SPECIES VERIFICATION AND FUNDAMENTAL DEMOGRAPHICS OF *LEPTASTREA PURPUREA* IN KĀNE‘OHE BAY, O‘AHU, HAWAI‘I

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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Abstract

Climate change is threatening coral reef health across the globe. Knowledge of the fundamental biology of coral species is essential to designing effective conservation strategies for coral reef ecosystems. *Leptastrea pupurea* is an encrusting coral that is resilient to elevated temperatures and eutrophication, yet overwhelmingly understudied. This research seeks to understand the fundamental biology of *L. purpurea* to better use this species in reef restoration efforts. DNA was extracted from 64 colonies distributed across three spatial scales in Kāneʻohe Bay, Oʻahu, Hawaiʻi to assess fine scale demographic relationships among colonies. Maximum likelihood phylogenetic trees were built using assembled mitochondrial genomes from these samples, as well as twelve previously published samples from across the Indo-Pacific from across the Indo-Pacific to determine the relative relationship of Hawaiian *Leptastrea purpurea* to those across the broader range. We found that putative *L. purpurea* exhibits unique genetic diversity in Hawaiʻi compared to the Indo-Pacific. These data suggest a potential undescribed species of *Leptastrea* in the Hawaiian islands, highlighting the importance of verifying the fundamental biology of species to design effective place-based conservation strategies.

Key words : Coral, Hawaii, Genetics

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1.0 Introduction

1.1 BACKGROUND

The health and ecosystem function of coral reefs across the world are declining due to anthropogenic stressors such as dredging, coastal development, runoff, unsustainable fishing, and elevated sea temperatures (Carpenter et al., 2008; De'ath et al., 2012; Hughes et al., 2017; Pandolfi et al., 2003). Coral reef resilience is strongly influenced by inter- and intraspecific diversity of corals, which enhances the ability of reef ecosystems to withstand stressors like disease and bleaching (Baskett et al., 2014; Nyström, 2006; Nyström et al., 2000). However, ongoing losses in coral diversity threaten the reef's resilience and ability to provide crucial ecosystem services. Effectively responding to these challenges requires broadening the scope of coral research beyond the most commonly studied reef-building species to include a wider range of coral taxa. This expanded focus ensures that all functional trait spaces of coral (Madin et al., 2023; McWilliam et al., 2018, 2020) are represented in research, providing the foundational knowledge to design effective conservation strategies.

The establishment of marine protected areas (MPA) is emerging as a promising strategy to mitigate these threats to coral reefs (Roberts et al., 2017). The long-term success of MPA's depends on understanding fundamental population connectivity of coral species, yet our understanding of larval recruitment and dispersal potential remains limited (Cowen & Sponaugle, 2009; Weersing & Toonen, 2009). Observational studies on larval recruitment are challenging due to the periodic and infrequent nature of coral spawning, often guided by celestial cues (Randall et al., 2020; Wolstenholme et al., 2018). Consequently, much of our understanding of larval recruitment relies on genetic inferences from adult colonies (Weersing & Toonen, 2009).

Hawai‘i represents a unique location for studying coral population genetics due to its geographic isolation from continental landmasses (Bhattacharya et al., 2022), which limits gene flow and contributes to the high levels of endemic species found in the region (Alison Kay & Palumbi, 1987). However, coral research in Hawai‘i has largely focused on three dominant species: *M. capitata*, *P. compressa* & *P. damicornis/acuta*. Given the high levels of endemism, research efforts must extend beyond the most commonly studied reef-building species to develop effective, place-based conservation strategies that reflect the unique species composition of the Hawaiian Islands.

Figure 1. Adult colony of *Leptastrea purpurea* taken in Kāne‘ohe Bay, O‘ahu.

Leptastrea purpurea (Figure 1) is an encrusting coral distributed from across the Indo-Pacific from the Red Sea to Rapa Nui (Easter Island), across shallow reef flats to depths of up to

40m (130 ft). This coral fills an ecological niche essential to the resilience of coral reefs. Encrusting corals like *L. purpurea* act as a glue, cementing reefs to ensure their structural resilience. This coral is resistant to both elevated temperatures and acidification (Bahr et al., 2016; Galanto et al., 2022), highlighting its important role in conservation.

Despite its ecological importance, this coral is overwhelmingly understudied, and little is known about its fundamental biology. Published research on *Leptastrea purpurea* has focused on its resilience and reproductive ecology, but its demography and taxonomy (particularly in Hawai‘i) remain unexplored. Arrigoni et al. (2020) examined the species delimitation of *Leptastrea* (including the species *Leptastrea purpurea*) across the entire Indo-Pacific, sampling corals from Saudi Arabia (Red Sea coast), Djibouti, Yemen (Gulf of Aden coast and Socotra Island), Madagascar, Mayotte Island, the Maldives, Papua New Guinea, New Caledonia, and French Polynesia. However, no corals were sampled from the Hawaiian Islands, leaving a research gap in understanding the distribution and taxonomy of this species.

Figure 2. Cluster of several adult *Leptastrea purpurea* colonies taken in Kāne‘ohe bay, O`ahu.

This coral often occurs in clusters of closely grouped, but distinct, colonies (Figure 2). In other species, this distribution might be explained by partial mortality or asexual fragmentation. This is unlikely to be the case for this species as it forms small encrusting colonies, hindering asexual reproduction through fragmentation as a dominant reproductive strategy. Moreover, dead skeletons are not visible between colonies, suggesting that each colony of *L. purpurea* is genetically distinct.

Alternatively, this distribution may be explained by its reproductive mode. *L. purpurea* is a brooding coral (Nietzer et al., 2018), and it is generally believed that brooding corals experience more genetic subdivision compared to broadcast spawners. For example, research on the brooding coral *Seriatopora hystrix* in Australia suggests that its larvae typically settle near parent colonies, indicating short-distance dispersal and weak population connectivity, though occasional long-distance dispersal across reefs has also been observed (Underwood et al., 2007, 2009). Thus, colonies of *L. purpurea* within this clustered distribution may exhibit parent-offspring relationships, influenced by the limited larval dispersal characteristic of brooding corals.

1.2 RESEARCH OBJECTIVE

In this study, we analyzed tissue samples from 64 colonies of *Leptastrea purpurea* across four reefs in Kāne‘ohe Bay, O‘ahu, Hawai‘i. Our goal was to verify the species distribution in Hawai'i and understand demographic relationships among colonies to support place-based conservation strategies in Hawaiʻi. We hypothesize that due to the shorter larval dispersal of brooding corals, *L. purpurea would* exhibit strong population structure between clusters, reefs, and across a 2-kilometer distance.

2.0 Methodology

2.1 FIELD COLLECTION

From June through September of 2023, 64 samples of *L. purpurea* were collected across four reefs in Kāneʻohe Bay, Oʻahu, Hawaiʻi, including the reef surrounding the Hawaiʻi Institute of Marine Biology (H.I.M.B), as well as patch reefs 3, 12, and 13 in the bay. A total of 16 individuals were sampled at each reef consisting of four singletons and three clusters of four colonies. The criteria for singletons was the presence of only one *L. purpurea* colony within a $0.5m^2$ area, while clusters were defined as four colonies within $0.5m^2$. These reefs were selected to assess the demographic population structure of *L. purpurea* at a reef scale (<1km), as well as across 2km. Corals were collected under H.I.M.B's Special Activity Permit 2023-2024

Figure 3. Satellite imagery of Kāneohe Bay, Oahu featuring the sampling scheme of *L. purpurea*. collections (A) Patch reef 3 and the reef surrounding H.I.M.B. (B) patch reef 12 and 13.

For each cluster of *L. purpurea*, a 0.5 m^2 quadrant was centered on the largest colony within the cluster, and the perimeter of the quadrat was used to ensure that there were exactly four *L. purpurea* colonies in the designated area. Agisoft Metashape® (v.2) targets were used to mark the corners of the quadrant, and 2 cm^2 scale bars were positioned near the center of the cluster. The quadrat was then removed to photograph the cluster using a Sony A7iii and Zeiss Batis 18 mm lens in a Ikelite 200 dl underwater housing. Photos (n>150) were taken in a lawnmower pattern to construct 3D models on Metashape®. If the cluster was located in a complex environment, additional photos were taken in an arch pattern to ensure high quality of the 3D model. For the collection of singletons, the same quadrant was used to ensure that the singleton was the only colony of *L. purpurea* within the 0.5m^2 area. 3D photogrammetry and the collection of singletons followed the same procedure outlined above. GPS coordinates and a photo of each individual were obtained during collection for identification. A 2 cm^3 sample of tissue from each colony was collected using a hammer and chisel, then samples were stored in salt-saturated imethyl sulfoxide (DMSO) buffer (Gaither et al., 2011) at -20 degrees celsius until processing.

2.2 3D MODELS AND DISTANCE MEASUREMENTS

Photos of each site were aligned using Metashape® (v.2) to create 3D models. Using a batch process, accuracy was set to ultra high for photo alignment, then markers were detected. A point cloud was constructed, and mesh was built with the settings faces:low and quality:high. Texture was added to each model to define the edges of *L. purpurea* colonies, and scale bars were created using the $2cm^2$ Metashape® markers to enable accurate measurements ($\pm 1.0x10^{-4}$ m). Individual colonies were identified on the 3D model based on reference photos taken from the collection site.

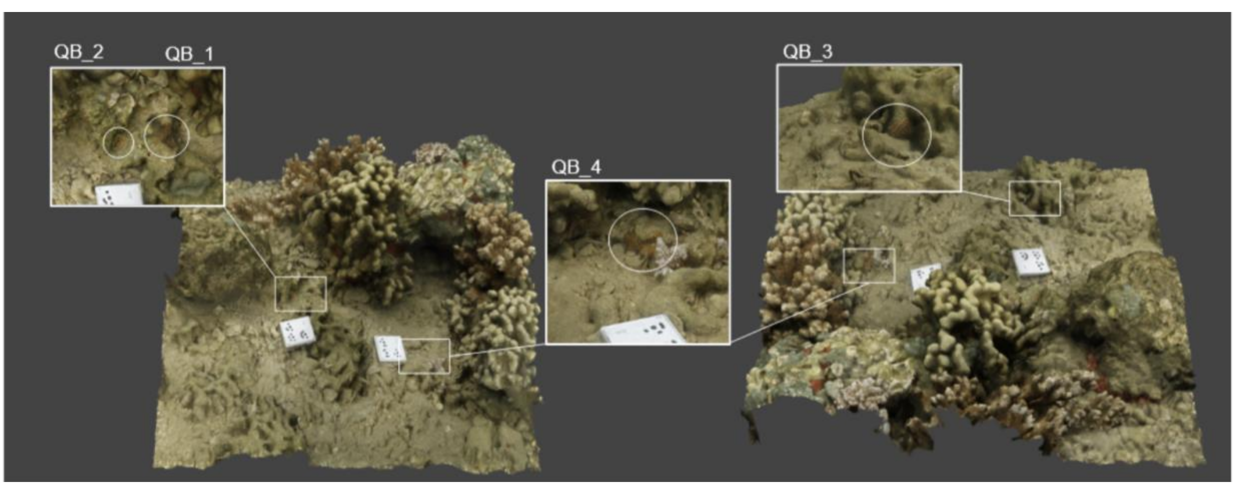


Figure 4. 3D model of Quadrant B collected on the reef surrounding H.I.M.B. featuring four colonies of *Leptastrea* purpurea collected (QB_1, QB_2, QB_3, QB_4) viewed from the front and back.

To measure the distance between colonies within a cluster, digital markers were placed on the nearest edge of a colony to the edge of the center colony, then connected with the measurement tool. Measurements were recorded with ± 1.0×10^{-4} m accuracy

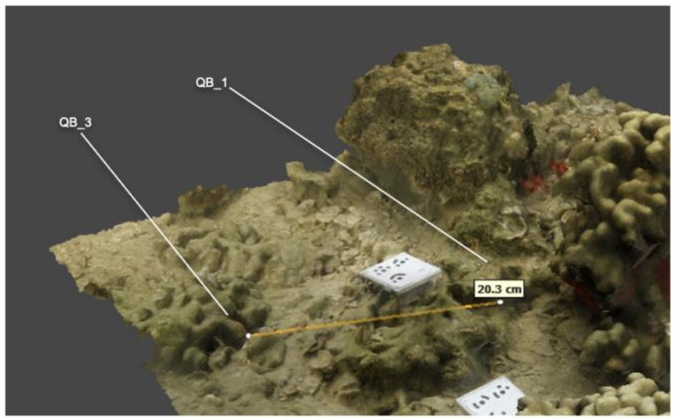


Figure 5. Distance measured between two colonies of *Leptastrea* purpurea in a cluster on Metashape using the measurement tool.

2.3 LIBRARY PREPARATION AND DNA SEQUENCING

DNA was extracted from a 1cm^3 coral fragment using an DNeasy® Blood and Tissue kit (Qiagen Inc., Hilden, Germany) with the KingFisher Flex (Thermo Fisher Scientific, Rocklin, CA, USA), and quantified using a Qubit ® Flex Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). All samples were diluted with nuclease-free water to 20ng/ul, and 250 ng of each sample was digested using MboI and Sau3AI for a restriction site associated digest (see detailed protocol - 6.1 Supplemental Materials).

Low coverage whole genome sequencing libraries were then prepared using a modified version of the Watchmaker DNA Library Prep Kit for Fragmented Double-Stranded DNA, without primers (Watchmaker Genomics, Boulder, CO), for all 64 samples of *L. purpurea*, as well as 9 technical replicates to establish a baseline for clonality upon data analysis. All reaction steps were performed in either ¼ or ½ of the recommended volumes (see detailed protocol - 6.1 Supplemental Materials) to lower costs.

Briefly, samples were End repaired and A-tailed, then ligated with 15uM xGen™ Stubby Adapter (Integrated DNA Technologies, Coralville, IA). All samples were cleaned with Mag-Bind® TotalPure NGS beads (Omega Bio-tek Inc, Norcross, GA) at a 1:1 ratio after ligation to remove fragments below 200 bp, then quantified using a Qubit ® Flex Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

We carried forward 200 ng of the post-ligation, bead-cleaned product, which was then amplified with xGen UDI (unique dual index) 10nt primer pairs for 9-12 cycles. Gel electrophoresis was used to determine if the target DNA insert (< 600bp) had been amplified.

Samples that did not have visible products in the gel electrophoresis after 12 cycles (n=6) were re-amplified following the procedure outlined above. Of these, three samples amplified with the standard 9-12 cycles, and three were not processed further. We quantified all amplified samples using a Qubit ® Flex Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Samples were then split into 2 equimolar pools based on high (>5ng/ul) or low (< 5ng/ul) post-amplification concentrations. Both pools were sent to UC Davis Genome Center where 9.9ng of DNA from the low pool received 5 additional PCR cycles using Kapa reagents. Both pools were cleaned using KAPA pure beads (Roche Molecular Systems, Inc.) to remove fragments below 300bp, and pooled with equal molarities for sequencing. The final pooled library was then sequenced as a paired-end 2x300 bp run on the AVITI 600 cycle flow cell HIGH (PE300) generating ~180 Gb of data (300 million read-pairs), or ~4 million read-pairs per sample on average.

2.4 Data Analysis

2.4.1 DEMOGRAPHIC ANALYSIS

Reads were processed to ensure high-quality alignments following the dDocent pipeline described in Puritz et al. (2014) using default parameters unless indicated here. Briefly, the dDocent v.2.9.8 pipeline (Puritz et al. 2014) merged the trimmed reads using PEAR v.0.9.6 (Zhang et al. 2013) and reads were assembled using BWA v.0.7.15 (Li and Durbin 2009). Reads were mapped to a de novo reference built with SPAdes (Bankevich et al. 2012) using dDocent with the standard settings. The bam files were then sorted and merged using SAMtools v.1.6 (Li et al. 2009) to create a single bam file. Variants were called using FreeBayes (Garrison and Marth 2012).

The resulting VCF files were further filtered by removing individuals with maximum missing data of 50%, minor allele counts less than 3, and a minimum quality score of 30. A minimum coverage depth of 3 was applied, keeping only 66 of the total 71 samples. INDELs, individuals with 50% or more missing data, and non-biallelic SNPs were removed. The data was then thinned to allow for only 1 SNP per 1000 base pairs. The SNPrelate package (Zheng et al., 2012) was used to verify technical replicates; however, their verification was unsuccessful, preventing further investigation of demographic relationships of *L. purpurea* using this dataset.

2.4.2 SPECIES VERIFICATION IN HAWAI‘I

Nearly complete mitochondrial genomes were obtained from the electronic supplementary materials of Arrigoni et al. (2020) of fourteen high-quality samples of *Leptastrea* (with the least amount of missing data) collected across the Indo-Pacific. This selection included one sample of each *Leptastrea* species *(L. gibbosa, L. magaloni, L.inneaqualis, L. transversa, L. bottae*), seven samples of *Leptastrea purpurea*, and *Lobactis scutaria* as the outgroup (Table 1).

Table 1. Voucher number, museum/institute of collection, species identification, molecular clade assignment from Arrigoni et al. (2020), and locality of 14 *Leptastrea* coral samples from the Indo-Pacific used to reconstruct the genetic species delimitation of *Leptastrea* (Arrigoni et al., 2020).

voucher number	museum/institute	ID	molecular clade	locality
HS3167	IRD	*Leptastrea gibbosa sp. n.*	I	New Caledonia
HS3915	IRD	*Leptastrea purpurea*	II	New Caledonia
Y716	UNIMIB	*Leptastrea transversa*	II	Yemen, Gulf of Aden
GA097	UNIMIB	*Leptastrea purpurea*	III	French Polynesia, Gambier Islands
MA101505	UNIMIB	*Leptastrea purpurea*	III	Maldives
MY247	UNIMIB	*Leptastrea purpurea*	III	Mayotte Island
HS3045	IRD	*Leptastrea purpurea*	III	New Caledonia
PFB776	UNIMIB	*Leptastrea purpurea*	III	Papua New Guinea
SA0264	KAUST	*Leptastrea purpurea*	III	Saudi Arabia, Red Sea
SO076	UNIMIB	*Leptastrea purpurea*	III	Yemen, Socotra Island
MD225	UNIMIB	*Leptastrea magaloni sp. n.*	IV	Madagascar
SA0043	KAUST	*Leptastrea inaequalis*	V	Saudi Arabia, Red Sea
SA0017	KAUST	*Leptastrea bottae*	VI	Saudi Arabia, Red Sea
SA2939	KAUST	*Lobactis scutaria*	outgroup	Saudi Arabia, Red Sea

The mitochondrial genomes of five high-quality samples of *Leptastrea purpurea* from Hawaiʻi were selected based on the highest read counts and distribution across sampling locations to add to the Indo-Pacific dataset. To assemble the mitochondrial genome, cleaned and trimmed forward and reverse reads from dDocent were paired using Geneious Prime (V2022.0.2). A reference-guided assembly was performed for each sample using Sandalolitha robusta (LC818214) (Yoshioka et al., 2024) as the best available reference. Unlike Arrigoni et al. (2020), who used *Polycyathus chaishanensis* as the reference, we used the fungiid coral *Sandalolitha robusta* because *Leptastrea* is more closely related to the family Fungiidae than to Caryophylliidae (Kitahara et al., 2010) resulting in better alignments and less missing data. The assembly was conducted with the Geneious assembler at medium to low sensitivity, and iterated up to 5 times.

A consensus was then generated for each of the five samples using a minimum quality threshold of 60%. For positions with no coverage, an ‘N’ was called, and for positions with coverage <3, a ‘?’ was called. The resulting consensus was trimmed to match the reference. The consensus sequences were aligned to the other *Leptastrea* sequences from Arrigoni et al. (2020) using the MUSCLE (v3.8.425) alignment plug-in in Geneious, with up to 8 iterations under default parameters, and the alignment was exported as a .phylip file. The alignment was then trimmed to remove large stretches of data missing from the Arrigoni et al. (2020) sequences to create a complete alignment and reduce artificial inflation of differences.

Maximum likelihood trees were constructed using the nearly complete mitochondrial genomes using IQ-TREE (V.2.3.2) using the CIPRES supercomputer (Miller et al. 2010), with DNA as the sequence type and 5000 bootstrap replicates, using *Lobactus scutaria* as the outgroup. The -m TEST option was employed to determine the best-fit model for each partition, followed by tree reconstruction using the identified best-fit model, which was HKY with empirically determined base frequencies and a proportion of invariant sites with 1000 ultrafast bootstrap replicates.

3.0 Results

3.1 DISTANCE BETWEEN CLUSTERS OF *L. PURPUREA*

The distances between coral colonies within the cluster were measured to analyze spatial demographic relationships. These data, along with the reef collection site, quantrat ID, type, sample code, and distance from the center colony, are summarized in Table 2.

Table 2. Summary of Distances Between Corals Within Clusters Using Metashape® (v.2) with Accuracy of $\pm 1.0\text{x}10^{-4}$ m

Reef	Quadrat	Type	SampleCode	Distance from Center Colony (cm)
HIMB	A	Quadrat	QA_1	0
HIMB	A	Quadrat	QA_2	4.93
HIMB	A	Quadrat	QA_3	8.03
HIMB	A	Quadrat	QA_4	5.47
HIMB	B	Quadrat	QB_1	0
HIMB	B	Quadrat	QB_2	3.01
HIMB	B	Quadrat	QB_3	20.4
HIMB	B	Quadrat	QB_4	20.8
HIMB	C	Quadrat	QC_1	0
HIMB	C	Quadrat	QC_2	9.9
HIMB	C	Quadrat	QC_3	23.7
HIMB	C	Quadrat	QC_4	9.87
Reef 12	E	Quadrat	QE_1	0
Reef 12	E	Quadrat	QE_2	1.32
Reef 12	E	Quadrat	QE_3	10.1
Reef 12	E	Quadrat	QE_4	5.83
Reef 12	F	Quadrat	QF_1	0
Reef 12	F	Quadrat	QF_2	15.9
Reef 12	F	Quadrat	QF_3	4.5
Reef 12	F	Quadrat	QF_4	10.6
Reef 12	G	Quadrat	QG_1	0
Reef 12	G	Quadrat	QG_2	18.3
Reef 12	G	Quadrat	QG_3	20.5
Reef 12	G	Quadrat	QG_4	30.3
Reef 13	H	Quadrat	QH_1	0
Reef 13	H	Quadrat	QH_2	1.7
Reef 13	H	Quadrat	QH_3	8.75
Reef 13	H	Quadrat	QH_4	21.2
Reef 13	I	Quadrat	QI_1	0
Reef 13	I	Quadrat	QI_2	4.7
Reef 13	I	Quadrat	QI_3	9.23
Reef 13	I	Quadrat	QI_4	11.5
Reef 13	J	Quadrat	QJ_1	0
Reef 13	J	Quadrat	QJ_2	6.04
Reef 13	J	Quadrat	QJ_3	17.4
Reef 13	J	Quadrat	QJ_4	25.3
Reef 3	K	Quadrat	QK_2	3.21
Reef 3	K	Quadrat	QK_3	17.6
Reef 3	K	Quadrat	QK_4	25.2
Reef 3	L	Quadrat	QL_1	0
Reef 3	L	Quadrat	QL_2	30
Reef 3	L	Quadrat	QL_3	25
Reef 3	L	Quadrat	QL_4	24.8
Reef 3	M	Quadrat	QM_1	28.5
Reef 3	M	Quadrat	QM_2	2.79
Reef 3	M	Quadrat	QM_3	28.4
Reef 3	M	Quadrat	QM_4	28.4

3.2 MAXIMUM LIKELIHOOD TREE

Maximum likelihood phylogenetic trees inferred from nearly complete mitochondrial genomes indicate that *L. purpurea* in Hawai‘i is genetically distinct from other *L. purpurea* across the Indo-Pacific (Figure 6). The Hawaiian *L. purpurea* forms a well-supported monophyletic clade, separate from *L. purpurea* collected across the Indo-Pacific. Bootstrap values (100) indicate strong statistical support for the clustering of Hawaiian *L. purpurea* individuals. The tree resolves Hawaiian *L. purpurea* as nested within the Indo-Pacific *L. purpurea* clade while more distantly related to other *Leptastrea* species.

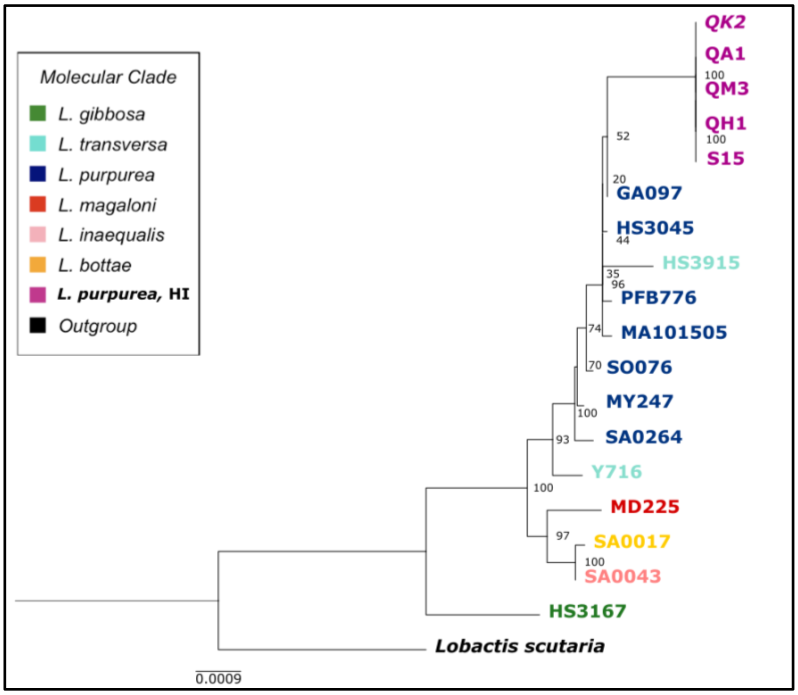


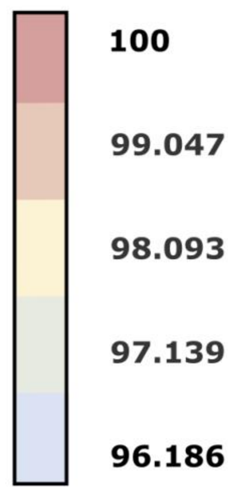
Figure 6. Maximum likelihood (ML) phylogeny tree of *Leptastrea* across the Indo-Pacific, including *L. purpurea* in Hawai‘i using nearly complete mitochondrial genomes (10,797 bp), generated from IQTree. Node values represent the bootstrap support value. Molecular clades are color coded according to the legend.

3.3 PAIRWISE SIMILARITY HEATMAP

Pairwise similarity values from the 10,797 bp alignment of nearly complete mitochondrial genomes indicate that *Leptastrea purpurea* in Hawai‘i is genetically distinct from other *L. purpurea* across the Indo-Pacific (Table 3). Within Hawai‘i, *L. purpurea* exhibited no genetic divergence, with pairwise similarity values of 100%. In contrast, *L. purpurea* from the Indo-Pacific showed pairwise similarity values of 99.48%, 99.56%, and 99.66% when compared to Hawaiian populations. The pairwise similarity values between Hawaiian and Indo-Pacific *L. purpurea* (99.481% to 99.495%) approaches or falls below the differences observed between recognized *Leptastrea* species including *L. bottae* and *L. magaloni* (99.72%) and *L. inaequalis* and *L. bottae* (99.86%), and between Indo-Pacific *L. purpurea* and *L. magaloni* (99.64%).

Table 3. Heatmap of pairwise similarity values of *Leptastrea* across the Indo-pacific obtained from the 10,775 bp alignment. Sample designations follow the legend in Figure 6.

	QK2	QA1	S15	QM3	QH1	SA0264	GA097	PFB776	Y716	HS3167	MD225	SA0017	SA0043	Lobactis
QK2		100	100	100	100	99.495	99.563	99.617	99.409	98.895	99.327	99.348	99.374	96.186
QA1	100		100	100	100	99.481	99.567	99.611	99.396	98.877	99.315	99.335	99.361	96.193
S15	100	100		100	100	99.481	99.567	99.611	99.396	98.877	99.315	99.335	99.361	96.193
QM3	100	100	100		100	99.481	99.567	99.611	99.396	98.877	99.315	99.335	99.361	96.193
QH1	100	100	100	100		99.481	99.567	99.611	99.396	98.877	99.315	99.335	99.361	96.193
SA0264	99.495	99.481	99.481	99.481	99.481		99.741	99.787	99.687	99.176	99.604	99.613	99.667	96.269
GA097	99.563	99.567	99.567	99.567	99.567	99.741		99.875	99.655	99.132	99.592	99.597	99.629	96.292
PFB776	99.617	99.611	99.611	99.611	99.611	99.787	99.875		99.694	99.173	99.639	99.634	99.667	96.325
Y716	99.409	99.396	99.396	99.396	99.396	99.687	99.655	99.694		99.208	99.604	99.664	99.669	96.32
HS3167	98.895	98.877	98.877	98.877	98.877	99.176	99.132	99.173	99.208		99.185	99.217	99.224	96.211
MD225	99.327	99.315	99.315	99.315	99.315	99.604	99.592	99.639	99.604	99.185		99.72	99.752	96.278
SA0017	99.348	99.335	99.335	99.335	99.335	99.613	99.597	99.634	99.664	99.217	99.72		99.863	96.327
SA0043	99.374	99.361	99.361	99.361	99.361	99.667	99.629	99.667	99.669	99.224	99.752	99.863		96.306
Lobactis	96.186	96.193	96.193	96.193	96.193	96.269	96.292	96.325	96.32	96.211	96.278	96.327	96.306	



4.0 Discussion

4.1 DEMOGRAPHIC RELATIONSHIPS OF *L. PURPUREA*

The technical replicates established during library preparation did not appear as clones or close relatives during data analysis. This discrepancy could have arisen from various sources of error, including incorrect assignment of sample IDs or barcodes, sequencing errors, or complications with the sequence alignment pipeline. The latter may have been particularly influenced by our use of a whole-genome low-coverage sequencing approach instead of true ezRAD-seq (Toonen et al., 2013), which resulted in fewer groups of overlapping sequence reads than dDocent (which utilizes FreeBayes for variant calling) typically expects. Consequently, we were unable to proceed with the analysis of the demographic relationships of *L. purpurea* using the data generated here.

To address this issue, future library preparation should pay close attention to barcode identification and demultiplexing, and follow the ezRAD protocol outlined in Toonen et al. (2013). This approach would generate high-coverage, better suited for interpreting fine-scale demographic relationships. Given this data limitation, the project instead shifted to verify the species identity of *Leptastrea purpurea* in Hawai‘i.

4.2 SPECIES VERIFICATION OF *L. PURPUREA* IN HAWAI‘I

The maximum likelihood (ML) phylogenetic analysis provides strong evidence that *Leptastrea purpurea* in Hawai‘i is genetically distinct from *L. purpurea* found across the rest of the Indo-Pacific. *L. purpurea* in Hawai‘i forms a well-supported monophyletic clade with high bootstrap values of 100 (Figure 6). The pairwise distance heatmap shows no genetic divergence within the Hawaiian population, while revealing substantial differentiation between Hawaiian and Indo-Pacific *L. purpurea* (Table 3). A monophyletic Hawaiian clade indicates divergence from Indo-Pacific populations of *L. purpurea,* with pairwise mitogenome differences ranging from 99.48% to 99.49%. Small pairwise similarity values are significant due to the long evolution period of mitochondrial genomes (Shearer et al., 2002). The observed mitochondrial divergence within what is currently recognized as the same species is comparable to or greater than that found between recognized *Leptastrea* species, suggesting that Hawaiian *L. purpurea* may represent a distinct evolutionary lineage.

Though best documented in terrestrial environments (Cowie & Holland, 2006; Meesakul et al., 2023), Hawaiʻi is well known for having high levels of endemic species, including reef fish, corals, mollusks, and algae (Alison Kay & Palumbi, 1987; Bowen et al., 2013; Hourigan & Reese, 1987; Hughes et al., 2002). The distinct Hawaiian clade of *L. purpurea* relative to the Indo-Pacific (Figure 6), along with genetic dissimilarity equivalent to that among other species of *Leptastrea* (Table 3), may indicate the presence of an undescribed cryptic species of *Leptastrea* in Hawaiʻi. However, these findings require further taxonomic investigation to clarify the evolutionary history and classification of this population.

Coral species have traditionally been classified based on the examination of morphological traits in their gross colony structure and soft tissue traits. However, since the advent of high-throughput sequencing, which can generate large genomic datasets capable of resolving complex phylogenetic relationships with greater precision, many studies have revealed a disconnect between molecular relationships and taxonomic classifications based on morphology (Romano & Cairns, 2000; Romano & Palumbi, 1996). These discrepancies can be attributed to factors such as environmentally induced phenotypic plasticity (Forsman et al., 2009;

Todd, 2008) and substantial intraspecific morphological variation (Paz-García et al., 2015; Shaish et al., 2007), among others, which complicate accurate taxonomic classification of corals.

This may be the case for *Leptastrea purpurea* in Hawai‘i, where its presence has been documented based on traditional morphological classification. However, our findings from analyzing genetics suggest a potential cryptic species, indicating that *L. purpurea* may have originally spread to the Hawaiian Islands and, through isolation from the broader Indo-Pacific, diverged into a distinct evolutionary lineage.

Further research is necessary to validate these observed patterns in Hawai‘i. All coral samples analyzed in this study were collected from Kāne‘ohe Bay, an ecologically distinct environment that has experienced significant natural and anthropogenic disturbances, and which is now home to a resilient reef ecosystem (Bahr et al., 2015; Hunter & Evans, 1995). These stressors may have exerted strong selection to drive the observed divergence of *Leptastrea purpurea* in Hawai‘i from its Indo-Pacific counterparts. To accurately assess the evolutionary relationships of *L. purpurea* within the Hawaiian archipelago, future studies should incorporate coral samples from multiple locations across the Hawaiian Islands in addition to those from Kāne‘ohe Bay. Furthermore, a comprehensive morphological analysis of skeletal characteristics in both Indo-Pacific and Hawaiian *L. purpurea* populations should be conducted to evaluate morphological variance between the Hawaiian and Indo-Pacific *L. purpurea* species.

As climate change continues to threaten the health of coral reefs, the design of effective reef restoration strategies is crucial to preserving coral reef biodiversity and their ecosystem services. Accurate species identification is foundational knowledge that is essential to the design of effective conservation strategies. This is especially true in Hawai‘i, one of the most isolated island archipelagos on the planet where many species are endemic and genetically distinct from distant populations.

5.0 Conclusion

Our findings provide strong genetic evidence that *Leptastrea purpurea* in Hawai‘i is genetically distinct from its Indo-Pacific counterparts, suggesting the potential for an independent evolutionary lineage. Future research should verify these results using nuclear DNA, analyzing skeletal morphology, and including samples from across the Hawaiian islands to understand how to best integrate this resilient species into conservation efforts. Given Hawai‘i’s high levels of endemism and the ecological significance of *L. purpurea*, accurate species identification will be crucial for informing targeted management strategies that support coral reef resilience amid ongoing environmental change in Hawai‘i.

6.0 Appendix

6.1 SUPPLEMENTARY MATERIALS

Modified Watchmaker PCR Protocol for Whole Genome Low Coverage Sequencing

Materials

Watchmaker DNA library prep kit cat # 7K0102-096 (https://watchmakergenomics.com/portfolio/dna_lpk/erat-ordering-information/)

IDT xGenTM Stubby Adapter cat # 10005924 and xGen UDI 10nt Primer pairs cat # 10008052 (https://www.idtdna.com/pages/products/next- generation-sequencing/adapters/xgen-stubby-adapter-and-udi-primer-pairs/)

Extraction

We recommend extracting with the Kingfisher robot (for more than 16-24 samples) and the Omega MagBind Blood and Tissue DNA HDQ kit. The robot can process 96 samples at once. Waiting to process as many samples together as you can is recommended to reduce costs on robot consumables.

- Qubit quanting extracted DNA and normalizing to 20 ng/uL if the DNA quantity is high.
    - If the quantity is below this amount but quality is still good, normalize to 8 or 10 ng/uL.

Safe stopping point- Extracted DNA can be safely stored in the freezer at -20 both before and after quantifying for extended periods of time.

Fragmentation

Fragmentation is performed using the restriction enzymes Sau3AI and MboI from NEB (product. After normalization (if necessary), DNA samples are digested as follows for ¼ reaction:

1⁄4 reaction:

Reagent	**Per Sample (µL)**
NEB CutSmart Buffer	2.5
Nuclease free water	9
MboI	0.5
Sau3AI	0.5
Genomic DNA	12.5

Genomic DNA Incubate digestion @ 37°C for 1 hour, then 65°C for 20 minutes.

Safe stopping point- digested DNA can be stored in the freezer at -20 for 1 month. Avoid repeated freeze-thaw cycles if possible.

End Repair/A-tailing

Keep enzymes on ice while setting up the reaction, and return them to the freezer when setup is complete. Mix ER/AT buffer and ER/AT enzyme mix as per the table below and vortex briefly. Spin down and keep on ice (this ER/AT master mix can be stored at 4°C for 2 hours). Add ER/AT master mix to samples.

¼ reaction:

Reagent	Per Sample (µL)
ER/AT buffer	1.75
ER/AT enzyme mix	0.75
Digested DNA	12.5

- Once ER/AT master mix has been added to digested DNA, transfer samples to thermal cycler with the following settings:
    - Lid temperature: 85°C 20°C for 30 minutes 65°C for 30 minutes, 4°C ∞

proceed immediately to adapter ligation when samples are at 4°C. Ligation reagents may be set up and prepped during ER/AT incubation.

Adapter Ligation

Master mix (MM) for adapter ligation can be set up, on ice, as follows:

¼ reaction:

Reagent	**Per Sample (µL)**
Stubby adapter* 15 uM	1.25
DNA Ligase	1.25
Ligation buffer	6.25
Input DNA (from ER/AT)	8.75

- Once the master mix has been made, vortex briefly and spin down. Keep on ice until the ER/AT reaction comes off the thermal cycler, then add the ligation MM directly to the ER/AT tubes. Pipette up and down 10 times to ensure mixing (try to avoid creating bubbles) and return to thermal cycler for ligation with the following settings:
    - Heated lid: OFF
      Incubate at 20°C for 15** minutes

After ligation, we recommend proceeding immediately to post-ligation cleanup.

Post-ligation cleanup

For this we recommend using the Kingfisher and a 1x concentration of beads (differing from 0.8X in the Watchmaker protocol), so 27.5 uL of ligation (for a 1⁄4 reaction) gets 27.5 uL Omega beads (as opposed to SPRI beads used in the Watchmaker protocol). We used the KingFisher bead cleaning protocol and eluted in a 40 uL elution buffer.

Safe stopping point

Amplification

Amplification is where unique barcodes are added to each sample. We use the IDT xGen UDI primer pairs (which come in plates) for unique dual indexing. The reactions are set up as shown below:

1⁄2 Reaction:

Reagent	**Per Sample (µL)**
Equinox amplification mix	12.5
Indices from UDI plate	2.5
Ligated DNA	10

Amplify on the thermal cycler as follows:

Initial denaturation: 98°C for 45 seconds

Denaturation: 98°C for 15 seconds *Annealing:* 60°C for 30 seconds *Extension:* 72°C for 45 seconds

Final extension: 72°C for 60 seconds *Hold at 12°C

(*If you're still troubleshooting and you may need to add cycles, we recommend holding at room temperature. Cycles can be added after a room temperature hold where you run a gel to determine the appropriate cycle number for a sample type.)

We recommend troubleshooting a subset of samples with 1⁄2 reactions in case you have to run several times on a gel- once your reactions are amplifying consistently across the board, we recommend switching to 1⁄4 reactions. We also recommend running all amplifications on a gel if

you have less than 100 samples, and a subset of samples (at least one from every row on every plate) if you have many samples. Consistent reactions should look like this:

In general, expect to do 6-8 cycles of amplification for normal amounts of DNA. Even low amounts of DNA will likely not take more than 8 cycles to amplify, but you can go up to 16 according to the kit. DON'T OVERAMPLIFY! Start with 6-8 cycles and stop as soon as you have clear bands that are no longer faint.

Safe stopping point- amplified DNA is very stable and can be stored in the freezer at -20 for several months. Avoid repeated freeze-thaw cycles if possible.

Note : we deviated from the Watchmaker protocol in that, after checking gel smears, we skipped post-amplification cleanup and instead quantified, pooled, and verified the 2 libraries before sending off for sequencing. UC Davis performed the final post-amplification cleanup on pooled libraries using Kapa pure beads with a 1.1 x reaction.

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