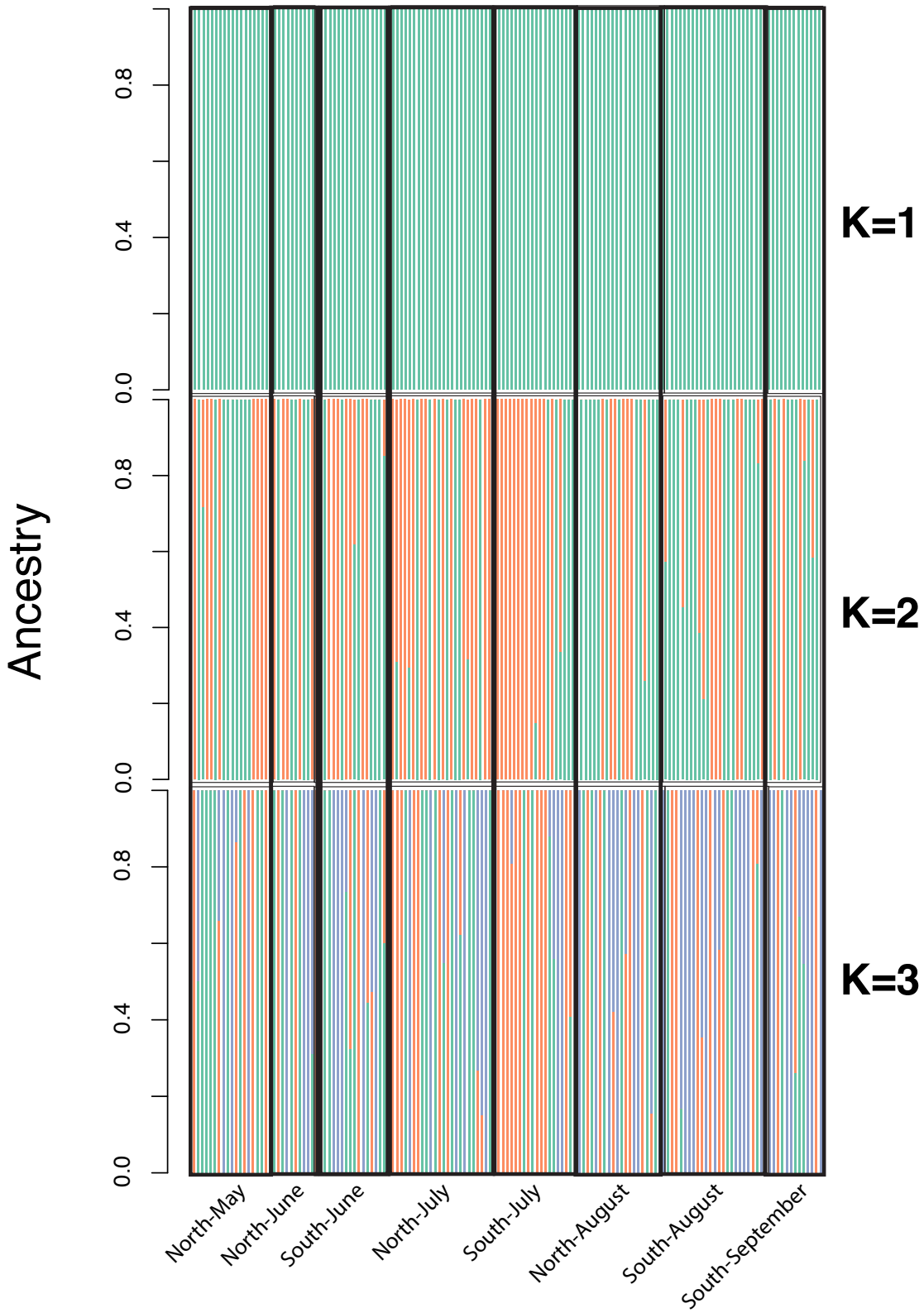


Figure 3.8. An individual based ancestry model (implemented in ADMIXTURE, Alexander et al. 2009) testing for likelihood of different numbers of populations (k=1 to k=3). The model with the lowest cross-validation error is k=1 over all sampling groups.

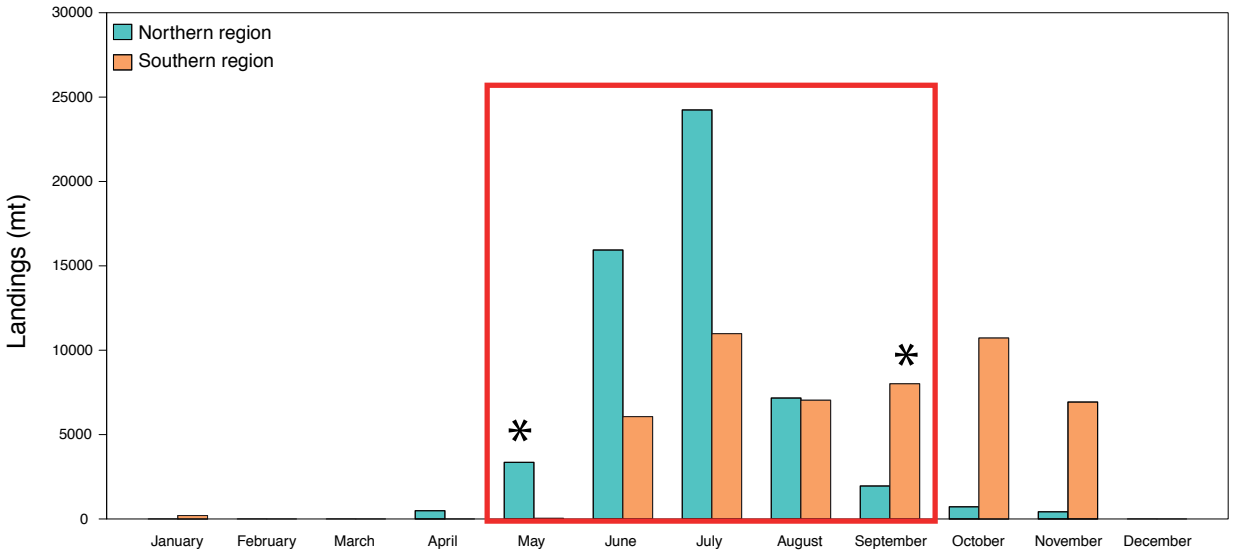


Figure 3.9. Total landings of market squid (mt) in northern and southern regions of the California fishery in 2014 derived from landing receipts publically available from California Department of Fish and Wildlife. Months sampled in this study are highlighted in the red box, asterisks represent samples taken only from one region during that month.

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