GENETIC SEX DETERMINATION IN *OCTOPUS RUBESCENS*

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This paper is firstly dedicated to my master's advisor, Dr. Kirt Onthank, my undergraduate advisor, Dr. Tammy Windfelder, and every other science teacher I have ever had the honor of knowing, as without them I would never have had the inspiration, bravery, and strength to continue to question the unknowns of the world.

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ABSTRACT

The sex determination system of octopuses has been completely unknown up until recently, even though many of their mollusk relatives have known genetic sex determination systems. The goal of this study was to determine the sex determination system of *Octopus rubescens*. I hypothesized that those octopuses have genetic sex determination using an XX-XY or similar ZW-ZZ system. This hypothesis was based on the knowledge that the majority of gonochoric mollusks that have known-sex determining systems have the XX-XY system. I extracted DNA from 28 *O. rubescens* and performed double-digest RAD sequencing (ddRAD-seq) using EcoRI and MspI enzymes. Based on the RAD-tag count analysis, my results were most consistent with a ZW-ZZ chromosomal system as 7 out of 10 females possessed a unique RAD-tag that only 1 out of 18 males had. My results are in contrast to other literature that suggested that octopuses have a ZO-ZZ chromosomal system.

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INTRODUCTION

Background and Significance

Sex determination is an incredibly varied and complex process that is directed by a multitude of systems throughout the animal kingdom. Some methods of sex determination include chromosomal variations, temperature dependance, and location dependance. Information about sex determination methods is important not only for research purposes, but also for conservation, which is becoming increasingly critical as global climate change continues.

Chromosomal Sex Determination

Nettie Stephens first discovered sex chromosomes in the early 1900s using male and female mealworm chromosomes. In these mealworms, Stephens discovered that the males had one chromosome that was smaller than the female chromosomes, which were all the same length (Hake 2008; Stephens 1905). These chromosomal differences, which were later named Y and X, are the most common sex determination method in animals (Hake 2008).

Since the time of Stephen's work in 1905, two different types of heterogametic chromosomal sex determinations have been discovered in the animal kingdom; the XY and and ZW systems. The XX-XY system is known as male heterogamety. In this system, the Y chromosome is male specific and is usually smaller than the X chromosome, which is present in both males and females. Within this system, XX individuals are considered typical females, while XY individuals are considered typical males (Doris 2011; Graves Marshall 2008). The XY system is found in most mammals (Dorris 2011), some insects (Graves Marshall 2008), and some gastropods such as the

knobbed whelk (*Busycon carica*; Avise 2004; Nakamura 1986), soft shell clams (*Mya arenaria*; Allen et al. 1986), and dwarf surf clams (*Mulinia lateralis*; Guo and Allen 1994).

The ZW system is known as female heterogamety, and usually functions as the opposite of the XY system; the homozygous individuals (ZZ) are considered typical males while heterozygous individuals (ZW) are considered typical females (Doris 2011; Smith 2009). Like the XY system, the chromosome present in both sexes (Z) is typically larger than the chromosome found in only females (W; Doris 2011). The ZW system is found in birds, snakes, and some insects such as the domestic silk moth (*Bombyx mori*; Graves Marshall 2008; Smith 2009; Kiuchi 2014).

Along with the XY and ZW systems, there are also chromosomal systems in which one sex is missing a chromosome. In the XX-XO system, females are XX while males have an X chromosome, are missing the typical Y chromosome, and are denoted as XO (Hake 2008). In the ZO-ZZ system, females have a Z chromosome, but are missing their typical W chromosome and are denoted as ZO while the males are ZZ (Traut 2007). These two systems are most commonly found in insects, such as grasshoppers and caddisflies (Hake 2008; Traut 2007).

Complicated Outliers

Although seemingly simple in explanation, genetic sex determination is often more complicated than just a combination of one or two Xs, Ys, Zs, and Ws. For example, in platypuses (*Ornithorhynchus anatinus*), males tend to have five X chromosomes and five Y chromosomes, while females have five pairs of X chromosomes. In the Japanese frog, *Rana rugosa*, different populations have different

determining chromosomal systems. Two known populations have male heterogamety but are homomorphic, meaning their two differing chromosomes look and act extremely similar. Another population has classic male heterogamety, while another has classic female heterogamety (Graves Marshall 2008).

Surprisingly, *Drosophila melanogaster*, one of the most common genetic models, has an uncommon and complicated chromosomal sex determination system. *Drosophila melanogaster* sex is determined by the balance between the female-determining factors in the X chromosome and the male-determining factors in the autosomes. Normally, *Drosophila* have one or two X chromosomes and two sets of autosomes. So, if the X:A ratio is one (2X:2A), the fly is female, while if there is only one X chromosome (1X:2A), the fly is male. In this species, the Y chromosome is considered an autosome, as it is not involved in sex determination, but does have genes that allow for the fly to form sperm. If a fly is XO, they are considered an infertile male as they only have one X chromosome and no autosomal factors allowing them to form sperm (Hake 2008; Gilbert 2000).

There are many other chromosomal combinations in *Drosophila* that result in male or female flies. Flies that have XXXYYY, XXXXYYYY, XXXYY, XXXXYYY are considered female, as they have equal or more female determining chromosomes than male determining factors. An X:A ratio of one or higher allows a female to develop due to the activation of the *Sxl* gene, which is a regulator of female development. Flies that have XYY or XYYY are considered male, as they have more male determining autosomes than female determining genes (Hake 2008; Gilbert 2000).

Some animal species, such as certain reptiles, frogs, and fish, do not have obvious sex chromosome differences even though their sex is determined genetically. An example

is the three-spined stickleback (*Gasterosteus aculeatus*) which has genetic sex determination with male heterogamety but no sex chromosomes, even though close relatives have the XY system. It was found that a sex-specific marker with many mutations, deletions, and insertions may be responsible for their genetic sex differences (Graves Marshall 2008). There is also one known mammal for which both sexes are XO, the Transcaucasian mole vole (*Ellobius lutescens*), which are described as having an "enigmatic" sex determination (Sharat Chandra 1999).

There is also haplodiploidy, in which one sex is haploid and has no father, while the other is diploid. This system is common in insects such as ants and bees. Typically, the females are diploid, or heterozygotes, while the males are haploid, or hemizygotes. In some cases there are diploid males that often are eliminated by other worker insects during their larval stage as they are produced at the expense of the workers due to their low viability (Wilgenburg 2006).

Non-Genetic Sex Determination

Sequential Hermaphroditism

Some organisms can be born presenting as one sex, but then change their sex at least once throughout their life via hormonal or social cues, usually for reproduction. These individuals are known as sequential hermaphrodites. There are three different types of sequential hermaphroditism: protandry, protogyny, and bidirectional. Protandry is the transition of an organism from male to female, protogyny is the transition of an organism from female to male, and bidirectional can occur multiple times and the organism can

transition in either direction (male to female or female to male; Warner 1975; Todd 2016).

Protandry can be found in a variety of animals, such as fish (de Mitcheson 2008), mollusks (Collin 2013), and crustaceans (Bauer 2006), but has not been seen in any terrestrial vertebrate (Henshaw 2017). Possibly the most well-known example of organisms that experiences protandry are clownfish. In clownfish groups, the largest individual fish is a female and once that female dies, the largest male will gain weight and transition into the new female for the group (Buston 2004). Some mollusks that are considered protandrous are the marine gastropods of the family *Calyptraeidae*, the genus *Coralliophila*, and the subclass *Patellogastropoda* (Baeuer 2006), none of which are cephalopods.

The most common type of hermaphroditism in fish is protogyny, as 75% of the 500 known sequentially hermaphroditic fish are protogynous (Pauly 2007). Among those protogynous fish, wrasses are the most common. In typical wrasse mating pairs, the larger fish is a male and the smaller fish is a female. The female wrasse can transition into a male if they grow larger than their male, or if there are no males present nearby (Todd 2019).

The least common type of hermaphroditism is bidirectional hermaphroditism. This is seen in some reef fish, such as the genus *Gobiodon*, as it can be risky for them to leave their reef to mate. If there are two males or two females in the reef, one of them can transition to the other sex in order to mate to mitigate their “risk-of-movement” (Munday 1998).

Location Dependent Sex Determination

Another non-chromosomal sex determination method is known as location dependence, and it is primarily found in marine animals. *Bonellia viridis*, a marine worm, has a sex determination system in which if larvae come into contact with a female, they become male due to the females producing bonellin, a male sex determining chemical. But, if the larvae land on the seafloor and do not come into contact with a female, and therefore do not come into contact with bonellin, the larvae either remains undifferentiated or turn into a female (Gilbert 2000).

The slipper snail, *Crepidula fornicata*, uses its place in the mound on the ocean floor to determine its sex. The young individuals of *C. fornicata* are always male, but then they have a life phase during which their male reproductive system degenerates. After that life phase, if the snail is attached to a female in the mound, it will become male. But, if a male snail is removed from its attachment to a female, it will become a female. Also, if there are too many males in the mound, some of them will transition to females. Once the snail is a female, it will never revert back to a male (Gilbert 2000).

Location dependence sex determination is unique from sequential hermaphroditism, as with sequential hermaphroditism, the individual's sex changes throughout its life typically solely based on the ratio of males and females in their group. With location dependent sex determination, the individual's sex changes due to their physical location regardless of already existing ratios; i.e. coming into direct contact with a female.

Temperature Dependent Sex Determination (TSD)

Along with genetics causing sex determination differences, the temperature at which eggs are incubated during certain periods of development can determine the sex of an animal, specifically in some species of reptiles and fish. This phenomenon is known as temperature dependent sex determination (TSD; Graves Marshall 2008).

TSD can be seen in some species of marine turtles, alligators, crocodiles, and lizards. In marine turtles specifically, eggs that are incubated in low temperatures (around 26°C) have a 100% chance of their sex being determined as male, while eggs that are incubated in high temperatures (around 33°C) have a 100% chance of their sex being determined as female; this is known as the MF-pattern (Graves Marshall 2008; Maldonado 2002).

In contrast, in some species of alligators, eggs that are incubated in low temperatures have a 100% chance of their sex being determined as female, while eggs that are incubated in high temperatures have a 100% chance of being determined as male; this is known as the FM-pattern or Pattern I. In addition to the FM-pattern, in some species of alligator, if incubated at extremely high temperatures, the eggs have a 100% chance of their sex being determined as female; this is known as the FMF-pattern or Pattern II (Graves Marshall 2008; Ospina-Alvarez 2008). Within the FMF-pattern, for the sex to be determined as male, the temperature has a much smaller window, causing the proportion of males to never reach 100% in some species of crocodiles and lizards (Graves Marshall 2008).

These different types of TSD are considered to be present strictly in reptiles, but there is evidence of one unique bird, the Australian bush turkey (*Alectura lathami*), that

can have different sexes depending on temperature. *A. lathami* is a mound building megapode, and it was found that eggs that were incubated in lower temperatures were more likely to be male while eggs that were incubated in higher temperatures were more likely to be female. In average, or optimal, temperature mounds, the male to female proportion ratio was 1:1 (Goth 2005).

Octopus Sex Determination

Genetic sex determination is well understood and documented in mammals and most other vertebrates, but not for invertebrates. Most genetic sex determination studies use limited model organisms, specifically bivalves such as hermaphroditic oysters and clams (Zhang 2014). However, it has been found that most gonochoric mollusks use the XX-XY chromosomal system, similar to mammals.

Mammals typically have XX and XY chromosomes leading to genetic sex differences, and the expression of certain hormones and transcription factors such as *Sry, Sox9, Dmrt1, FoxL2, ß-catenin,* and *Wnt4* leads to physical sex differences (Matson and Zarkower 2012). Some studies have discovered sex determination genes in Pacific oysters (*Crassostrea gigas*), which have *Sry*-like genes involved in sex determination (Zhang 2014). Knobbed whelk (*Busycon carcica*) have mammalian-like X-linked sex determination (Avise 2004). Many researchers also found that gastropods (Nakamura 1986), soft shell clams (*Mya arenaria*; Allen et al. 1986), and dwarf surf clams (*Mulinia lateralis*; Guo and Allen 1994) have the XX-XY sex determining system. There is also known XO sex determination in the snail family Neritidae (Nakamura 1986). At the onset of this current study, no sex determination system had been discovered in any cephalopods.

Overall Importance

Most information regarding genetic sex-determination comes from a small number of organisms, meaning there is a lot of information that is unknown about the majority of organisms. It is incredibly important to study non-model organisms, such as octopuses, because external features for identifying sex can be unreliable, especially during early life stages, such as in larvae and paralarvae (Yusa 2009). For example, in octopuses, to phenotypically identify an individual as a male one needs to locate either enlarged suckers or a modified third right arm.

Enlarged suckers are considered a secondary sexual characteristic of male octopuses, but they have a lot of size variation within a population. They could be compared to human breast size or facial hair, which exist on a spectrum and cannot reliably be used to identify the sex of a human. Because sucker size exists on such a broad spectrum, identifying an octopus as male solely based on a large sucker can be unreliable, as some females may have larger suckers and some males may have smaller suckers. Also, relying on solely locating the modified third right arm is unreliable as it may not be present. Even further, these two secondary sex characteristics are only present in adults and not hatchlings, so it may be impossible to properly sex an octopus until they are an adult. Knowing whether there is a sex determining system, what it is, and how to identify it would greatly assist in the accuracy of sexing octopuses.

Sexing octopuses is vital for basic octopus research, as knowing their sex can help explain behavioral or size differences. Also, knowing their genetic sex will allow more complicated sex-related octopus research. For example, being able to genetically sex octopuses will allow researchers to determine if any specific sex proportion of octopuses

exists in the wild, especially among eggs and paralarvae, and if there are any factors that may affect their sex proportions, such as climate change or ocean acidification.

Accurately sexing octopuses is vital not only for research, but also for conservation. Octopuses are key features of marine ecosystems as they are found in all depths in almost all marine habitats and are important prey for seabirds, cetaceans, and fish (Culler-Juarez 2021). They also have high gross conversion efficiencies, near 50%, showing that they are a very important link between the different trophic levels in marine ecosystems (Culler-Juarez 2021). Due to their importance in ecosystems, conservation efforts may soon need to be made for these organisms, and knowing their genetic sex can assist with captive mating efforts.

Hypothesis

For this project, I hypothesized that *Octopus rubescens* will have an XX-XY or similar ZW-ZZ system. This hypothesis was based on the knowledge that the majority of gonochoric mollusks that have known-sex determining systems have the XX-XY system (Nakamura 1986; Allen 1986; Guo 1994). By using ddRAD-seq and computational analyses, I expected to find specific genetic sequences present in one sex of octopuses but completely absent in the other. If I found that *O. rubescens* had an XX-XY sex determination system, I would expect unique genetic sequences only found in males due to their unique Y chromosome, and the opposite result for a ZW-ZZ system.

METHODS

Octopus Collection

Twenty-eight *Octopus rubescens* were collected via SCUBA from Driftwood Park, Whidbey Island, Island County, Washington (48.16397, -122.63746). After they were located in glass bottles on the ocean floor at a depth of 15-18m, the octopuses and the glass bottles they were found in were sealed in resealable plastic bags and brought to the surface. Once at the surface, each octopus was removed from their glass bottle and placed in a red plastic Nalgene bottle and safely transported to Rosario Beach Marine Laboratory (RBML) in Anacortes, Washington, in a cooler filled with oxygenated seawater.

Once at RBML, the octopuses were weighed and sexed by myself and two others according to their basic anatomy. Male octopuses typically have enlarged suckers close to their beak and they are expected to have a modified third right arm with a hectocotylus (Robson 1926). The hectocotylus can be difficult to locate on a moving octopus especially if the arm tip is curled and/or close to the body. Typical females are expected to have neither of those characteristics. Most females were in or close to senescence, but not brooding eggs, when collected. It is clear when an octopus is in senescence as they tend to have looser skin, and they do not eat as regularly as non-senescent octopuses. Female senescent octopuses behaviorally are more likely to remain in one location (typically the back of their bottle) unless forced to move, while senescent males typically move around more often than non-senescent males (pers obs). In despite of these usual difficulties in sexing, I was confident the octopuses were phenotypically sexed accurately due to the fact that three people assisted in sexing them. Having more than one person

confirm the phenotypic sex, takes away the chance that a hectocotylus was not seen, or an enlarged sucker led to a female inaccurately being marked as a male rather than a female.

After massing and sexing, the octopuses were housed in individual three-liter tanks with flowing seawater. They were fed either one collected purple shore crab (*Hemigrapsus nudu*s), wrinkled purple whelk (*Nucella lamellosa*), bay mussel (*Mytilus trossulus*) or one store-bought manila clam (*Venerupis philippinarum*) daily. Once tissue extraction was completed, all healthy octopuses were returned back to the collection location via SCUBA. Octopuses that were unhealthy, such as those deep in senescence, were humanely euthanized and preserved for future research.

Sample Collection

To collect the octopus tissue for DNA extraction, each octopus was removed from its three-liter tank and placed in a one-liter tank with seawater where 22.5mL of absolute ethanol was added slowly over a period between five to thirty minutes depending on the individual octopus' sedation rate (Culler-Juarez, 2021). The time range for the addition of ethanol is large as some octopuses, specifically larger males, do not succumb to the ethanol quickly, usually due to a combination of their size and their attempts to climb out of the ethanol-seawater mix. Smaller females usually succumbed to the ethanol faster, as they were usually much smaller than the males and less likely to climb out of the ethanol-seawater mix. While adding the ethanol, the octopus' breathing and coloration were monitored to assess for signs of physiological distress, such as hypoxia-induced harm. When an octopus has difficulty breathing, they tend to become pale and their mantle stops rhythmically moving, indicating impaired ventilation through their gills.

Once fully anesthetized, as recognized by a lack of reaction to touch, they were removed from the ethanol-seawater and placed on a clean dissection tray. On that clean dissection tray, phenotypic sex was once again confirmed, with multiple people present, by locating either a hectocotylus or a lack of one on the third right arm. There, I cut approximately 2cm from the tips of their first right (R1) and second right (R2) arms using a clean scalpel. They were then swiftly placed into a well-oxygenated seawater tank to recover, where their breathing, coloration, and reaction to touch was monitored. After octopuses appeared to fully recover from anesthesia (5-10 minutes), as evidenced by coordinated arm movement in relation to the head and eyes, normal coloration, and normal responses to touch, they were returned to their original three-liter tank.Their cut arm tips were placed in labeled microcentrifuge tubes and stored in a -80°C freezer.

DNA Extraction

One of the frozen arm tips per individual was slightly defrosted, approximately 0.05g was removed with a clean scalpel, and the remaining arm tip was returned to the -80°C freezer. The outer layer of the skin was cut away using a clean razor blade to reduce possible bacterial contamination. Once the skin was removed, the rest of the inner muscular tissue was finely chopped, using the same razor blade after it had been cleaned with ethanol. The finely chopped tissue was weighed and stored in a new labeled microcentrifuge tube in the -80°C freezer to prevent degradation. A new, clean, razor blade was used for each octopus to reduce cross contamination.

DNA extraction was performed on the thawed finely chopped tissue using the *Monarch® Genomic DNA Purification Kit* and by following the supplied “DNA extraction via tissue” methods.

DNA Sequencing

The extracted DNA was stored in labeled microcentrifuge tubes in the -80°C freezer. Half of each DNA sample (a range between 7 to 50.5ng/uL) was sent to Admera Health where they performed double digest restriction-site associated DNA sequencing (ddRAD-seq) via Illumina sequencing using EcoRI-MspI restriction enzymes. EcoRI-MspI enzymes were used previously to extract DNA from a closely related octopus species, *O. vulgaris*, with success (García-Fernández 2017). The other half of each DNA sample was stored at -80°C as backup.

ddRAD-seq is a type of high-throughput sequencing that sequences a large number of sample locations throughout an entire genome. The sequences are largely shared between individuals, meaning the same locations in the genome will be sequenced for each individual. Overall, this method allows for sequencing a larger number of individuals in a genome-wide search for markers that would otherwise not be possible due to monetary and time constraints. Studies using ddRAD-seq also do not require a previously sequenced genome; no published *O. rubescens* genome existed when this project began.

The first step of ddRAD is to digest the DNA with one of the restriction enzymes to create fragments, in this case EcoRI (Fig 1a). Restriction enzymes cleave DNA at a specific, short DNA sequence that is typically 4-8 base pairs long, known as the restriction site. For instance, EcoRI cleaves DNA at the sequence GAATTC between the first G and A. Because the sequences recognized by the restriction enzymes are short, they often occur thousands to hundred thousands of times throughout the genomes of eukaryotes. After EcoRI cleaves the DNA, a barcoded adaptor is ligated to the fragments.

The DNA is then digested by another restriction enzyme, in this case, MspI. MspI cleaves between the first and second C in a CCGG sequence, and again an adaptor is ligated to the fragments. After the second digestion, the fragments are size-selected (in my case for the insert to be around 250 base pairs long), purified, and then amplified for analysis (Figure 1a; Peterson 2012).

Once the DNA is cut into sections by the restriction enzymes, the first 150-300 base pairs of the inserts are sequenced. For this project, my sequencing was "paired end"; Illumina sequencing is known as "paired end" when an insert is sequenced in both the forward and reverse directions. A sequenced insert is then known as a "read."

Data Analysis

To analyze my reads I merged my forward and reverse sequences together using the tool *NGmerge* (Figure 1b) (Gasper 2018). As mentioned above, my insert size was around 250 base pairs and my reads were around 150 base pairs each, or around 300 base pairs total in the combined forward and reverse sequences; I had around 25 base pairs of overlap for each insert. In Illumina sequencing a single read is typically 150 to 300 base pairs long, but inserts are usually longer to prevent overlap of the forward and reverse reads, and therefore wasting sequencing. If my reads were around 150 base pairs each, the sequences merged at their 25 base pairs of overlap. If the insert was much larger than 250 base pairs, or if there were significant sequencing errors that prevented finding the overlap, the read would be removed and thrown out as merging would no longer be accurate.

After merging the two reads, I quality checked the reads using *Trimmomatic* (Bolger 2014). This program trims off any bases in the reads, typically at the ends of the

read, that fall below the per-base PHRED quality score of 15, or drop whole reads for which the average quality score are less than 36 bases after trimming. Similar to merging, this process also shortened some of the sequences, due to the nature of trimming off low quality reads and base pairs. High-quality reads did not receive trimming as all bases in the read would have a PHRED score higher than 15.

RAD-tag Presence Analysis

The program *RADsex* (Feron 2021) did not find any sex-associated RAD-tags. A RAD-tag is a specific segment, or insert, of DNA that contains genetic information that one is interested in. Therefore, I tried a more manual method to identify sex-associated RAD-tags by creating a library of representative RAD-tag sequences, then tallying RAD-tag count between octopuses with the goal of being able to compare individual sequences between octopuses. With this method, I expected to find any differences in the presence or absence of RAD-tags that would point to possible sex association chromosomes.

To create a library of RAD-tag sequences, I used the data from the male with the most sequenced reads, Octopus 07, and the data from the female with the most sequenced reads, Octopus 25, and I concatenated those sequences into a single FASTQ file. I then used the program *CD-HIT-EST* to cluster those reads together; a "cluster" is a group of reads that had the same, or very nearly the same, sequence (Weizhong 2006). I set the necessary minimum sequence similarity to 97% for sequences to be considered the same cluster; the default is 90% similar. *CD-HIT-EST* generated a FASTQ file which contained the representative sequence for each cluster. Next, I converted this FASTQ file into a FASTA file, and reformatted the headers so the file would work with downstream

programs. I indexed the FASTA file using *bwa index*, which makes a new file that enables more efficient search of the FASTA, speeding up further processing (Li 2009a). I aligned the merged and quality checked sequence reads of every individual octopus to the RAD-tag library using *bwa mem*, which produced a SAM (sequence alignment map) file for each octopus (Li 2013).

To take up less storage space, I converted each SAM file to a BAM file. Once the files were in BAM (binary alignment map) format, I sorted and indexed the BAM file using *samtools* (Li 2009b; Danecek 2021). Next, I used *idxstats* to tally the number of reads that aligned to each specific RAD-tag sequence, or marker, for each octopus sample (Li 2009b; Danecek 2021). I then tallied how many male and how many female octopuses each RAD-tag was present in. I performed a proportion test to determine if any RAD-tag was present in significantly more of one sex than the other.

Variant Analysis

Next, I searched for genetic variations that would be sex specific. To do this, I used *bcftools* to convert BAM files for each octopus to a BCF (Danecek 2021). A BCF is a binary file type that is used to record single nucleotide polymorphisms (SNPs) and inserts and deletions (indels) in the reads as variations from a reference sequence without having to record an entire sequence. I used the library of representative clusters as the reference for this analysis. I then found tallies of variants for each octopus using *bcftools stats* (Danecek 2021).

After I tallied the variants for each octopus sample, I merged each sample's clusters together into a single file. I used *pLink2* to test if there are any SNPs or indels that were significantly associated with one sex or the other (Chen 2019). When

calculating the total number of SNPs, if there was a SNP location that was the same in multiple octopuses, it was entered as one SNP, as opposed to counting each individual SNP in each octopus. For example, if I was looking at two octopuses who each had 3 SNPs: Octopus 1 has SNP1, SNP2, and SNP3 and Octopus 2 has SNP1, SNP2, and SNP4. One way of counting these SNPs is to count each SNP from each octopus; meaning there would be 6 total SNPs. Alternatively, I could only count the unique SNPs; meaning there would be a total of 4 SNPs. In my case, I did the latter as I was focused on finding the *unique* genes in the population, as opposed to the total number of the genes. I did the same to calculate the total number of indels. This analysis could potentially allow me to detect if there was a non-chromosomal genetic variant associated with sex.

RAD-tag Count Analysis

I used *DEseq2*, to determine if a potential RAD-tag read quantity was associated with sex. *DEseq2* is designed to detect significant differences in sequence read counts between two groups of samples, and is normally used with RNAseq data to determine mRNA expression differences (Love 2014). This approach could potentially allow me to detect if chromosome copy number was associated with sex, such as a XX-XO or ZZ-ZO system, as one would expect approximately half as many copies of RAD-tag reads from loci on a haploid chromosome compared to a diploid chromosome. However, ddRAD-seq is not ideal for this type of analysis due to the high variance in RAD-tag counts.

For this analysis I used the RAD-tag counts per octopus previously created using *idxstats* (Danecek 2021). I then used the function DESeq with the default settings to calculate p-values for the expression difference of RAD-tags between male and female samples. DESeq uses the Wald significance test to determine the p-value, and the

Benajamini-Hochberg method to adjust p-values for multiple comparisons. I used BLAST to determine potential identity of significantly differentially expressed RAD-tags identified by this method.

RESULTS

I extracted DNA using the *Monarch® Genomic DNA Purification Kit* from 10 female and 18 male *O. rubescens* for ddRAD-sequencing (ddRAD-seq) using Illumina sequencing.

I first looked for sex-associated RAD-tags with the program RADsex (Feron 2021), which did not return any significantly segregated RAD-tag between sexes.

I then developed my own pipeline to attempt to find sex-associated RAD-tags. To do this, I merged, trimmed, and mapped my raw paired end reads to reference files. In total I obtained 16,253,301 total raw paired end reads, ranging from 194,209 to 1,537,165 per octopus (Table 2). After merging forward and reverse reads, 13,741,213 total reads remained (Table 2), and after quality control trimming 13,585,312 total reads remained. I used merged, quality-controlled reads from the male with the most sequencing (octopus 07) and the female with the most sequencing (octopus 25) to construct a library of representative RAD-tags, of which 254,528 were found. Those RAD-tags in total covered 54,283,914 base pairs, or around 1.4% of the ~3.82 billion base pairs of the complete *O. rubescens* genome (Wright 2024). Finally, 13,365,150 total reads, ranging from 155,034 to 1,318,578 per each individual octopus, were mapped to specific RAD-tags (Table 2). By looking at the reads mapped from each individual octopus, I did not find any tags that were present in all of one sex and none of the other, nor any tag that was significantly found more frequently in one sex or the other (Figure 2). There were, however, over 20,000 RAD-tags that occurred in 1 female and 0 males, but only approximately 6,000 that occurred in 1 male and 0 females, despite males out numbering females 18 to 10 (Figure 2).

To see if there were any copy number differences in RAD-tags between the sexes, I used *Deseq2*. Using this method I found two significant RAD-tags (Table 4; Table 5; Appendix I). A BLAST search of RAD-tag LH00150:205:22F3JTLT3:3:1107:16626:17503 revealed the highest similarity to sequences from bacterial species in the genus *Mycoplasmopsis* (Table 6; Appendix I). RAD-tag LH00150:205:22F3JTLT3:3:1183:40148:9972 had top sequence matches to multiple predicted transcripts from *Octopus bimaculoides*, including an E3 ubiquitin-protein ligase XIAP and several uncharacterized non-coding RNAs (Table 7; Appendix I). When looking at the results from each individual octopus, I found that this RAD-tag was found in 7 out of the 10 female octopuses with read counts of 4 to 34 (106 reads total) in those females in which it occurred, and in 1 out of 18 males with a single read count (Figure 3).

To search for sex-specific SNPs or indels, I used *pLink2*. I found 1,113,504 total SNPs, ranging from 53,192 to 239,442 per individual octopus, and 101,501 total indels, ranging from 5,581 to 21,133 per individual octopus. Neither the SNPs nor indels were found to be sex specific (Fisher's Exact Tests, Benjamini-Hochberg corrected p-value range: 0.95-1).

DISCUSSION

Search for Sex Associated RAD-tags

I merged the forward and reverse quality-controlled reads and constructed a library of representative RAD-tags, or clusters, and determined the presence or absence of each RAD tag in the sequence data from each octopus. In doing so, I did not find any significant tags that were present in all of one sex and none of the other (Figure 2).

Search for Sex Associated SNPS and Indels

Because I did not find any significant tags that were present in either sex and not the other, I used *pLink2* to search for sex-specific SNPs or indels (Table 2). I once again did not find any SNPs or indels that were associated by sex. This method is a powerful method to find nucleotide-level differences in the genome that may be present, so if the sex-associated differences between male and female octopuses was based on a few different alleles of the same gene, I should have been able to find it.

Tallying for Sex-Associated RAD-tags

As searching for differences via SNPs and indels was unsuccessful, I continued my search by assessing if read count was significantly different between sexes of any RAD-tag using DESeq2. This method yielded two significant RAD-tags. One, when BLASTed closely matched bacteria of the genus *Mycoplasmopsis* and therefore I considered this RAD-tag likely originated from microbial contamination. The other RAD-tag with read counts significantly different between sexes most closely matched *O. bimaculoides* with 85% identity match. This was to be expected, as most *O. rubescens* genes are not present in BLAST. This RAD-tag was found significantly more often in female octopuses than males (Figure 3), as 7 out of 10 females had this RAD-tag present

and only 1 out of 18 males had it present. These findings suggest that there is a female-associated sex determination method, as females have one specific RAD-tag more often than males.

Overall Findings

My overall results were largely inconclusive, but best fit with a ZZ-ZW sex determination system due to the large number of RAD-tag sequences found only in females and the potential sex-associated RAD-tag found in mostly females. Finding one RAD-tag present in most of the females, but in virtually none of the males suggested that there may have been genetic sequences present in females that are absent in males. These findings match with how ZW-ZZ chromosomal systems tend to function in typical individuals; one chromosome (Z) chromosome is present in both sexes, but specifically females have one different chromosome (W) that has different genetic information than any of the male chromosomes. Because a single RAD-tag was found in most of the 10 females but was represented by a single read in only one of the 18 males, I conclude that females may have had a unique chromosome where that RAD-tag was found. Also, the large number of RAD-tag sequences (20,000) only found in females corroborated this conclusion.

Despite the suggestive nature of these results, they do not provide sufficient evidence to conclusively support a ZW-ZZ sex determination system in octopuses. If octopuses employed a ZW-ZZ sex determination system, I would have expected to find substantially more RAD-tags unique to females considering the methods I used. Given that I identified over 250,000 RAD-tags, that octopuses have 30 chromosomes (Destanović 2023), and that more than 13 million reads mapped to those RAD-tags, I

would have expected to find hundreds to thousands of RAD-tags consistently present in one sex and absent in the other if *O. rubescens* employed a ZZ-ZW or XX-XY sex determination system while searching for unique RAD-tag read counts. Also, searching for differences in RAD-tag read counts should have identified more than one RAD-tag that was present only in females, if a distinct sex chromosome was present. Regardless, the statistically significant RAD-tag I found that was present in most of my females was linked to an E3 ubiquitin-protein ligase XIAP and several uncharacterized non-coding RNAs (Appendix IV). Ubiquitin is a small protein found in all cells that is essential for protein degradation (Hershko 1998), and is likely not a sex determining gene.

I selected these methods with the hypothesis that octopuses would have had a chromosomal system similar to other mollusks, such as XX-XY chromosomal system, as described earlier. Using ddRAD-seq is an incredibly cost effective method as it is a reduced representation sequencing, meaning not every sequence is analyzed.

Likely Octopus Chromosomal System

Coffing et al. 2025 suggested that octopuses have a ZO-ZZ chromosomal system. They used the California two-spot octopus (*O. bimaculoides*), the species from which the first cephalopod genome was sequenced (Albertin 2015) and one of the the first to be placed into scaffolds (Albertin 2022). A “scaffold” is a collection of contigs that are on the same chromosome, and their order is known, but they are separated by an unknown gap in length.

Coffing et al. 2025 used PacBio’s long high-fidelity (HiFi) sequencing and chromosomal conformation capture (Hi-C) to construct their own chromosomal-scale scaffolds of a female *O. bimaculoides*, revealing a genome assembly size of 2.3Gb and 30

chromosomal scaffolds. Coverage of Illumina short read data and the mapping of Hi-C contacts in the female octopus showed that chromosome 17 had reduced representation in the sequencing compared to other chromosomes. In contrast, this reduction was not observed in male octopuses, indicating there was only a single copy of chromosome 17 present in female octopuses, making it a putative Z chromosome. Coffing et al. 2025 also located a comparable Z chromosome in other octopuses (*Octopus sinensis*, *Octopus minor*, and *Hapalochlaena maculosa*), and within other cephalopod species (*Sepia esculenta*, *Euprymna scolopes*, *Architeuthis dux,* and *Nautilus pompilius*). Considering that the last common ancestor for *Nautilus* and coleoids was ~482 million years ago, it is likely that this Z chromosome is the oldest known sex chromosome (Coffing 2025). This high level of conservation of the Z chromosome means it is likely that *O. rubescens* has the same sex determining system as *O. bimaculoides*.

A primary reason my data did not indicate the same conclusion is that I did not sequence an entire genome, but instead did ddRAD-seq. ddRAD-seq is sufficient to find sequence differences between populations, but not differences in chromosomal copy number, which appears to be the factor causing sex determination in octopuses. If there were any differences in the sequences between males and females, such as is the case in XX-XY or ZZ-ZW systems, I likely would have found it. I also did not initially assume that octopuses could have a ZO system since it is rare compared to other chromosomal sex determination systems and since most published literature regarding sex determination in mollusks noted heterogametic chromosomes. According to the Tree of Sex (last updated May 27th 2025; Bachtrog 2025), out of 11,915 invertebrates ZZ-ZW chromosomal system is present in 37 animals (0.31%), the ZO chromosomal system is

present in 121 animals (1.02%), the XX-XY system is present in 5,038 animals (42.28%), and the XO system is present in 2,062 animals (17.31%), with the rest being “other”. Coffing (2025) did mention that a small W chromosome might exist, but it could not be identified with current sequencing data due to its possible small size and/or inability to be scaffolded. However, my significant RAD-tag (Figure 3) maps to chromosome 2 of the *Octopus bimaculoides* genome, so even if a small W chromosome does exist, I did not find it here.

The reasoning behind choosing ddRAD-seq was due to my limited funds, and ddRAD-seq seemed like a promising choice as most animals use sex determination systems that employ two distinct sex chromosomes. I expected that octopuses would likely employ the same due to our current understanding of mollusk sex determination, most of which have an XX-XY chromosomal system (Zhang 2014; Avise 2004; Allen 1986; Guo and Allen 1994).

Fortunately, due to my findings regarding the large amount of RAD-tags sequences found in mostly females, I was correct in my assumption that males are ZZ, but I was not correct in my assumption of females being ZW, as it is likely they are ZO due to missing one of their chromosome pairs.

Even though my RAD-seq data did not unravel the sex determination methods in *Octopus rubescens*, they can be useful for other projects, such as finding variance between the sex chromosomes for individual octopuses, as I have 28 individual octopuses to compare. Future research could also use my data to compare whether phenotypic sex differs from genotypic sex in any way and figure out where in the genome this is occurring.

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LITERATURE CITED

Albertin, Caroline B. et al. "Genome and Transcriptome Mechanisms Driving Cephalopod Evolution." *Nature Communications*, vol. 13, 2022, doi:10.1038/s41467-022-29748-w.

Albertin, Caroline B. et al. "The Octopus Genome and the Evolution of Cephalopod Neural and Morphological Novelties." *Nature*, vol. 524, 2015, pp. 220-24, doi:10.1038/nature14668.

Allen, Standish K. et al. "Abnormal Gametogenesis and Sex Ratio in Triploid Soft-Shell Clams (*Mya Arenaria*)." *Biological Bulletin*, vol. 170, 1986, pp. 198-210, doi:10.2307/1541803.

Avise, John C. et al. "Genetic Sex Determination, Gender Identification and Pseudohermaphroditism in the Knobbed Whelk, *Busycon Carica* (Mollusca: Melongenidae)." *The Royal Society*, vol. 271, no. 1539, 2004, doi:10.1098/rspb.2003.2533.

Bachtrog, Doris et al. "Are All Sex Chromosomes Created Equal?" *Trends in Genetics*, vol. 27, no. 9, 2011, pp. 350-57, doi:10.1016/j.tig.2011.05.005.

Bachtrog, Doris et al. “Tree of Sex” Online Database, 2014, last Accessed 05/27/2025, URL: https://www.treeofsex.org/.

Bauer, Raymond T. "Same Sexual System but Variable Sociobiology: Evolution of Protandric Simultaneous Hermaphroditism in *Lysmata* shrimps." *Integrative and Comparative Biology*, vol. 46, no. 4, 2006, pp. 430-38, doi:10.1093/icb/icj036.

Bolger, Anthony M. et al. "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data." *Bioinformatics*, vol. 30, no. 15, 2014, pp. 2114-20, doi:10.1093/bioinformatics/btu170.

Bull, J. J. "Sex Determination in Reptiles." *The Quarterly Review of Biology,* vol. 55, 1980, doi:10.1086/411613.

Buston, Peter. "Does the Presence of Non-Breeders Enhance the Fitness of Breeders? An Experimental Analysis on the Clown Anemonefish *Amphiprion Percula*." *Behavioral Ecology and Sociobiology*, vol. 57, 2004, pp. 23-31, doi:10.1007/s00265-004-0833-2.

Chen, Zhen-Lin et al. "A High-Speed Seach Engine Plink2 with Systematic Evaluation for Proteosome-Scale Identification of Cross-Linked Peptides." *Nature Communications*, vol. 10, 2019, doi:doi.org/10.1038/s41467-019-11337-z.

Coffing, Gabrielle C. et al. "Cephalopod Sex Determination and Its Ancient Evolutionary Origin." *Current Biology*, vol. 35, no. 4, 2025, doi:10.1016/j.cub.2025.01.005.

Collin, Rachel. "Phylogenetic Patterns and Phenotypic Plasticity of Molluscan Sexual Systems." *Integrative and Comparative Biology*, vol. 53, no. 4, 2013, pp. 723-35, doi:10.1093/icb/ict076.

Culler, Monica Elise. "Temperature and Ocean Acidification Effects on the Immune System of *Octopus Rubescens*." Biology, Master of Science, Walla Walla University, 2019.

Culler-Juarez, Monica E. and Kirt L. Onthank. "Elevated Immune Response in *Octopus Rubescens* under Ocean Acidification and Warming Conditions." *Marine Biology*, vol. 168, no. 137, 2021, doi:10.1007/s00227-021-03913-z.

Danecek, Petr et al. "Twelve Years of Samtools and Bcftools." *GigaScience*, vol. 10, no. 2, 2021, doi:10.1093/gigascience/giab008.

de Mitcheson, Yvonne Sadovy and Min Liu. "Functional Hermaphroditism in Teleosts." *Fish and Fisheries*, vol. 9, no. 1, 2008, pp. 1-43, doi:10.1111/j.1467-2979.2007.00266.x.

Destanović, Dalila et al. "A Chromosome-Level Reference Genome for the Common Octopus, *Octopus Vulgaris* (Cuvier, 1797)." *G3 Genes|Genomes|Genetics*, vol. 13, no. 12, 2023, doi:10.1093/g3journal/jkad220.

Feron, Romain et al. "Radsex: A Computational Workflow to Study Sex Determination Using Restriction Site-Associated Dna Sequencing Data." *Molecular Ecology Resources*, 2021, pp. 1-17, doi:10.1111/1755-0998.13360.

Fraser, James A. and Joseph Heitman. "Chromosomal Sex-Determining Regions in Animals, Plants, and Fungi." *Current Opinion in Genetics & Development*, vol. 15, no. 6, 2005, pp. 645-51, doi:10.1016/j.gde.2005.09.002

García-Fernández, Pablo et al. "Epigenetic Dna Methylation Mediating *Octopus Vulgaris* early Development: Effect of Essential Fatty Acids Enriched Diet." *Frontiers*, vol. 8, 2017, doi:10.3389/fphys.2017.00292.

Gaspar, John M. "Ngmerge: Merging Paired-End Reads Via Novel Empirically-Derived Models of Sequencing Errors." *SpringerNature Link*, vol. 19, 2018, doi:10.1186/s12859-018-2579-2.

Gilbert, Scott F. "Chromosomal Sex Determination in *Drosophila*." *Developmental Biology*, 6th edition ed., 2000, https://www.ncbi.nlm.nih.gov/books/NBK10025/.

Goth, Ann and David T. Booth. "Temperature-Dependent Sex Ratio in a Bird." *Biology Letters*, vol. 1, no. 1, 2005, pp. 31-33, doi:10.1098/rsbl.2004.0247.

Graves Marshall, Jennifer A. "Weird Animal Genomes and the Evolution of Vertebrate Sex and Sex Chromosomes." *Annual Review of Genetics*, vol. 42, 2008, pp. 565-86, doi:10.1146/annurev.genet.42.110807.091714.

Guo, X. and S. K. Allen. "Sex Determination and Polyploid Gigantism in the Dwarf Surfclam (*Mulina Lateralis*)." *Genetics*, vol. 138, no. 4, 1994, pp. 1199-206, doi:10.1093/genetics/138.4.1199.

Hake, Laura and Clare O'Connor. "Genetic Mechanisms of Sex Determination." *Nature Education*, vol. 1, no. 1, 2008.

Henshaw, Jonathan M. "Protandrous Hermaphroditism." *Encyclopedia of Animal Cognition and Behavior*, 2017, pp. 1-6, doi:10.1007/978-3-319-47829-6_1972-1.

Hershko, Avram and Aaron Ciechanover. "The Ubiquitin System." *Annual Review of Biochemistry*, vol. 67, 1998, pp. 425-79, doi:10.1146/annurev.biochem.67.1.425.

Kiuchi, Takashi et al. "A Single Female-Specific Pirna Is the Primary Determiner of Sex in the Silkworm." *Nature*, vol. 509, 2014, pp. 633-36, doi:10.1038/nature13315.

Li, Heng. "Aligning Sequence Reads, Clone Sequences and Assembly Contigs with Bwa-Mem." *Cornell University*, 2013, doi:10.48550/arXiv.1303.3997.

Li (a), Heng and Richard Durbin. "Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform." *Bioinformatics*, vol. 25, no. 14, 2009, pp. 1754-60, doi:10.1093/bioinformatics/btp324.

Li (b), Heng et al. "The Sequence Alignment/Map Format and Samtools." *Bioinformatics*, vol. 25, no. 16, 2009, pp. 2078-79, doi:10.1093/bioinformatics/btp352.

Li, Weizhong and Adam Godzik. "Cd-Hit: A Fast Program for Clustering and Comparing Large Sets of Protein or Nucleotide Sequences." *Bioinformatics*, vol. 22, no. 13, 2006, pp. 1658-59, doi:10.1093/bioinformatics/btl158.

Love, Michael I. et al. "Moderated Estimation of Fold Change and Dispersion for Rna-Seq Data with Deseq2." *bioRxiv*, 2014, doi:10.1101/002832.

Maldonado, L.C. Torres et al. "Expression Profiles of Dax1, Dmrt1, and Sox9 during Temperature Sex Determination in Gonads of the Sea Turtle *Lepidochelys*

Olivacea." *General and Comparative Endocrinology*, vol. 129, no. 1, 2002, doi:10.1016/S0016-6480(02)00511-7.

Matri, Heini M. et al. "The Evolution of Sex Determination Associated with a Chromosomal Inversion." *Nature Communications*, vol. 10, no. 145, 2019, doi:10.1038/s41467-018-08014-y.

Matson, Clinton K. and David Zarkower. "Sex and the Singular Dm Domain: Insights into Sexual Regulation, Evolution and Plasticity." *Nature*, vol. 13, no. 3, 2012, pp. 163-74, doi:10.1038/nrg3161.

Munday, Philip L. et al. "Bi-Directional Sex Change in a Coral-Dwelling Goby." *Behavioral Ecology and Sociobiology*, vol. 43, 1998, pp. 371-77.

Nakamura, Hiroshi K. "Chromosomes of Archaeogastropoda (Mollusca: Prosobranchia), with Some Remarks on Their Cytotaxonomy and Phylogeny." *Publications of the Seto Marine Biological Laboratory*, vol. 31, 1986, pp. 191-267.

Ospina-Alvarez, Natalia and Francesc Piferrer. "Temperature-Dependent Sex Determination in Fish Revisited: Prevalence, a Single Sex Ratio Response Pattern, and Possible Effects of Climate Change." *PLoS One*, vol. 3, no. 7, 2008.

Pauly, Daniel. “Darwin's Fishes: An Encyclopedia of Ichthyology, Ecology, and Evolution.” *Cambridge University Press*, 2007.

Pekar, Katherine. "What Do Deep-Sea Octopuses Eat in Shallow Waters? Dietary Analysis of *Muusoctopus Leioderma* using Stable Isotopes and Fecal Analysis." Biology, Walla Walla University, 2019, p. 78.

Peterson, Brant K. et al. "Double Digest Radseq: An Inexpensive Method for De Novo Snp Discovery and Genotyping in Model and Non-Model Species." *PLOS One*, 2012, doi:10.1371/journal.pone.0037135.

Rigby, P. Robin and Sakurai Yasunori. "Multidimensional Tracking of Giant Pacific Octopuses in Northern Japan Reveals Unexpected Foraging Behaviour." *Marine Technology Society Journal*, vol. 39, 2005, pp. 64-67, doi:10.4031/002533205787521730.

Robson, G. C. "On the Hectocotylus of the Cephalopoda - a Reconsideration." *Journal of Molluscan Studies*, vol. 17, no. 2-3, 1926, pp. 117-22, doi:10.1093/oxfordjournals.mollus.a063896.

Sharat Chandra, H. "Another Way of Looking at the Enigma of Sex Determination in *Ellobius Lutescens*." *Indian Academy of Sciences*, vol. 76, no. 8, 1999, pp. 1072-73.

Smith, Craig A. et al. "The Avian Z-Linked Gene *Dmrt1* is Required for Male Sex Determination in the Chicken." *Nature*, vol. 461, 2009, pp. 267-71.

Sonner, Sofia Camille. "Metabolic Cost of *Octopus Rubescens* Chromatophore System." Biology, Walla Walla University, 2021, p. 172.

Stevens, Nettie Maria. “Studies in Spermatogenesis Part I”. 1905.

Todd, E.V. et al. "Bending Genders: The Biology of Natural Sex Change in Fish." *Sexual Development*, vol. 10, 2016, pp. 223-41, doi:10.1159/000449297.

Todd, Erica V. et al. "Stress, Novel Sex Genes, and Epigenetic Reprogramming Orchestrate Socially Controlled Sex Change." *Organismal Biology*, vol. 5, no. 7, 2019, doi:10.1126/sciadv.aaw7006.

Traut, W. et al. "Sex Chromosomes and Sex Determination in Lepidoptera." *Sex Dev*, vol. 1, 2007, pp. 332-46, doi:10.1159/000111765.

Van Heukelem, William Fred. "Growth, Bioenergetics and Life-Span of *Octopus Cyanea* and *Octopus Maya*." Zoology, University of Hawaii, 1976.

Warner, R.R. "The Adaptive Significance of Sequential Hermaphroditism in Animals." *The American Naturalist*, 1975, doi:10.1086/282974.

Watts, Philip C. et al. "Parthenogenesis in Komodo Dragons." *Nature*, vol. 444, 2006, pp. 1021-22, doi:10.1038/4441021a.

Wilgenburg, Ellen van et al. "Single Locus Complementary Sex Determination in Hymenoptera: An "Unintelligent" Design?" *Frontiers in Zoology*, vol. 3, no. 1, 2006.

Wright, Ricky Orion. "Characterizing a-to-I Rna Edits in Nervous Tissue of *Octopus Rubescens* in Response to Ocean Acidification." Biology, Walla Walla University, 2024, p. 57.

Yusa, Yoichi. "Causes of Variation in Sex Ratio and Modes of Sex Determination in the Mollusca - an Overview." *American Malacological Bulletin*, vol. 23, no. 1, 2007, pp. 89-98, doi:10.4003/0740-2783-23.1.89.

Zhang, Na et al. "Genomic Analysis of the Pacific Oyster (*Crassostrea Gigas*) Reveals Possible Conservation of Vertebrate Sex Determination in a Mollusc." *Genes, Genomes, Genetics*, vol. 4, no. 11, 2014, pp. 2207-17, doi:10.1534/g3.114.013904.

TABLES AND FIGURES

Table 1. Number of raw paired sequences, merged sequences, trimmed sequences, and reads mapped to the reference file for each octopus.

Octopus	Octopus phenotypic sex	Raw paired sequences	Merged sequences	Trimmed sequences	Reads mapped
01	Male	675,079	581,191	575,351	566,020
02	Female	845,175	725,596	718,861	706,885
03	Female	284,054	242,029	239,473	234,903
04	Male	485,156	414,487	409,915	399,873
05	Male	854,136	734,584	726,428	714,645
06	Female	296,487	254,305	251,589	246,831
07	Male	874,173	750,407	743,645	743,630
08	Male	701,639	607,370	602,664	592,279
09	Male	754,005	645,542	639,540	627,435
10	Male	514,444	438,944	434,402	424,654
11	Male	684,540	588,943	584,416	573,798
12	Female	612,301	524,534	519,087	509,036
13	Female	677,394	550,371	543,831	534,964
14	Female	340,764	279,877	276,289	271,347
15	Male	706,692	582,626	576,467	566,189
16	Male	349,318	289,497	286,530	279,777
17	Male	379,894	312,629	309,145	303,233
18	Female	415,233	345,369	341,499	334,749
19	Male	816,669	697,937	690,324	673,566
20	Male	238,429	188,361	185,674	181,096
21	Male	548,715	435,528	429,870	420,574
22	Male	255,548	206,777	204,092	200,034
23	Female	709,668	589,500	581,885	562,131
24	Male	786,923	672,908	655,578	649,239
25	Female	1,537,165	1,331,893	1,318,494	1,318,478

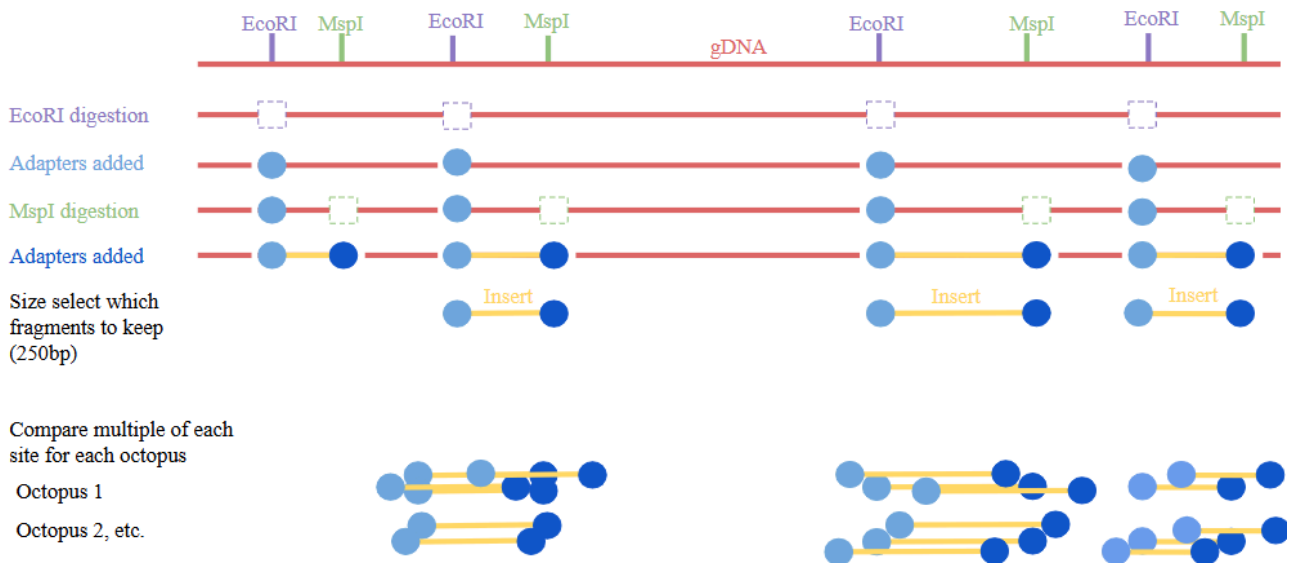
Octopus	Octopus phenotypic sex	Raw paired sequences	Merged sequences	Trimmed sequences	Reads mapped
26	Male	194,209	159,067	156,968	155,034
27	Male	391,220	323,098	318,904	315,182
28	Female	324,271	267,843	264,391	259,568

Table 2. Per octopus statistics collected during variant calling.
This table shows total sequences (after merging and trimming), the reads mapped to the reference file, and the number of loci, SNPs, and indels per octopus.

Octopus number	Octopus phenotypic sex	Total sequences	Reads mapped	Loci count	SNP count	Indel count
01	Male	575,351	547,025	172,928	159,389	13,539
02	Female	718,861	688,387	185,668	170,910	14,758
03	Female	239,473	228,293	120,251	112,186	8,065
04	Male	409,915	388,267	158,091	146,622	11,469
05	Male	726,428	696,001	180,914	166,435	14,479
06	Female	251,589	240,045	122,337	114,097	8,240
07	Male	743,645	743,637	59,714	53,192	6,522
08	Male	602,664	575,843	180,020	166,078	13,942
09	Male	639,540	608,771	190,261	175,395	14,866
10	Male	434,402	412,125	160,569	148,870	11,699
11	Male	584,416	557,491	179,831	165,860	13,971
12	Female	519,087	494,656	170,377	157,136	13,241
13	Female	543,831	521,075	169,007	156,111	12,896
14	Female	276,289	263,888	130,969	122,340	8,629
15	Male	576,467	550,193	177,987	164,584	13,403
16	Male	286,530	271,706	135,171	126,241	8,930
17	Male	309,145	293,445	138,021	128,535	9,486
18	Female	341,499	325,503	144,735	134,619	10,116
19	Male	690,324	645,148	193,216	178,576	14,640
20	Male	185,674	175,534	106,323	100,191	6,132
21	Male	429,870	408,818	159,608	148,065	11,543
22	Male	204,092	193,571	112,565	105,949	6,616
23	Female	581,885	545,127	183,101	170,012	13,089
24	Male	665,578	617,657	182,768	169,047	13,721
25	Female	1,318,494	1,107,383	260,575	239,442	21,133
26	Male	156,968	150,230	96,797	91,216	5,581

Octopus number	Octopus phenotypic sex	Total sequences	Reads mapped	Loci count	SNP count	Indel count
27	Male	318,904	304,699	136,354	126,730	9,624
28	Female	264,391	252,409	127,405	118,861	8,544

a)



b)

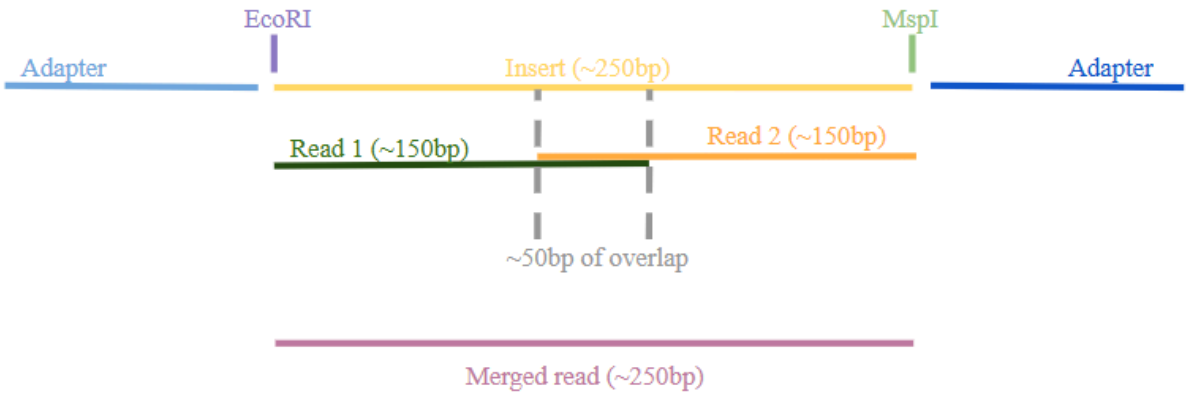


Figure 1. Visualization of process to get RAD-tags from gDNA.
a) Visualization of the ddRAD process. b)Visualization of size selection.

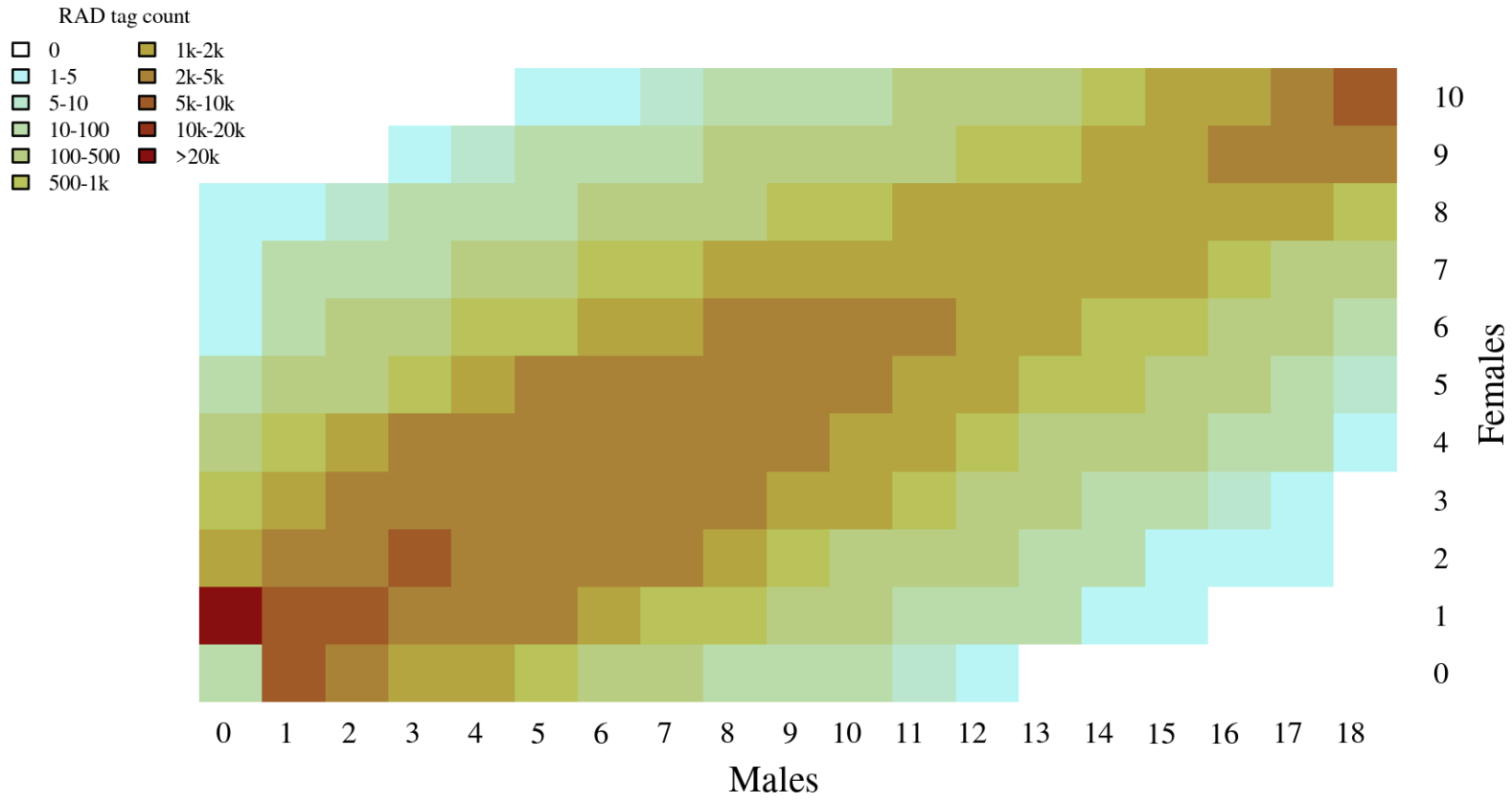


Figure 2. Distribution of RAD-tags between the male and female octopuses.

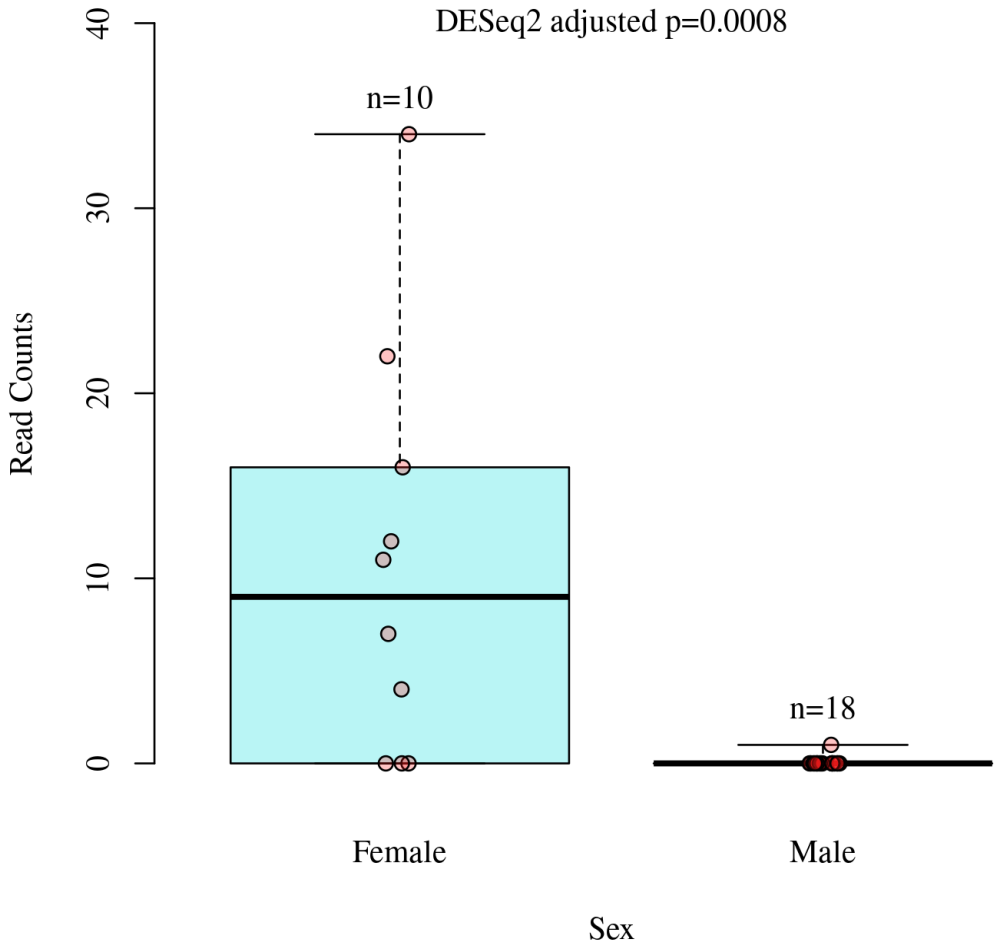


Figure 3. Read counts of significant sex associated RAD-tag identified using DESeq2 (Wald test, Benjamini-Hochberg adjusted p-value = $8.11968 \times 10^{-4)}$.

APPENDIX I: Significantly Differentially Expressed RAD-tags

>LH00150:205:22F3JTLT3:3:1107:16626:17503

AATTCTTCTTTCGCCTCAAGGAAAACCGTTTAATCAAAATAAAGCATTAGAATT
GTCTAAAGAAGATGAAATAACATTTATTTCAGGACGTTATGAAGGGTTTGATGA
AAGAGTAAGATTTTTGGTTGATGAAGAACTCTCAATAGGTGATTATGTATTAAC
TGGAGGTGAATTACCATCAATGGTAATAGCGGATTCAATTATTAGATTAATACCC
G

>LH00150:205:22F3JTLT3:3:1183:40148:9972

AATTCAAGACTGGTGAGAAATGAAAGCAGTATGCTCCATTGGATGTGCAACTC
AAGTGCACACATAGACCAAAGTATGAGTGCACTGAGAGAAACGTTAAGCATA
AGAGGAATCGAATGTAGCATACAAGAGAGAAGACTACGGTGGTTTGGAAATG
TGAGGCGTATGAATGAAGATAGTTGCATAAAGAAGTGCCGATTACTGAGGGTG
GAAGGTACCCG

APPENDIX II: *Deseq2* results for the two significant RAD-tags.
The p-value was found by conducting a Wald test, which is the program default.

RAD-tags	log2Fold Change	lfcSE	stat	pvalue	padj
LH00150:205:22F3JTLT3:3:1107:16626:17503	22.50999	3.079892	7.3069	2.69751E-13	6.82869E-08
LH00150:205:22F3JTLT3:3:1107:16626:17504	-5.66112	0.975119	-5.80556	6.41497E-09	8.11968E-04

APPENDIX III: Top 10 BLAST results of RAD-tag LH00150:205:22F3JTLT3:3:1107:16626:17503.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
Mycoplasmopsis adleri strain G145 chromosome, complete genome	*Mycoplasmopsis adleri*	206	206	91%	0	82.91	1130687	CP174162.1
Mesomycoplasma moatsii strain NCTC10158 chromosome, complete genome	*Mesomycoplasma moatsii*	187	187	99%	0	79.17	737131	CP146987.1
Mesomycoplasma moatsii strain MK 405 chromosome, complete genome	*Mesomycoplasma moatsii*	187	187	99%	0	79.17	737137	CP174166.1
Mycoplasma mobile 163K complete genome	*Mycoplasma mobile* 163K	183	183	99%	0	78.7	777079	AE017308.1
Mycoplasmopsis meleagridis strain W24_02583 chromosome	*Mycoplasmopsis meleagridis*	178	178	95%	0	78.85	639577	CP182213.1
Mycoplasma anserisalpingitidis strain ATCC:BAA-2147 chromosome, complete genome	*Mycoplasma anserisalpingitidis*	178	178	99%	0	78.8	959110	CP042295.1
Mycoplasma meleagridis strain NCTC10153	*Mycoplasmopsis meleagridis*	178	178	95%	0	78.85	644173	LR215042.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
genome assembly, chromosome: 1								
Mycoplasmopsis anatis strain NCTC 10156 chromosome, complete genome	*Mycoplasmopsis anatis*	177	177	100%	0	78.54	956094	CP030141.1
Mycoplasma anatis strain NCTC10156 genome assembly, chromosome: 1	*Mycoplasmopsis anatis*	177	177	100%	0	78.54	978241	LR215035.1
Mycoplasmopsis phocirhinis strain 852 chromosome, complete genome	*Mycoplasmopsis phocirhinis*	175	175	68%	0	85.91	865472	CP034841.1

APPENDIX IV: Top 10 BLAST results of RAD-tag LH00150:205:22F3JTLT3:3:1183:40148:9972.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
PREDICTED: *Octopus bimaculoides* E3 ubiquitin-protein ligase XIAP (LOC106872101), transcript variant X5, mRNA	*Octopus bimaculoides*	154	154	61%	0	85.19	5827	XM_052978443.1
PREDICTED: *Octopus bimaculoides* uncharacterized LOC128249677 (LOC128249677), ncRNA	*Octopus bimaculoides*	147	147	85%	0	77.54	7757	XR_008265789.1
PREDICTED: *Octopus bimaculoides* uncharacterized LOC106871636 (LOC106871636), ncRNA	*Octopus bimaculoides*	124	124	96%	0	74.42	10821	XR_001409681.2
PREDICTED: *Octopus sinensis* uncharacterized LOC118768469 (LOC118768469), ncRNA	*Octopus sinensis*	115	115	50%	0	82.88	160	XR_005004326.1
PREDICTED: *Octopus bimaculoides* uncharacterized LOC128249071 (LOC128249071), transcript variant X2, ncRNA	*Octopus bimaculoides*	96	96	82%	0	73.22	11079	XR_008265172.1
PREDICTED: *Octopus sinensis* uncharacterized LOC118765574	*Octopus sinensis*	95.1	95.1	57%	0	76.38	12203	XR_005001432.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
(LOC118765574), transcript variant X1, ncRNA								
PREDICTED: *Octopus bimaculoides* AN1-type zinc finger protein 3-like (LOC106880241), transcript variant X6, misc_RNA	*Octopus bimaculoides*	89.7	89.7	92%	0	69.61	9896	XR_008265179.1
PREDICTED: *Octopus bimaculoides* AN1-type zinc finger protein 3-like (LOC106880241), transcript variant X3, misc_RNA	*Octopus bimaculoides*	89.7	89.7	92%	0	69.61	11088	XR_008265176.1
PREDICTED: *Octopus bimaculoides* AN1-type zinc finger protein 3-like (LOC106880241), transcript variant X5, misc_RNA	*Octopus bimaculoides*	89.7	89.7	92%	0	69.61	10098	XR_008265178.1
PREDICTED: *Octopus bimaculoides* AN1-type zinc finger protein 3-like (LOC106880241), transcript variant X2, misc_RNA	*Octopus bimaculoides*	89.7	89.7	92%	0	69.61	12555	XR_008265175.1