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Determining the reproductive patterns of the Titan Acorn Barnacle (Megabalanus coccopoma) in its introduced range

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Determining the reproductive patterns of the Titan Acorn Barnacle (Megabalanus coccopoma) in its introduced range

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By

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Under the mentorship of *Dr. Scott Harrison*

ABSTRACT

Invasive species are a significant conservation concern given their contribution to native species decline. The barnacle, *Megabalanus coccopoma,* is a common invasive species in tropical and subtropical regions of both the Pacific and Atlantic oceans. Little is known about the life history and ecology of *M. coccopoma,* and data on reproductive biology could provide valuable insight into its propensity to establish introduced populations. Most species of barnacle (including *M. coccopoma*) are hermaphroditic, but self-fertilization is rare in species studied to date. A recent genetic study of introduced *M. coccopoma* populations in the southeastern US showed high levels of genetic variation but more homozygosity than expected. One explanation for this pattern is that self-fertilization may be induced when individuals settle where no potential mates are available. The purpose of this study is to test for self-fertilization and multiple paternity in *M. coccopoma* using highly variable genetic markers. Larvae were collected from the mantle cavity of mature barnacles in clusters and adults isolated from any potential mates. Multi-locus genotypes of larvae were compared with maternal genotypes to detect the presence or absence of non-maternal alleles, and to determine the number of potential sires of a brood. Data revealed that the offspring of both isolated and grouped adults had allelic contributions from at least one father, rejecting self-fertilization as the method of reproduction and providing support for mechanisms such as spermcasting and sperm competition in this population of *M. coccopoma.*

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Introduction

Introduced species are considered one of the main threats to the economic stability and biodiversity of the ecosystem they are introduced to (Bax 2003). The spread of disease, increase in competition for resources, and disruption of native species' niches are all impacts caused by an introduced population that threaten the viability of native species (Roman & Darling 2007). An introduced species is not always harmful to an ecosystem, but is considered invasive if it negatively impacts native species and the environment it is introduced to (Bax 2003). Aquatic and marine invasive species are of particular concern due to their ability to spread rapidly on the hulls of ships traveling overseas, through fisheries activities, or canal and channel disruption by humans. The abundance of an invasive marine species can increase at a remarkable rate due to their ability to reproduce quickly, adapt to various environments, and the potential to disperse rapidly over large areas due to stages in a species' reproductive cycles (Baxter 2013). Efforts to control an introduced species' impacts on its target environment have become crucial to the survival of native species and ultimately the ecosystem they inhabit.

The titan acorn barnacle, *Megabalanus coccopoma,* is native to the tropical eastern Pacific Ocean and ranges from Mexico to Peru. This species of barnacle has also established introduced populations in the western Atlantic colonizing inshore and offshore areas from Brazil to the Carolinas of the United States, as well as northwestern European waters and the western Indian Ocean (Kerckhof 2010). In 2006, *M. coccopoma* was recorded for the first time in the southeastern United Sates in St. Augustine, FL, Brunswick, GA, and Charleston, SC. (Tibbetts 2007; Gilg *et al*. 2010; Spinuzzi *et al*. 2013). It has become an increasing concern that the quick reproductive cycle of this introduced barnacle will threaten and eventually outcompete native invertebrates and

planktonic species of the southeastern United States coastline. The lack of information available about the reproductive biology of *M. coccopoma* creates issues for population biologists who aim to address the problems that invasive species cause for native populations. Therefore, monitoring the reproduction patterns and increasing widespread occurrence of this barnacle is important to maintain the integrity of the eastern seaboard ecosystem (Reigel *et al*. 2015).

Megabalanus coccopoma is a large, filter-feeding barnacle that reaches up to 5 cm in height and width, has a characteristic pink, purple, and white coloration on its plates, and is found regularly on boats, buoys, ships, and other various man-made structures present in the ocean (Foffonoff 2003). As is characteristic of most barnacles, *M. coccopoma* are simultaneous hermaphrodites. Little is known about the reproductive biology of *M. coccopoma*, but the few studies of balanoid barnacle mating behaviors that exist suggest that self-fertilization rarely occurs and outcrossing is common (Barnes and Crisp 1956; Kelly *et al.* 2012). A recent population genetic study found that the *M. coccopoma* populations collected off of the Georgia coast had very high allelic diversity but exemplified high deviation from Hardy-Weinberg equilibrium due to lower than expected number of heterozygotes (Reigel 2015). One possible explanation for this genetic pattern is high amounts of inbreeding through selfing. There is limited research available on the reproductive biology of *M. coccopoma*, and especially the invasive populations that have quickly colonized the southeastern United States in the past nine years (Masterson, 2007). The purpose of this study is to use genetic data to determine if self-fertilization and/or multiple paternity occurs in introduced populations of *M. coccopoma.*

Methods

In September 2016, 11 adults were collected from the introduced *Megabalanus coccopoma* population found on the rock jetties on Tybee Island, GA (32.024 N, 80.842 W). The specimens collected included both isolated and clustered adult barnacles in order to determine whether proximity to potential mates affected the reproduction method utilized. The mode of reproduction and nature of paternity in an introduced *M. coccopoma* population was accomplished through the genetic analysis of adults and their respective offspring broods. In order to extract the DNA from the adult barnacle specimens, I cracked open the shell and removed the organism, inspected for the presence of larvae, and then preserved the adult in individual containers in 90% ethanol until DNA extraction was performed. The larvae extracted from the adult specimens were placed in individual containers in 20µL of lysis buffer(10mM Tris pH 8.3, 50mM KCl and 0.5% Tween 20 and 200 µg/ml proteinase k). Larval DNA was extracted by incubating individual larva in lysis buffer at 65^{o}C for one hour followed by 100^{o}C for 15 minutes. DNA was extracted from adults using the DNeasy Blood and Tissue Kit (QIAGEN) following manufacturer protocol.

Polymerase chain reaction (PCR) was performed using primers for the seven genetic markers that are known to be highly variable in this barnacle species (Reigel 2015; Reigel *et al.* 2015). The markers used were microsatellite markers MERC13, MERC15, MERC24, MERC26, MERC27, and MERC29 (Reigel *et al.* 2015). The PCR reactions were performed for each sample in a total volume of 20µL and included 10µL of Apex Taq Master Mix (Genessee Scientific), 6.5µL deionized water, 0.125µM

fluorescently-labeled forward primer, 0.25μM reverse primer, and 2 μL of DNA. The Thermocycling protocol was as follows: 95ºC for 5 minutes; 38 cycles of 95ºC for 10 s, 60 ºC for 15 s and 72 ºC for 20 s; and 72ºC for 5 min. PCR products were analyzed using an ABI 3500 Genetic Analyzer. Alleles were sized at each locus in relation to an internal size standard using GeneMapper 3.0 software, revealing the alleles present in the adult and offspring genotypes.

Four barnacles were collected living attached in a group, and were labeled specimens N, O, P, and Q. The genotypes of the larvae broods of both adults N and Q were examined at 6 microsatellite loci, and compared to the genotypes of their respective mother. If the genotype of each offspring from a brood only had alleles at each locus that are identical to the alleles present in the mother's genotype, then self-fertilization is concluded to be the method of fertilization. If any of the offspring have non-maternal alleles in their genotype, selfing can be excluded. If the individuals in a brood are a product of cross-fertilization, then each locus will have a maternal and non-maternal allele. A method of determining paternity is through comparing the non-maternal alleles in the genotypes of the offspring against the genotypes of the other adults in the group of four. If an adult in that group of four has a genotype that matches the non-maternal alleles for every locus in the offspring brood, then it may be the sire of the brood. If an adult doesn't have the alleles that are considered non-maternal in the brood, then it can be excluded as a potential father. I calculated the probability of each adult genotype using allele frequencies from a study on the genetics of this introduced population of *Megabalanus coccopoma* (Reigel 2015). Each locus genotype probability was calculated using Hardy-Weinberg expectations. These probabilities were used to assess our confidence in assignment of paternity, as it would indicate that the probability of another

barnacle having the exact same genotype. Each barnacle's multilocus genotype was assigned a probability of it being present in the population by multiplying all of the individual loci genotype probabilities. This allowed us to determine the overall probability of sampling the individual's genotype. If an adult was a candidate for the sire of the brood because of his genotype, it could be supported by the low calculated frequency of his genotype belonging to a different adult. Multiple paternity was determined by the presence of more than two alleles among larvae in the brood for any loci which would suggest a minimum number of fathers greater than one.

Two adult barnacles collected in isolation of any potential mates (samples B and C) had offspring broods. The offspring were genotyped at five (B) or six (C) microsatellite loci and the resulting allelic composition of the brood was compared to their respective mother. If the brood contained only maternal alleles at the microsatellite loci tested, then the data would indicate self-fertilization as the likely mode of reproduction. The probability of misidentification could then be calculated as described above. If the offspring genotypes contained non-maternal alleles, then outcrossing is supported as the mode of reproduction. A brood's collective number of non-maternal alleles at each locus allows us to calculate the minimum number of fathers to the brood. The number of fathers can be calculated by assuming one father per two alleles; two non-maternal alleles at a locus indicate at least one father, three or four non-maternal would indicate at least two fathers, and so on. The data allows us to construct a possible genotype for the father, which can be compared to adult genotypes in the study.

Results

Adults N, O, P, and Q were in a group and individuals N and Q contained offspring broods. The genotypes of the eight larvae that belonged to adult N had non-maternal alleles at each locus (Table 2), which indicates that self-fertilization was not the method of reproduction. The minimum number of fathers for brood N was 1. Upon comparison against the other adult genotypes present in the grouping, adult O was the only individual that contained the alleles that were found in the offspring brood of N at every locus. The absence of alleles in the genotypes of adults P and Q at 4 of the 5 loci in the offspring brood allowed us to exclude them as sires of the brood. The reconstructed paternal multilocus genotype matched adult O. The calculated probability of O's genotype according to the allele frequencies from the population study by Reigel (2015) is 5.21×10^{-15}, suggesting a low probability of misidentification. The other individual in the group of four that contained offspring was adult Q. The genotypes of adult Q's eight larvae had non-maternal alleles at every locus, which rules out self-fertilization as the method of reproduction (Table 3). The minimum number of fathers for brood Q was 1. The comparison of the entire brood's set of alleles against the genotypes of the other adults in the group revealed that adult N and adult O could be excluded as potential fathers for brood Q. Adult N only shared alleles with the brood at three of the five loci. Adult O only shared alleles with the brood at two of the five loci. The multilocus genotype of adult P matched the paternal genotype reconstructed from larval data. The probability calculated for P's overall genotype for the five markers analyzed was 8.12×10^{-16}, which indicates that it is highly unlikely for there to be a barnacle nearby with an identical genotype to P. This provides strong support to the hypothesis that P is the father of Q's brood.

The broods of isolated adults B and C were genotyped, and compared against their mother's genotype to determine how the isolated individuals were successfully reproducing. The results in Table 3 indicate the presence of non-maternal alleles at each locus. The presence of non-maternal alleles in the genotype of each larva in the brood allows us to exclude selfing as the mode of reproduction for the isolated adults. The total collection of alleles for the six loci from the brood of C contained two non-maternal alleles at four of the loci, and one non-maternal allele at two of the loci. This suggests that the minimum number of fathers was 1 for the brood and that multiple paternity was unlikely. The reconstructed paternal genotype to brood C did not match any adults in our sample.

Adult B and seven of its larvae were analyzed at five microsatellite loci. Self-fertilization was ruled out due to the presence of two non-maternal alleles at four of the loci, and one non-maternal allele at one locus, as illustrated in Table 4. This combination of non-maternal alleles indicates that there is at least one sire to the brood and that multiple paternity was unlikely. The reconstructed paternal genotype to brood B did not match any adults in our sample.

Table 1. Genotypes and calculated frequencies of the 11 *Megabalanus coccopoma* individuals collected and analyzed at six highly variable microsatellite loci.

	Individual										
	B	C	D	E	F	I	M	N	O	P	Q
MC13 Genotype	192	282	212	176	184	238	208	192	246	192	192
	192	282	212	220	278	250	292	388	266	192	320
Genotype Probability	1.13E-03	1.37E-05	4.24E-04	6.34E-03	3.18E-04	3.18E-04	1.37E-03	7.55E-04	1.97E-04	1.13E-03	4.41E-04
MC15 Genotype	293	313	325	325	313	301	295	331	297	293	313
	321	337	325	329	329	321	325	341	325	321	325
Genotype Probability	4.58E-03	4.85E-03	6.74E-03	1.29E-02	8.48E-03	8.84E-03	3.11E-04	1.17E-03	9.19E-03	4.58E-03	4.44E-03
MC24 Genotype	188	188	222	188	192	188	196	188	196	188	188
	196	188	270	188	196	188	200	188	196	196	196
Genotype Probability	1.41E-02	3.70E-03	7.22E-06	3.70E-03	7.98E-02	3.70E-03	4.19E-02	3.70E-03	1.35E-02	1.41E-02	7.05E-03
MC26 Genotype	235	231	259	235	217	235	243	221	209	235	221
	239	255	263	271	243	267	279	243	263	239	263
Genotype Probability	3.87E-03	3.90E-03	1.05E-02	2.58E-03	3.29E-03	.004348	3.86E-03	1.93E-03	3.17E-04	3.87E-03	1.64E-03
MC27 Genotype	291	269	266	301	273	259	329	301	265	291	335
	309	269	298	321	277	285	329	317	301	309	377
Genotype Probability	3.38E-04	3.59E-03	7.22E-06	6.75E-04	3.75E-02	2.35E-04	6.86E-04	4.50E-04	6.75E-04	3.38E-04	3.61E-06
MC29 Genotype	184	160	176	180	-	-	-	-	-	184	172
	212	196	184	220	-	-	-	-	-	212	192
Genotype Probability	8.47E-03	1.14E-03	1.81E-02	3.18E-03	-	-	-	-	-	8.47E-03	2.28E-03
Overall Genotype Probability	**8.12E-16**	**3.92E-18**	**2.85E-20**	**1.67E-16**	**2.65E-11**	**1.06E-14**	**4.74E-14**	**2.84E-15**	**5.21E-15**	**8.12E-16**	**1.87E-19**

Table 2. The genotypes of adult N. Eight larvae and the constructed paternal genotype. Potential paternity was assigned by comparing non-maternal alleles in brood genotype with alleles belonging to other adults in the grouping of four.

Individual	MC-13	MC-15	MC-24	MC-26	MC-27
N adult	**192** **388**	**331** **341**	**188** **188**	**221** **243**	**301** **317**
NL01	192 246	297 331	188 196	209 221	301 301
NL02	192 266	297 331	188 196	209 243	301 301
NL03	266 388	325 341	188 196	221 263	265 317
NL04	192 266	325 331	188 196	243 263	265 301
NL05	246 388	325 341	188 196	209 243	265 317
NL06	266 388	297 331	188 196	243 263	301 301
NL07	192 246	297 331	188 196	209 243	265 301
NL08	192 246	325 341	188 196	243 263	265 317
Alleles in Brood	192, 388 246, 266	331, 297 325, 341	188, 196	221, 243, 209, 263	301, 317, 265
Non-maternal Alleles in Brood	246, 266	297, 325	196, 196	209, 263	265, 265
Paternity Match	O	O	O	O	O

Table 3. The genotypes of adult Q's eight larvae and the constructed paternal genotype. Potential paternity was assigned by comparing non-maternal alleles in brood genotype with alleles belonging to other adults in the grouping of four.

Individual	MC-13	MC-15	MC-24	MC-26	MC-27
Q adult	**192**	**313**	**188**	**221**	**335**
	320	**325**	**196**	**263**	**377**
QL01	192	313	188	239	273
	226	325	196	263	377
QL02	192	297	188	221	273
	320	325	196	307	335
QL03	192	297	188	221	273
	226	313	188	239	377
QL04	192	313	188	263	273
	192	325	188	307	377
QL05	192	297	188	221	273
	320	325	188	307	335
QL06	192	297	188	221	273
	192	325	196	307	335
QL07	192	325	188	239	273
	320	325	188	263	335
QL08	192	313	188	221	273
	192	325	188	307	377
Alleles in Brood	192, 226,320	313, 325, 297	188, 196	221, 263 239, 307	335, 377, 273
Non-maternal Alleles in Brood	226	297	188	239, 307	273
Paternity Match	P	P	P	P	P

Table 4. Isolated individuals and offspring genotypes at highly-variable microsatellite loci. Non-maternal alleles present indicate the paternal contribution to the brood genotype.

Individual	MC-13	MC-15	MC-24	MC-26	MC-27	MC-29
C adult	**282**	**313**	**188**	**231**	**269**	**160**
	282	**337**	**188**	**255**	**269**	**196**
CL01	242	301	188	255	269	196
	282	337	192	255	293	236
CL04	242	319	188	229	269	160
	282	337	192	231	293	164
CL05	246	301	188	229	269	196
	282	337	192	255	293	236
CL06	246	313	188	255	269	164
	282	319	196	255	293	196
Alleles in brood	282, 242, 246	313, 337, 301, 319	188, 192, 196	231, 255, 229	269, 293	160, 196, 236, 164
Non-maternal alleles in brood	242, 246	301, 319	192, 196	229	293	236, 164
B adult	**192**	**293**	**188**	**235**	**291**	**184**
	192	**321**	**196**	**239**	**309**	**212**
BL01	-	309	188	239	301	194
	-	321	204	267	309	212
BL02	-	293	196	239	277	184
	-	309	204	295	309	194
BL03	-	293	188	239	277	194
	-	293	212	267	291	212
BL04	-	293	196	235	301	194
	-	321	212	295	309	212
BL05	-	293	188	235	277	194
	-	321	204	267	291	212
BL06	-	293	196	235	291	184
	-	321	212	267	301	194
BL07	-	309	188	239	291	180
	-	321	212	295	301	184
Alleles in brood	-	293, 321, 309	188, 196, 204, 212	235, 239, 267, 295	291, 309, 301, 277	184, 212, 194, 180
Non-maternal alleles in brood	-	309	204, 212	267, 295	301, 277	194, 180

Discussion

Comparative analysis of the genotypes of individuals and their offspring gave insight into the reproductive behavior of *Megabalanus coccopoma* in its introduced range. Microsatellite loci genotypes revealed that both the broods from clustered barnacles and isolated barnacles were a product of cross-fertilization, and there was no evidence for self-fertilization. Genetic analysis of one isolated adult and her offspring showed that her brood had four markers with two non-maternal alleles, and two markers with one non-maternal allele. The second isolated barnacle had four markers with two non-maternal alleles, and one marker with one non-maternal allele. The presence of alleles that are not found in the mother's genotype not only provides evidence against self-fertilization, but supports that cross-fertilization in isolated barnacles was likely with a single father given heterozygosity estimates in this population.

The presence of non-maternal alleles in the broods of isolated barnacles challenges common hypotheses regarding barnacle reproduction: internal fertilization through direct copulation or self-fertilization. Although hermaphrodism is the rule in barnacles, very few species are known to self-fertilize (Barnes and Crisp 1956, Furman and Yule 1990, Dasai *et al*. 2006). *Balanus amphitrite* and *B. improvisus* have been reported as balanoid species that can be induced to self-fertilize, but no species of *Megabalanus* has been reported to do so (Furman and Yule 1990, Dasai *et al*. 2006). The majority of reports of barnacle species that are potential self-fertilizers are based on observations of individuals isolated from potential mates carrying larval broods and assumed to have self-fertilized rather than genetic confirmation (Barnes and Crisp 1956, Furman and Yule 1990, Dasai *et al*. 2006). Genetic evidence of cross-fertilization in isolated barnacles reported here as well as in other recent studies contradicts the idea that

hermaphroditic barnacles may self-fertilize in the absence of mates (Barazandeh *et al.* 2013, 2014; Ewers-Saucedo *et al.* 2016**.**).

A tentative hypothesis as to how isolated barnacles manage to outcross is spermcast mating, or spermcasting (Barazandeh *et al*. 2013). Spermcasting is the process by which a sessile aquatic organism releases sperm into the water, with the hopes of a nearby barnacle using the free-floating sperm to fertilize the eggs present in its mantle cavity (Bishop &Pemberton 2006). Barazandeh *et al.* (2013) observed that sperm capture was used by 100% of Pacific intertidal gooseneck barnacles, *Pollicipes polymerus,* that were located outside of the copulation range between mates (Barazandeh *et al.* 2013). A subsequent study on *Balanus glandula* and *Chthamalus dalli* determined that spermcast mating does occur in these two intertidal acorn barnacles, although at lower rates than the stalked barnacle (Barazandeh *et al.* 2014). Spermcasting mating has only recently been acknowledged as a fertilization mechanism in barnacles, and the frequency and extent to which barnacles use it is not established (Barazandeh *et al.* 2013). The isolated location of the barnacles in this study provides strong evidence for the case that *M. coccopoma* might employ spermcast mating in order to successfully establish an introduced population.

Multiple paternity and thus varying gamete combinations in a brood increases reproductive success and offspring survival. When adults are in close range to several mates it isn't uncommon to have multiple sires to a single brood (Plough *et al.* 2014). Multiple paternity has been recorded in up to 79% of broods in high-density populations of the Pacific gooseneck barnacle, *Pollicipes elegans* (Plough *et al.* 2014). Although the reproductive biology of barnacles allows for broods with multiple sires, there are many

studies that indicate it is not as common in clustered barnacles as expected (Kelly *et al.* 2012).

Paternity determination of a cluster of *M. coccopoma* adults in this study allowed for the identification of sires to their broods. Adults N, O, P, and Q were attached in a cluster, and both N and Q had larvae. The genetic analysis of adult N and her larvae revealed that her offspring were sired by adult O. The genotypes of N's brood contained at least one non-maternal allele at each marker, each of which could be found in adult O's genotype. The other adult in the grouping that contained offspring upon collection was adult Q. The genotypes of Q's larvae contained alleles that were absent from Q's genotype for the marker, but could be found in the genotype of P. The consistent allelic contribution of alleles from P to the brood of Q, and O to the offspring of N provides evidence that this *Megabalanus coccopoma* does reproduce by outcrossing through direct copulation with nearby mates. The single sire broods observed in this *M. coccopoma* population didn't provide evidence for multiple paternity. An explanation for this in a cluster of barnacles that were capable of being fertilized by multiple males with respect to location and reproductive activity might be sexual selection mechanisms in place by *M. coccopoma.*

There are numerous species-specific behavioral and physiological mechanisms in place to prevent another male's sperm from fertilizing the eggs of a male's mate, but one of the most frequently seen is sperm competition. Sperm competition can prevent another male's sperm from fertilizing his mate's eggs through mechanisms such as sperm storage or the removal of other mate's gametes (Simmons 2005). The exact mechanisms used by *M. coccopoma* to prevent multiple sires in a brood are unknown, methods that require movement such as mate or territory guarding can be ruled out (Simmons 2005). The close

proximity of the group of four barnacles that have participated in pseudo-copulation with one another would be an ideal environment for multiple sires to share paternity over a brood. Similarly, if sperm capture was utilized by this population's isolated barnacles to produce their cross-fertilized broods, the chance of the mother grabbing free-floating sperm in the water from just one sire is low. The findings of this study suggest little to no multiple paternity in *Megabalanus coccopoma*. The lack of multiple paternity broods of both clustered and individual adults give rise to questions regarding the pre- or post-fertilization mechanisms that are in place by this species to lead to single-sired broods.

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