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Isolation of Microsatellites for an Endemic Species of Freshwater Mussel Using Next-Generation Sequencing Technology

Aldo Vazquez

ISOLATION OF MICROSATELLITES FOR AN ENDEMIC SPECIES OF FRESHWATER MUSSEL USING NEXT-GENERATION SEQUENCING TECHNOLOGY

A Thesis

By

ALDO VAZQUEZ

Submitted to Texas A&M International University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCES

May 2016

Major Subject: Biology

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Approved as to style and content by:

Chair of Committee,	Michael R. Kidd
Committee Members,	Ruby A. Ynalvez
	Monica O. Mendez
	Alfred Addo-Mensah
Head of Department,	Dan Mott

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ABSTRACT

Isolation of Microsatellites for an Endemic Species of Freshwater Mussel Using Next-Generation Sequencing Technology

Aldo Vazquez, B.S., Texas A&M International University;

Chair of Committee: Dr. Michael R. Kidd

Texas is home to 53 species of freshwater mussels. Unfortunately, factors associated with an increase in urbanization, such as decreasing water levels in lakes and rivers, have greatly affected the populations of many species. Currently, 15 species are classified as threatened by the state. Of these species, 11 have been petitioned to be enlisted under The Endangered Species Act (ESA), and one is currently a candidate for future protection. In this project, we have used next generation sequencing technology to develop a suite of species-specific genetic markers, known as microsatellites, to assess the genetic diversity of the populations of *Popenaias popeii*, the candidate for future listing under the ESA. Microsatellites are regions of DNA distributed across a species genome that are comprised of 1-6 nucleotides repeated in tandem. To date, we have tested the utility of 20 microsatellite markers and have optimized the PCR conditions for 4 of these. These markers will provide genetic data on population structure, gene flow, and existing levels of genetic diversity for this critically threatened species endemic to Southwest Texas and the lower portion of Pecos River in New Mexico.

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INTRODUCTION

North America is known for its great diversity of freshwater mussels hosting around 300 species (Williams *et al.*, 1993). Unfortunately, the populations of several species have been declining in the past decades (Williams *et al.*, 1993). Over 70% of the native freshwater mussel species in the United States and Canada are considered endangered, threatened, or of special concern (Williams *et al.*, 1993). In comparison with other animals in North America, freshwater mussels have exhibited higher rates of extinctions in the 20th century, and these extinctions rates are estimated to increase in the near future (Ricciardi & Rasmussen, 1999). This decline in freshwater mussel diversity can be attributed to factors associated with human activity, such as siltation, the introduction of invasive species, habitat destruction due to channel modification or dam construction, and commercial harvesting (Fuller, 1974; Williams *et al.*, 1993). In addition, lack of research efforts on invertebrates and mollusks often have caused freshwater mussels to be overlooked in conservation policies (Lydeard *et al.*, 2004; Neves *et al.*, 1997; Regnier *et al.*, 2008; Williams *et al.*, 1993). However, as the imperiled state of freshwater mussels garners more attention, greater research efforts have been allocated towards assessing the status of freshwater mussel species in North America (Neves *et al.*, 1997; Williams *et al.*, 1993).

Of the almost 300 freshwater mussel species in North America, 53 can be found in the state of Texas (Winemiller *et al.*, 2010). The state of Texas considers 15 of these species to be threatened (Winemiller *et al.*, 2010). Of these 15 species, 11 have been petitioned to be listed under The Endangered Species Act (ESA), and one is currently listed as a candidate (Winemiller *et al.*, 2010). The candidate species is *Popenaias popeii*, otherwise known as Texas hornshell. The listing of *P. popeii* as a candidate means that the United States Fish and Wildlife Service

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(USFWS) has sufficient information on its threatened status to propose the species as endangered or threatened under the ESA, but its listing is precluded by other species of higher priority.

The species *P. popeii* is endemic to the southwest region of Texas and the lower portion of the Pecos River in New Mexico (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007; USFWS, 2013). In recent years, live *P. popeii* have only been found in the Rio Grande and the Devils River in Texas and the Black River in New Mexico (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007; USFWS, 2013). In the Rio Grande, *P. popeii* can be found in two locations: in the area downstream of Big Bend and in Webb County in the city of Laredo, where the biggest population is currently located (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Karatayev *et al.*, 2012).

Ecological Importance

Freshwater mussels can provide several environmental services to freshwater ecosystems (Anderson & Kreeger, 2010). Dense assemblages of mussels can stabilize stream channels and prevent bed transport during times of high water flow (Anderson & Kreeger, 2010). Mussels can sequester nutrients from the water column and cycle them back into sediments through their biodeposits (Anderson & Kreeger, 2010). They improve water quality by removing particulates through filter-feeding, and they can serve as food for a variety of animals (Howells, 2010). Additionally, freshwater mussels can serve as an indicator species of environmental problems (Fuller, 1974; Waykar & Deshmukh, 2012). Considering its endemism, *P. popeii* could serve as a potential indicator for environmental problems within the Rio Grande and the Black River, where extant populations are currently located (Burlakova & Karatayev, 2012; Carman, 2007).

As an endemic species, *P. popeii* is a major contributor to the biodiversity and uniqueness of freshwater mussel communities (Burlakova *et al.*, 2011). The removal of endemic species can lead to homogenization of distinct mussel communities, and as a result, decrease biodiversity within an ecosystem (Burlakova *et al.*, 2011). Unfortunately, endemic species are sensitive to environmental changes (Burlakova *et al.*, 2011). Human alterations of rivers and streams, for instance, favor common species but drastically affect endemics (Burlakova *et al.*, 2011).

Taxonomy

The species *P. popeii*, otherwise known by its common name Texas hornshell, is a type of freshwater mussel that belongs to the *Unionidae* family (Turgeon *et al.*, 1998). Originally described as *Unio popeii* by Lea in 1857, the scientific name for this species has since undergone several revisions (Strenth *et al.*, 2004). It was placed in the genus *Elliptio* by Ortmann (1912), and it was given its own subgenus by Frierson (1927). Heard and Guckert (1970) elevated *Popenaias* to genus and created the subfamily *Popenaiadinae* to accommodate the genera *Cyrtonaias* and *Popenaias*. *Popenaiadinae* was eventually dropped as a taxon (Heard, 1974). Texas hornshell is currently classified under the subfamily *Ambleminae* (Campbell *et al.*, 2005).

Species Description

As a member of the unionid family, *P. popeii* is considered a bivalve mollusk and is characterized by two hard shells made up of crystallized calcium carbonate (Howells *et al.*, 1996). These shells, or valves, are attached together by a strong and flexible ligament, called the adductor muscle, located at the dorsal edge within each shell (Howells *et al.*, 1996). To prevent the shells from moving out of alignment with each other, *P. popeii* possesses an arrangement of

tiny processes, also located at the dorsal edge, called the hinge teeth (Howells *et al.*, 1996). The shell is produced and maintained by the mantle, which is a soft tissue that covers the interior surface of the shell (Howells *et al.*, 1996). The mantle covers a region above the foot made up of soft tissue called the visceral mass, which is where all the internal organs are located (Howells *et al.*, 1996).

The shells of *P. popeii* have an elongated trapezoidal shape (Howells *et al.*, 1996). They are compressed laterally with a round anterior and a slightly truncated, wider posterior (Howells *et al.*, 1996). The lengths, widths, and heights of live captures have been reported to range from 33.2 to 114 mm, 14.8 mm to 56.2 mm, and 25.6 mm to 57.7 mm, respectively (Carman, 2007; Karatayev *et al.*, 2012). The outside surface of the shell varies from a dark, olive green to a dark brown, while the inside surface of the shell can be glossy, dull-white, blue-white, or purple-gray (Howells *et al.*, 1996).

The larval stage of *P. popeii* is called glochidia. During this stage, *P. popeii* possesses semi-elliptical shells with pitted valves (Smith *et al.*, 2003). The hinge is long with a slightly convex shape and a well developed ligament (Smith *et al.*, 2003). The anterior margin of the shell is flat with a convex shape and the posterior is more flattened and truncated (Smith *et al.*, 2003). Near the edge of the shells in the inner surface, there are rows of conical denticles that measure up to 2 um long (Smith *et al.*, 2003). The largest denticles can be found in the outermost rows with the size decreasing in subsequent rows (Smith *et al.*, 2003).

Reproductive Biology and Life Cycle

Members of the species *P. popeii* are considered asynchronous breeders, which means individuals may be at different stages of reproduction during breeding seasons (Smith *et al.*,

2003). Gametogenesis appears to take place from January through September with concentrations of ova and sperm being the highest from January through July (Smith *et al.*, 2003). During late September, empty acini start becoming apparent (Smith *et al.*, 2003). From late October to December, gonads of *P. popeii* cease active production of gametes (Smith *et al.*, 2003). Spawning may extend up to 8 months or more, but individual broods of developing zygotes may only require 4 to 6 weeks before they can be released as glochidia (Smith *et al.*, 2003). This suggests that a single female may be able to reproduce more than once during a breeding season (Smith *et al.*, 2003).

Females produce ova that are stored in the gill mantle chamber (Smith *et al.*, 2003). Fertilization of the ova is made possible by males releasing sperm into the water column making the sperm accessible to nearby females (Fuller, 1974; Howells *et al.*, 1996). The sperm is then taken in through the female incurrent siphon (Fuller, 1974; Howells *et al.*, 1996). After the ova are fertilized, the developing zygotes are stored in brooding pouches called marsupia, located in the demibranchs of the gills (Smith *et al.*, 2003). The demibranch is the V-shaped structure of the gills in bivalves (Smith *et al.*, 2003). Each gill is composed of two demibranchs located laterally to the foot (Smith *et al.*, 2003). As an ectobranchous brooder, only the outer demibranchs contain marsupial structures (Smith *et al.*, 2003). These marsupial demibranchs exhibit thicker and more densely packed septa that contain layers of expanded epithelial cells (Smith *et al.*, 2003). In the event the marsupia of the outer demibranchs become unable to fit anymore larvae, the inner demibranchs may act as facultative larval incubators (Smith *et al.*, 2003). This occurs despite their lack of specialization, such as containing thinner, less densely packed septa and epithelial cells (Smith *et al.*, 2003).

Glochidia are not free swimming organisms, so parasitism of a host fish is necessary for survival during metamorphosis and for dispersal of juvenile mussels (Fuller, 1974; Howells *et al.*, 1996; Smith *et al.*, 2003). *Ambleminae* species rely in the production of conglutinates to increase the probability of glochidia coming into contact with a host (Barnhart *et al.*, 2008). Conglutinates are clusters of eggs that aggregate in the water tubes of the female demibranchs (Lefevre & Curtis, 1912). Glochidia are released as conglutinates suspended in mucus, which eventually break apart releasing the glochidia (Barnhart *et al.*, 2008). Once released, glochidia are able to parasitize nearby host fish by coming into contact with the head, gills, or skin (Barnhart *et al.*, 2008). Host fish may attempt to feed on conglutinates, which can aid in the release of glochidia as well as bringing them into contact with the host (Barnhart *et al.*, 2008).

Glochidia attach to a fish host by clamping the valves of the shells on fin, gill, or face tissue (Barnhart *et al.*, 2008; Fuller, 1974). Once attached, the host epithelial cells begin encapsulating the glochidia creating a cyst around them (Barnhart *et al.*, 2008; Fuller, 1974). After metamorphosis is complete, juvenile mussels initiate rupture of their cysts and proceed to drop from the host (Barnhart *et al.*, 2008; Fuller, 1974). The development of juvenile mussels into adults is contingent on finding a suitable substrate (Barnhart *et al.*, 2008; Fuller, 1974). A suitable substrate is often described as being firm and stable yet yielding enough to allow for juveniles to burrow into it (Barnhart *et al.*, 2008; Fuller, 1974).

Mussels experience little growth during the glochidial stage (Howells *et al.*, 1996). Most of their growth and development takes place during the juvenile stage and slows down as they reach maturity (Howells *et al.*, 1996). Shell measurements of recaptured adults suggest the growth of *P. popeii* is less than 1 mm per year (Lang, 2001). Although the life span of *P. popeii*

is not known, mussels belonging to the *Ambleminae* subfamily commonly live for over 20 years (Stansbery, 1967).

Historical Distribution

As a regional endemic, *P. popeii* is known to have historically occurred in the lower portion of the Pecos River in New Mexico downstream to the lower Rio Grande and its tributaries in Texas (Carmen, 2007; Howells *et al.*, 1996; Neck & Metcalf, 1988). Additionally, this mussel has been reported in several Mexican tributaries of the Rio Grande (Johnson, 1999; Strenth *et al.*, 2004).

This species was commonly found in New Mexico in the lower portion of the Pecos River from North Spring River, Roswell, Chaves County downstream to Texas, including the Black and Delaware Rivers in Eddy County (Cockerell, 1902; Taylor, 1983). Until 1937, live specimens could be found in the lower Pecos River near Carlsbad, New Mexico (Metcalf, 1982). In 1996, umbonal shell fragments of fossilized *P. popeii* were collected from the Pecos River in the Salt Creek Wilderness of the Bitter Lake National Wildlife Refuge and the Delaware River in Eddy County (Lang, 2001).

In Texas, specimens of *P. popeii* had been reported from several locations. In the Rio Grande, collections of *P. popeii* suggest the species has historically inhabited the river from San Francisco Creek in the Big Bend area, Brewster County, downstream to Brownsville, near the Gulf of Mexico (Howells *et al.*, 1996). Historical collections, as well as live specimens, have also been reported from Las Moras Creek and Devils River, which are tributaries to the Rio Grande (Howells *et al.*, 1996; Strecker, 1931). Along the Pecos River in Texas, *P. popeii* has

been found in Ward County and near the Rio Grande confluence in Val Verde County (Metcalf, 1982; Strecker, 1931).

Specimens reported from Mexico originate from Rio Salado, Rios Panuco, and Rios Valles (USFWS, 2013). The validity of these reports, however, remains questionable due to confusion regarding the classification and biology of freshwater mussels in Mexico (USFWS, 2013). Consequently, knowledge of the historical distribution of *P. popeii* in Mexico is incomplete and unreliable (USFWS, 2013).

Current Distribution and Status

The current distribution of *P. popeii* represents only a small fraction of its historical range with surviving populations occurring in only a few locations in New Mexico and Texas combined. From 2002 to 2011, the presence of *P. popeii* in Texas had only been confirmed by the discovery of 7 live specimens in the Devils River and 45 live specimens in the Rio Grande downstream of the Big Bend National Park (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Karatayev *et al.*, 2012). In 2011, however, 604 live *P. popeii* were recorded from a large population discovered in Laredo (Burlakova & Karatayev, 2011). A conservative estimate of more than 8000 individuals makes this population the largest ever reported from Texas, New Mexico, or Mexico (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Karatayev *et al.*, 2012). This population is comprised of multiple age classes, which suggest proper recruitment of juvenile mussels (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Karatayev *et al.*, 2012). This indicates that this is a healthy reproducing population and that a healthy host population also inhabits this area of the river, which is important for this

population to survive in the future (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Karatayev *et al.*, 2012).

In New Mexico, *P. popeii* represents the last remaining species of native freshwater mussels (Carman, 2007; Lang, 2001; USFWS, 2013). The current distribution of *P. popeii* in the State has been reduced to about 12% of its historical range with only one population currently present in the Black River (Carman, 2007; Lang, 2001). This population is located within Eddy County and extends from Black River Village downstream to the U.S. Highway 285 bridge crossing (Lang, 2001). This area covers approximately 14 km of the Black River between two low-head dams (Lang, 2001). This portion of the Black River possesses proper water flow and quality as well as suitable substrates (Lang, 2001). Collectively, these attributes provide proper conditions for the survival of this population (Lang, 2001).

Threats

As a freshwater mussel, *P. popeii* is a filter-feeding, sessile organism that relies on good water quality and proper physical habitat conditions for survival and reproduction (Fuller, 1974; Howells *et al.*, 1996). In addition, *P. popeii* requires an environment that can sustain populations of fish that can serve as hosts during the early stages of its life (Fuller, 1974; Howells *et al.*, 1996; Schwalb *et al.*, 2012). These characteristics contribute to the sensitivity of its sensitivity to habitat alterations.

Water Quality

A future increase in the intensity of land use within the Black River watershed presents a possible threat to the population of *P. popeii* inhabiting the river (Carman, 2007). Deforestation

and poor agricultural practices can lead to the accumulation of silt or sediments in rivers and lakes and have been associated with declines in local mussel populations (Fuller, 1974; Howells *et al.*, 1996; Williams *et al.*, 1993). Silt deposition from the water column can irritate and clog the gills of mussels reducing feeding and respiratory efficiency (Anderson & Kreeger, 2010; Fuller, 1974; Houp, 1993; Howells *et al.*, 1996). Sediments can suffocate the eggs and larvae of host fish populations and reduce the availability of food for both the fish and the mussels (Anderson & Kreeger, 2010; Fuller, 1974; Howells *et al.*, 1996). Silt settling in the interstitial space between sand and gravel can alter the flow of water, food, and oxygen (Anderson & Kreeger, 2010; Fuller, 1974; Howells *et al.*, 1996). This makes the space uninhabitable for young host fish and juvenile mussels who need it for spawning and survival (Anderson & Kreeger, 2010; Fuller, 1974; Howells *et al.*, 1996).

Runoff from agricultural land can carry dissolved minerals and lead to salinization of freshwater ecosystems (Kaushal *et al.*, 2005). Although adult mussels can acclimate to small increases in salinity, high increases can lead to reduced metabolic rates and higher mortality (Blakeslee *et al.*, 2013). In glochidia, salinization affects viability by decreasing attachment success to host fish and by interfering with metamorphosis (Blakeslee *et al.*, 2013). In Texas, agricultural runoff has led to significant increases in salinity levels throughout much of the historical range of *P. popeii* in the Rio Grande (Howells, 2010), a problem further exacerbated by the over extraction of water (Karatayev *et al.*, 2012). In laboratory studies, *P. popeii* exhibits behavioral signs of physiological stress, followed by death, at saline levels of 7.0 ppt (Lang, 2001). In the Pecos River, salinity levels can range from 6.0-7.0 ppt (Lang, 2001). This may have contributed to the local extirpation of *P. popeii* in the Pecos River, as well as in the Rio Grande below the confluence with the Pecos River (Burlakova & Karatayev, 2012).

In addition, pollution from point and nonpoint sources can have immediate impacts on the quality of water (USFWS, 2013). Changes in the chemical composition of water as a result of dissolved chemicals or toxins in aquatic environments can create poor conditions for which freshwater mussels may be particularly vulnerable to (Fuller, 1974). Mussels filter large volumes of water to feed and respire, which directly exposes them to contaminants dissolved in the water, such as pesticides or heavy metals (Bringolf *et al.*, 2007; Gundacker, 1999; Gundacker, 2000). These chemicals are readily absorbed by soft tissues, such as the viscera and the gills, and can be sequestered for long periods of time (Bringolf *et al.*, 2007; Gundacker, 1999; Gundacker, 2000).

Habitat Destruction

Dam construction and channel modification can alter conditions both upstream and downstream of a location extensively enough to destroy 30% to 60% of mussel fauna (Layzer *et al.*, 1993; Williams *et al.*, 1993). Velocity of water flow in altered rivers decreases, and as a result water currents become too weak to carry sediments (Isom, 1969). This creates sediment build up, which can smother mussels that are not well adapted to soft substrates (Isom, 1969). These sediments can also cause organic material to be retained in the hypolimnion of the channel leading to oxygen depletion and poor abundance of nutrients (Ellis, 1936). Decreases in temperature can also occur from either sediment build up or water discharge from water impoundments (Watters, 1999). Mussels require sustained exposure to warm waters in order for gametogenesis to take place (Watters, 1999). Constant exposure to cold waters, especially for individuals living in the hypolimnion, can cause mussels to reproduce less frequently or not at all (Watters, 1999).

Dams can present physical barriers that inhibit the movement of host fish (Collier *et al.*, 1996). This decreases the likelihood of encounters between mussels and hosts, as well as preventing parasitized fish from dispersing glochidia (Watters, 1999). Healthy, reproducing populations of mussels can therefore become isolated and their distribution becomes limited (Watters, 1999). Even without the physical presence of a dam, the turbid and anoxic water in an impoundment may isolate mussels and fishes in tributaries (Layzer *et al.*, 1993).

Shallow waters and heterogenous environments, such as those found in free-flowing streams and rivers, promote diversity in unionid communities (Burlakova *et al.*, 2011; Watters, 1999). Construction of water reservoirs can increase the depth of waterbodies as well as eliminate heterogeneity from the environment (Burlakova *et al.*, 2011; Watters, 1999). These conditions favor generalist and invasive species but severely reduce habitats for rare and endemic species (Burlakova *et al.*, 2011). The latter is a critical contributor to the uniqueness and diversity of unionid communities (Burlakova *et al.*, 2011). The loss of rare or endemic species, such as *P. popeii* can therefore lead to homogenization of entire unionid communities creating a decrease in species diversity (Burlakova *et al.*, 2011). In Texas, almost all endemics species can be found exclusively in streams and rivers, where diversity is almost double that of reservoirs (Burlakova *et al.*, 2011).

Invasive Species

The introduction and rapid expansion of the exotic bivalves *Corbicula fluminea* and *Dreissena polymorpha* in North America poses a threat to indigenous freshwater mussels (Strayer, 1999; Williams *et al.*, 1993). These two species have the potential to affect the community structures of native bivalves and the environment in freshwater ecosystems (Cherry

et al., 2005; Gardner *et al.*, 1976; Hakenkamp *et al.*, 2001; Karatayev *et al.*, 2005; Schloesser *et al.*, 2005; Strayer, 1999).

Since its discovery in the United States in 1938 (Burch, 1944), *C. fluminea* has spread over 44 states (Foster *et al.*, 2016). In Texas, *C. fluminea* was first discovered in 1958 in the Neches River (Howells *et al.*, 2004), and by the 1970s it had spread to all the major Texas drainage basins (Karatayev *et al.*, 2005). In Texas and New Mexico, *C. fluminea* is known to already inhabit several locations within the historical range of *P. popeii* (Carman, 2007; Howells, 1999). Because of its ability to dominate habitats, *C. fluminea* may present a threat to extant populations of *P. popeii* as a competitor for natural resources and habitat availability (Karatayev *et al.*, 2003; Sousa *et al.*, 2008). In addition, the large number of periodic die-offs of *C. fluminea* may affect water quality in the water column by increasing ammonia toxicity and decreasing oxygen levels (Cherry *et al.*, 2005; Cooper *et al.*, 2005). The potential threats of *C. fluminea*, however, remain for the most part speculative and little is known of the effect of *C. fluminea* on native bivalve populations (Strayer, 1999).

Within a few years since it was first detected in 1986 in Lake Erie (Carlton, 2008), *D. polymorpha* had managed to colonize the Great Lakes and spread into eight river systems in the United States (Ludyanskiy *et al.*, 1993). This rapid expansion has been followed by a decline in native bivalve populations in several of the invaded lakes and rivers (McNickle *et al.*, 2006; Ricciardi *et al.*, 1998). In addition, the regional extinction rates of North American freshwater mussels have been estimated to have increased 10-fold following the invasion of *D. polymorpha* (Ricciardi *et al.*, 1998). There are currently no official reports indicating the presence of *D. polymorpha* near the historical or current distribution of *P. popeii* (Benson *et al.*, 2015). However, extant populations of *P. popeii* are located within geographical regions that are at risk

for *D. polymorpha* invasion (Drake & Bossenbroek, 2015). The invasion of *D. polymorpha* could mark the extinction of *P. popeii* in the near future (Drake & Bossenbroek, 2015; Ricciardi *et al.*, 1998).

Microsatellites as Genetic Markers

The threatened status of *P. popeii* creates a need for a comprehensive approach to evaluating extant populations of this species. The current methodology used in the assessment of *P. popeii* populations are mark-and-recapture methods and timed searches (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007). Mark-and-recapture methods are used to estimate the population sizes of *P. popeii*, and timed searches are used to measure species diversity of unionid communities (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007). However, neither approach provides information on population structure or genetic diversity of *P. popeii*. This type of genetic information can be evaluated by researchers through the use of microsatellites as genetic markers.

Microsatellites are codominant sequences of DNA that contain 1-6 nucleotides that repeat in tandem, and they are found at several locations within a eukaryotic genome (Selkoe & Toonen, 2006; Tautz & Renz, 1984). Microsatellites are remarkably polymorphic, so individuals within a given species can possess different alleles for each microsatellite locus (Jehle & Arntzen, 2002; Selkoe & Toonen, 2006). This variability in microsatellite loci allows for the detection of genetic differences between any two individuals in a population. This eliminates any ambiguity that may arise from classifying individuals based on merely physical traits or tagging methods. Using microsatellite markers, a genetic profile can be generated for a particular population by using a representative sample (Pritchard *et al.*, 2000; Selkoe & Toonen, 2006).

This genetic information can then be used to determine several characteristics about the population (Jehle & Arntzen, 2002; Pritchard *et al.,* 2000; Selkoe & Toonen, 2006).

In the case of *P. popeii,* the use of microsatellite markers can help determine effective population size, population bottlenecks, migration patterns, gene flow between populations, and genetic diversity. The populations in the Black River in New Mexico and in the Rio Grande in Webb County, Texas, are assumed to be properly reproducing based on juvenile mussel recruitment (Carmen, 2007; Karatayev *et al.,* 2012). Measuring effective population size using microsatellite markers should help to verify this assumption. Using theoretical models for microsatellite alleles coupled with historical records, could help detect population bottlenecks that may have contributed to the current distribution of *P. popeii* (Marshall *et al.,* 2009). Microsatellite markers have been used to determine migration patterns and gene flow between populations of mountain plovers that were previously thought to be isolated from each other (Oyler-McCance *et al.,* 2008). Using a similar approach, researchers could detect migration patterns and gene flow between the current populations of *P. popeii,* which appear to be isolated from each other (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carmen, 2007; USFWS, 2013). The use of microsatellites could help measure genetic diversity within a population and among different populations (Oishi *et al.,* 2011; Oyler-McCance *et al.,* 2008). Information on genetic diversity can help identify subpopulations within the large population located in Webb County, Texas (Burlakova & Karatayev, 2012; Karatayev *et al.,* 2012; Oishi *et al.,* 2011). In addition, information on the genetic diversity of each population in Texas and New Mexico can be valuable when establishing conservation priorities.

Isolation and amplification of microsatellites requires the use of a polymerase chain reaction (PCR) (Selkoe & Toonen, 2006). The enzymatic replication of a microsatellite

necessitates the use of primers complementary to the flanking regions (Selkoe & Toonen, 2006). For this reason, flanking regions must be highly conserved within a population in order to prevent misrepresentation of microsatellite alleles (Selkoe & Toonen, 2006). Each primer should be tested for successful amplification of the microsatellite product (Selkoe & Toonen, 2006). In addition, each PCR reaction should be optimized for proper analysis of the amplified product (Selkoe & Toonen, 2006).

Traditional methods of designing PCR primers consisted of constructing whole genome libraries to develop primers de novo or testing already developed primers from related species (Abdelkrim *et al.*, 2009; Csencsics *et al.*, 2010). However, these approaches were time consuming and expensive (Abdelkrim *et al.*, 2009; Csencsics *et al.*, 2010). The availability of next-generation, shotgun sequencing technology allows for the production of large numbers of DNA sequences at reduced cost and time expenditure in comparison to traditional sequencing methods (Abdelkrim *et al.*, 2009; Csencsics *et al.*, 2010). According to Abdelkrim *et al.* (2009), the use of next-generation, shotgun sequencing technology can reduce the cost of primer development to 1/3-1/5 the price of most commercial suppliers who employ traditional methods.

In this study, we employed next-generation, shotgun sequencing technology to identify microsatellite loci and their respective flanking regions. Primers complementary to the flanking regions were developed and subsequently tested for successful isolation and amplification of microsatellite loci.

METHODS

Tissue Collection and DNA Extraction

A total of 15 specimens of *P. popeii* were collected over the course of 3 years from the Rio Grande in La Bota Ranch in the city of Laredo, Texas. Initially, tissues were obtained from mantle biopsies, and genomic DNA was extracted using an E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek). Due to their invasive and potentially lethal nature, mantle biopsies were replaced by hemolymph extractions as a means of tissue sampling. Hemolymph extractions were performed using a technique described by Gustafson *et al.* (2005). Field and lab studies have demonstrated this technique to be safe, even with repeated sampling on the same individuals (Gustafson *et al.*, 2005; McCartney *et al.*, 2009). Genomic DNA was isolated from hemolymph tissue using a Gentra Puregene Kit (Qiagen). DNA concentrations were quantified using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific).

Preparation of Genomic DNA Libraries for Sequencing

Shotgun sequencing of genomic DNA libraries was performed using an Ion Torrent Personal Genome Machine (PGM™) System (Life Technologies). Genomic DNA libraries are necessary to properly perform a shotgun sequencing run. The preparation of genomic DNA libraries is outlined in the following paragraphs.

Preparation of Adapter-Compatible DNA

In this step, genomic DNA was enzymatically fragmented using Ion Shear™ Plus Reagents provided in the Ion Xpress™ Plus Fragment Library Kit. The manufacturer's protocol was followed for a 200 base-read library size with 1 μg input of genomic DNA.

DNA Adapter Ligation and Purification

Adapter ligation was performed using the Ion Plus Fragment Library Kit following the manufacturer's protocol for 1 µg input of fragmented DNA. Purification of the adapter-ligated DNA was performed by following the manufacturer's protocol for a 200 base-read library.

Size Selection of Ligated DNA

An E-Gel® iBase™ unit, an E-Gel® Safe Imager™, and an E-Gel® SizeSelect™ 2% Agarose gel were assembled and prepared. The wells on the E-Gel® SizeSelect™ 2% Agarose gel were loaded following the manufacturer's protocol for 1 µg input of DNA samples. The reaction run time was set for 12-14 min. Once the reaction had started, the appropriately sized ladder band for a 200 base-read library was monitored periodically. The reaction was stopped once the desired band had reached the reference line on the gel. The collection wells were refilled to 25 µL with approximately 10 µL of nuclease-free water, and the reaction run was resumed. The middle marker well was monitored frequently for the desired fragment length. The reaction was stopped again once the 350 bp ladder band came into contact with the top edge of the collection well. The solutions were collected from the collection wells, and the wells were washed with 10 µL of nuclease-free water. The resulting solution was collected and pooled with the rest of the solutions.

Preparation of Template-Positive Ion Sphere Particles (ISPs)

An Ion OneTouch™ Instrument was properly assembled and prepared. An amplification solution was prepared using an Ion OneTouch™ 200 Template Kit following the manufacturer's

protocol for 200 base-read libraries. The Ion OneTouch™ Plus Reaction Filter Assembly was assembled by fitting an Ion OneTouch™ Reaction Tube onto the Ion OneTouch™ Plus Reaction Filter. The amplification solution and the Ion OneTouch™ Reaction Oil were added to the Ion OneTouch™ Plus Reaction Filter Assembly following the guidelines in the manufacturer's manual. The Ion OneTouch™ Plus Reaction Filter Assembly was carefully inverted and inserted into the ports located on the top stage of the Ion OneTouch™ Instrument. The "Assisted" setting was chosen on the screen of the instrument and the run was initiated. Once the run was finalized, the Recovery Tubes were removed and the template-positive ISPs were recovered following the manufacturer's guidelines.

Enrichment of Template-Positive ISPs with the Ion OneTouch™ System

A "Melt-Off" solution was prepared according to the manufacturer's manual and set aside for later use. Dynabeads® MyOne™ Streptavidin C1 (DMSC) Beads were resuspended and washed according to the manufacturer's protocol. An 8-well strip was removed from packaging, and the wells were filled with template-positive ISPs, DMSC Beads, Ion OneTouch™ Wash Solution, and freshly prepared Melt-Off solution according to the guidelines in the manufacturer's manual. The 8-well strip was properly inserted into the slot on the tray of the Ion OneTouch™ ES machine. An open 0.2 mL PCR tube was installed in the hole of the Tip Loader, and a new tip was placed in the Tip Arm. The run on the Ion OneTouch™ ES machine was initiated. Immediately after the run was completed, the PCR tube containing the enriched ISPs was securely closed and removed. The wash and removal of the enriched ISPs was performed according to the manufacturer's protocol.

Sequencing Using an Ion Torrent Personal Genome Machine™

Sequencing of genomic DNA was performed using an Ion Torrent Personal Genome Machine™ (PGM) System for the Ion 316™ Chip. In this step, Control Ion Sphere™ Particles were added to the enriched ISPs followed by annealing of the Sequencing Primer and binding of the Sequencing Polymerase. The Ion 316™ Chip was tested for functionality, washed, and loaded according to the manuals' guidelines. The experimental setup was confirmed, and the chip was loaded and clamped onto the stage of the instrument. The option "Next" was pressed to begin calibration of the chip. After calibration was completed, the option "Next" was pressed on the touchscreen initiating the sequencing run.

File Conversion Using Galaxy

Data output from the Ion Torrent PGM™ System is generated with FASTQ formatting. However, MSATCOMMANDER requires data to be inputted with FASTA formatting. Galaxy Version 16.01.rc1 (www.usegalaxy.org) was used to convert the FASTQ file from the sequencing run into a FASTA file. Unlike FASTA formatting, FASTQ assigns each letter in a nucleotide sequence a quality score using characters from the American Standard Code for Information Interchange (ASCII).

Microsatellite Detection and Primer Design with MSATCOMMANDER

Once converted, the FASTA files were processed by MSATCOMMANDER (Faircloth, 2008). The search parameters for the program were set to identify dinucleotide and tetranucleotide perfect repeats. The options "Design Primer" and "Repeats" were chosen before initiating the run.

Primer Selection Using Data from MSATCOMMANDER

A total of 20 primers were chosen based on the differences in melting temperatures between the forward and reverse sequences, their probability to lose their secondary structural integrity, their sequence motif, and their estimated size product. The chosen primers had no more than a 1^{o} C estimated difference between the reverse and forward sequences. These primers had probabilities of 0 for the primers to self anneal or anneal with the sequence of the opposite direction. The chosen primers possessed sequence motifs of CA and estimated product sizes ranging from 100-300. The chosen primers were ordered from Integrated DNA Technologies (IDT) (www.idtdna.com).

Microsatellite Isolation and Detection

Polymerase chain reactions (PCR) using a PrimeStar Kit (CloneTech) were performed to isolate and amplify each microsatellite sequence. A 1% agarose gel electrophoresis was performed after each PCR run to verify amplification of the microsatellite sequences. The initial conditions for a single PCR reaction consisted of 14.1 μL of nuclease-free water, 5 μL of PrimeStar Buffer, 1.2 μL of forward primer sequence, 1.2 μL of reverse primer sequence, 0.5 μL of PrimeStar Polymerase, and 1 μL of DNA sample. The initial thermal cycler conditions consisted of an initial preheating step at 95^{o}C for 60 s, a denaturing step at 95^{o}C for 45 s, an annealing step at temperatures that varied for each primer pair for 45 s, an elongation step at 72^{o}C for 30 s, a final extension step at 72^{o}C for 5 min, and a final holding time of 4^{o}C. Each PCR consisted of 40 amplification cycles. Initial annealing temperatures for each primer pair were determined by subtracting 5^{o}C from their respective melting temperatures. Results from the gel

electrophoresis were used to guide the optimization of the PCR conditions for each primer pair. To increase yield of PCR products, annealing times and amplification cycles were increased while annealing temperatures were decreased. To increase specificity of PCR products, annealing times and amplification cycles were decreased while annealing temperatures were increased.

Capillary Electrophoresis

An Experion™ DNA 1k Analysis Kit (Bio-Rad) was used to quantify the allele sizes of the PCR products. The DNA samples, DNA ladder, and gel-stain solution in the kit were prepared for the procedure following the manufacturer's protocol. An Experion™ DNA chip was primed for a reaction run by pipetting 9 μL of the gel-stain solution into the GS-labeled well and setting the chip on an Experion™ priming station. The pressure on the Experion™ priming station was set to C, and the time was set to 1. The chip was loaded according to the user's manual using nuclease-free water for wells not containing a DNA sample. The loaded chip was vortexed by using the Experion™ vortex station. The chip was placed into the Experion™ electrophoresis station within 5 minutes of vortexing the chip.

In the Experion™ software toolbar, the option "New Run" was clicked. In the "New Run" screen, the option "DNA > DNA 1K" was selected from the "Assay" pulldown list. Once a project folder was created and a name for the run was chosen, the option "Start Run" was clicked to initiate the procedure.

RESULTS

DNA Extractions from Tissue Collections

DNA samples obtained from mantle biopsies had a mean DNA concentration of 113.25 ng/µL. DNA samples obtained from hemolymph extractions had a mean DNA concentration of 23.38 ng/µL.

Sequencing Run with an Ion Torrent Personal Genome Machine™

The sequencing on the Ion Torrent PGM™ yielded 672, 986 DNA reads of fragments with a mean length of 190 bp (Fig. 1; Fig. 2). ISPs were present in 26% of the total addressable wells (Fig. 1). Of the wells containing ISPs, 79% were determined to be live ISPs (Fig. 1). The ISPs containing DNA libraries showed an enrichment of 77% (Fig. 1). The Ion Torrent PGM™ filtered a total of 28% of ISPs containing polyclonal reads, <1% of ISPs containing primer dimers, and 14% of ISPs containing low quality reads (Fig. 1). The size distribution of the sequencing reads is demonstrated in Fig. 3 with ~225 bp being the most common read.

	Count	Percentage
Total Addressable Wells	6,337,656	
▸ Wells with ISPs	1,658,556	26%
▸ Live ISPs	1,306,492	79%
▸ Test Fragment ISPs	139,059	11%
▸ Library ISPs	1,167,433	89%

	Count	Percentage
Library ISPs / Percent Enrichment	1,167,433	77%
▸ Filtered: Polyclonal	327,090	28%
▸ Filtered: Primer dimer	114	<1%
▸ Filtered: Low quality	167,243	14%
▸ **Final Library Reads**	**672,986**	**58%**

Figure 1 Ion Torrent PGM™ sequencing results. Of the 6,337,656 wells in the Ion 316™ Chip, 672,986 (10.62%) contained ISPs with high-quality library reads. The rest of the wells (89.38%) were empty, contained ISPs that that were not bound to DNA libraries, or contained ISPs bound to DNA libraries but were not suitable for analysis.

Total Number of Bases [Mbp]	127.80
▸ Number of Q17 Bases [Mbp]	92.35
▸ Number of Q20 Bases [Mbp]	81.66
Total Number of Reads	672,986
Mean Length [bp]	190
Longest Read [bp]	398

Figure 2 Ion Torrent PGM™ total base pair (bp) and library read results. The sequencing run yielded a total of 127.80 (Mbp) and 672,986 DNA libraries. The mean length was 190 (bp) with the longest read measuring 398 (bp).

Microsatellite Identification by MSATCOMMANDER

The program MSATCOMMANDER identified a total of 17, 905 microsatellite loci. The identified loci were comprised of 39.56% dinucleotide repeats and 60.44% of tetranucleotide repeats (Fig. 4). The microsatellite motifs with the highest occurrence were ACAT (54.72%), AC (19.69%), AG (15.52%), AT (4.25%), and AGAT (2.54%) (Fig. 5). Of the total number of microsatellite loci identified, 4.12% possessed flanking regions suitable for primer design.

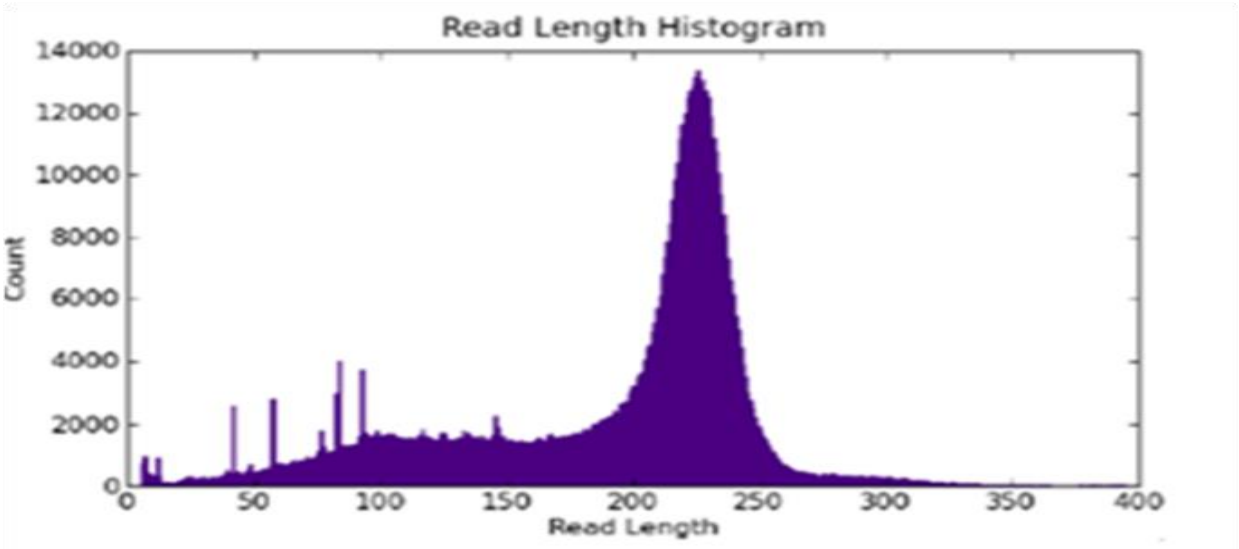

Figure 3 Size distribution of sequencing reads. The mean length of the library reads was 190 (bp) with the most common length being ~225 (bp).

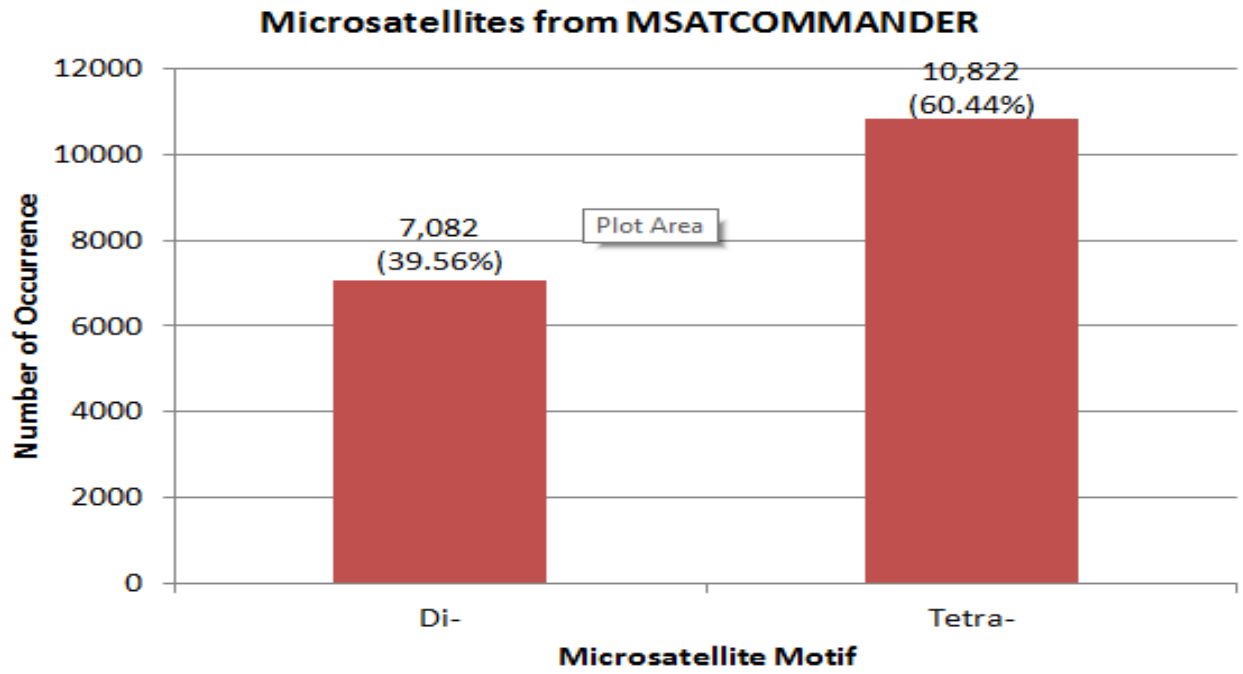

Figure 4 Total number of di- and tetra- microsatellite motifs identified by MSATCOMMANDER. Tetranucleotides comprised 60.44% of the microsatellite search while dinucleotides comprised 39.56%.

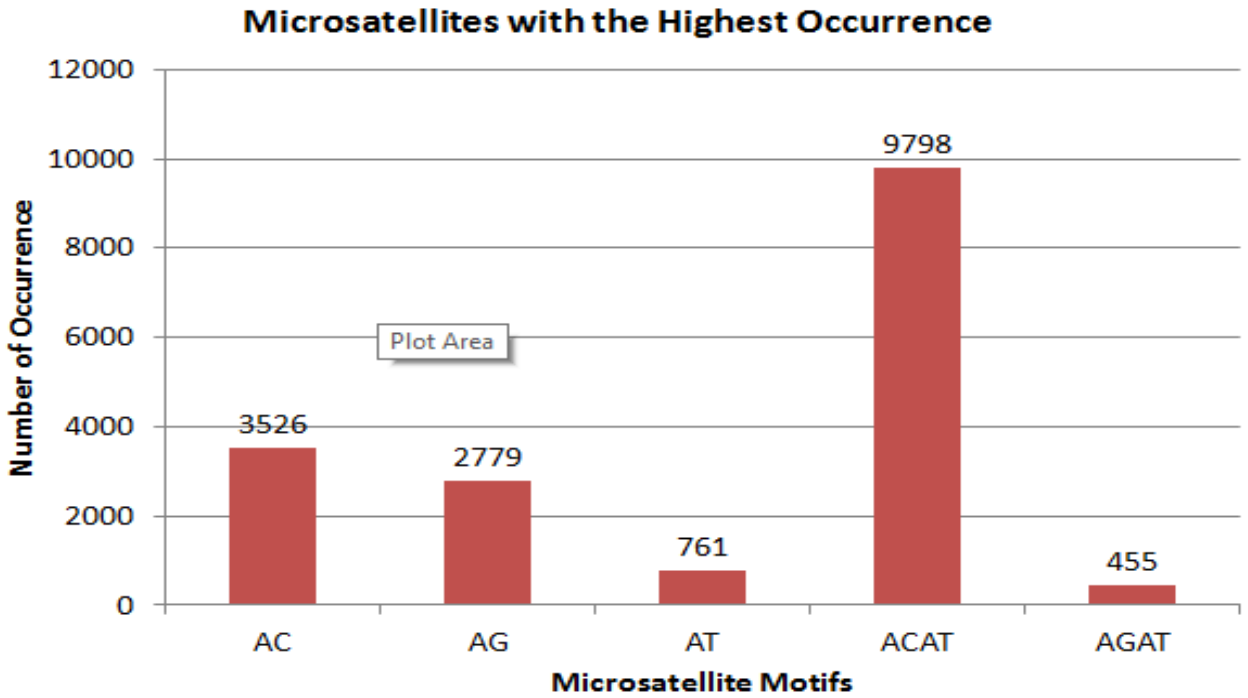

Figure 5 Most common microsatellite motifs in the *P. popeii* genome. ACAT sequence motifs comprised 54.72% of all sequences. AC sequence motifs comprised 19.69% of all sequences. AG sequence motifs comprised 15.52% of all sequences. AT sequence motifs comprised 4.25% of all sequences. AGAT sequence motifs comprised 2.54% of all sequences.

Microsatellite Isolation and Detection

Of the 20 primers ordered, 4 were able to isolate and amplify their respective microsatellites locus. Table 1 outlines the forward and reverse primer sequences for each microsatellite locus, as well as motifs and estimated product sizes of microsatellite sequences as indicated by MSATCOMMANDER. The PCR conditions for these 4 primers have been optimized (Table 2). The annealing temperatures ranged from 54.0-57 ^{o}C (Table 2). Thermal cycler conditions shared by all primers were the following: Pre-heating step at 95 ^{o}C for 60 s, denaturing step at 95 ^{o}C for 60 s, final extension at 72 ^{o}C for 30 s, and final holding time at 4 ^{o}C. As determined by a 1% agarose gel electrophoresis, the actual sizes of microsatellites Ppo004 and Ppo020 differed from the estimated sizes by MSATCOMMANDER while microsatellites Ppo005 and Ppo018 were similar to their estimated sizes (Fig. 6; Fig. 7; Fig. 8; Fig. 9).

Table 1 Characterization of microsatellite primers: forward and reverse sequences. Motifs and estimated products sizes were indicated by MSATCOMMANDER.

Microsatellites	Primer Sequence (Forward)	Primer Sequence (Reverse)	Motif	Estimated Product Size (bp)
Ppo004	ACAAGCGAACACTGAAAGACG	TGAGTAGCCAGTTCCAGCAG	CA	~100
Ppo005	CGCCAGGAGAAGAGTTTGTG	AGACCACCAGTAGCGATATGAC	CA	~100
Ppo018	TTATTTGCCATCGCCAAGGG	CTCAGCTCCTCTACGGCATC	CA	~200
Ppo020	TGCATCGACATTCAAACCCG	CCGAAAGATTGTTAAAGCGACG	CA	~200

Table 2 Final thermal cycler conditions after optimization for each pair of microsatellite primer.

Microsatellites	Annealing Temp. (^{o}C)	Annealing Time (s)	Elongation Temp. (^{o}C)	Elongation Time (s)	Cycles
Ppo004	57	30	72	30	35
Ppo005	56	35	72	30	35
Ppo018	56.2	45	72	30	40
Ppo020	54	45	72	30	40

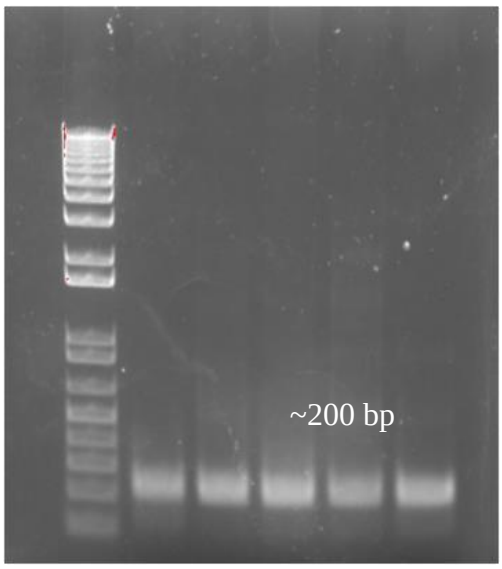

Figure 6 PCR amplification results with a 1% agarose gel for microsatellite Ppo004. Product size is ~200 (bp). Wells 1-5 contain specimens H1, H2, H3, H4, and H5.

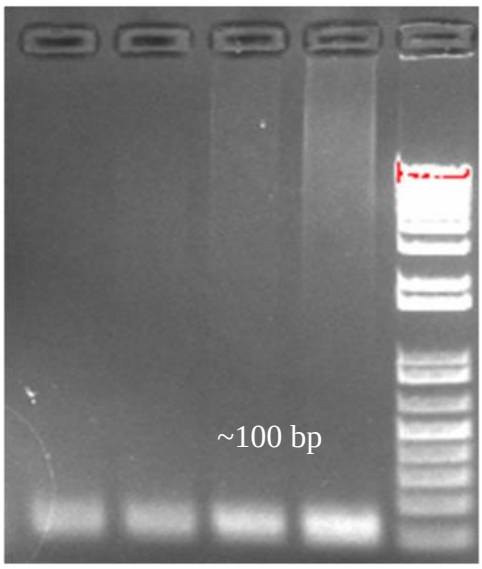

Figure 7 PCR amplification results with a 1% agarose gel for microsatellite Ppo005. Product size is ~100 (bp). Wells 1-4 contain specimens D-0043, D-0044, D-0045, and D-0046.

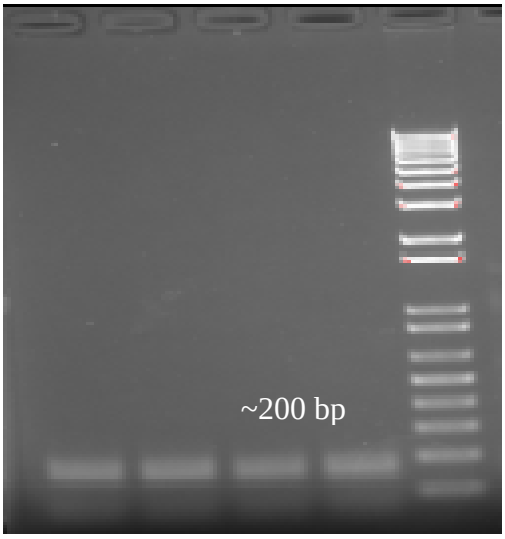

Figure 8 PCR amplification results with a 1% agarose gel for microsatellite Ppo018. Product size is ~200 (bp). Wells 1-5 contain specimens H1, H2, H3, H4, and H5.

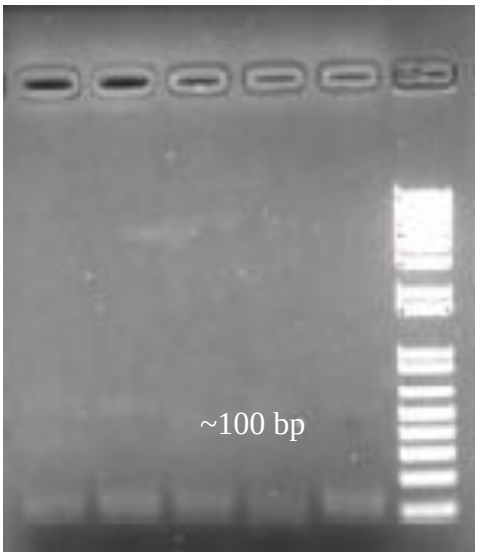

Figure 9 PCR amplification results with a 1% agarose gel for microsatellite Ppo020. Product size is ~100 (bp). Wells 1-4 contain specimens D-0043, D-0044, D-0045, and D-0046.

Microsatellite Characterization using Capillary Electrophoresis

Microsatellite alleles of sizes 211-217 bp, 106-131 bp, 137-173 bp, and 102-120 bp were isolated by loci Ppo004, Ppo005, Ppo018, and Ppo020, respectively (Table 3). A homozygote was identified at locus Ppo004 (Fig. 10). Heterozygotes were identified at loci Ppo005, Ppo018, and Ppo020 (Fig. 11; Fig. 12; Fig. 13). Of the individuals screened, 30% demonstrated heterozygosity at locus Ppo005, 25% demonstrated heterozygosity at locus Ppo018, and 40% demonstrated heterozygosity at locus Ppo020.

Table 3 Total number of alleles currently identified for each pair of primers and their respective allelic size range.

Microsatellites	Alleles Detected	Allelic Size Range (bp)	Potential Heterozygotes (%)
Ppo004	5	211-217	0
Ppo005	7	106-131	30
Ppo018	5	137-173	25
Ppo020	4	102-120	40

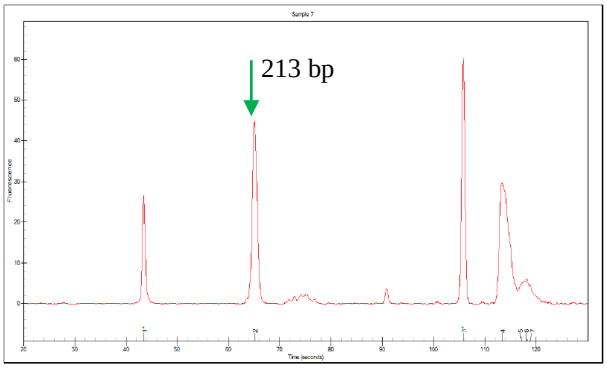

Figure 10 Experion™ capillary electrophoresis results for a homozygous Ppo004 microsatellite locus. Green arrow indicates allele of interest of 213 (bp) in size. This allele was isolated from specimen H7.

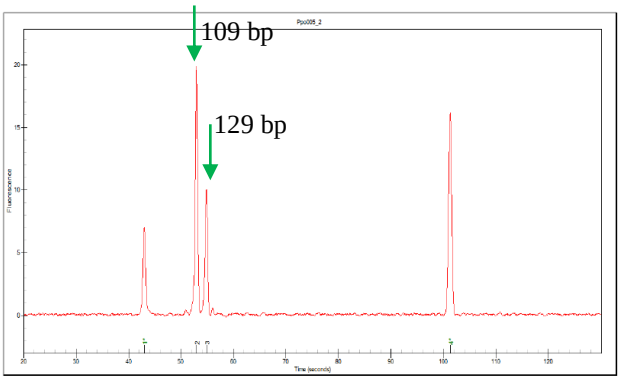

Figure 11 Experion™ capillary electrophoresis results for a heterozygous Ppo005 microsatellite locus. Green arrows indicate alleles of interest of sizes 109 (bp) and 129 (bp). These alleles were isolated from specimen H7.

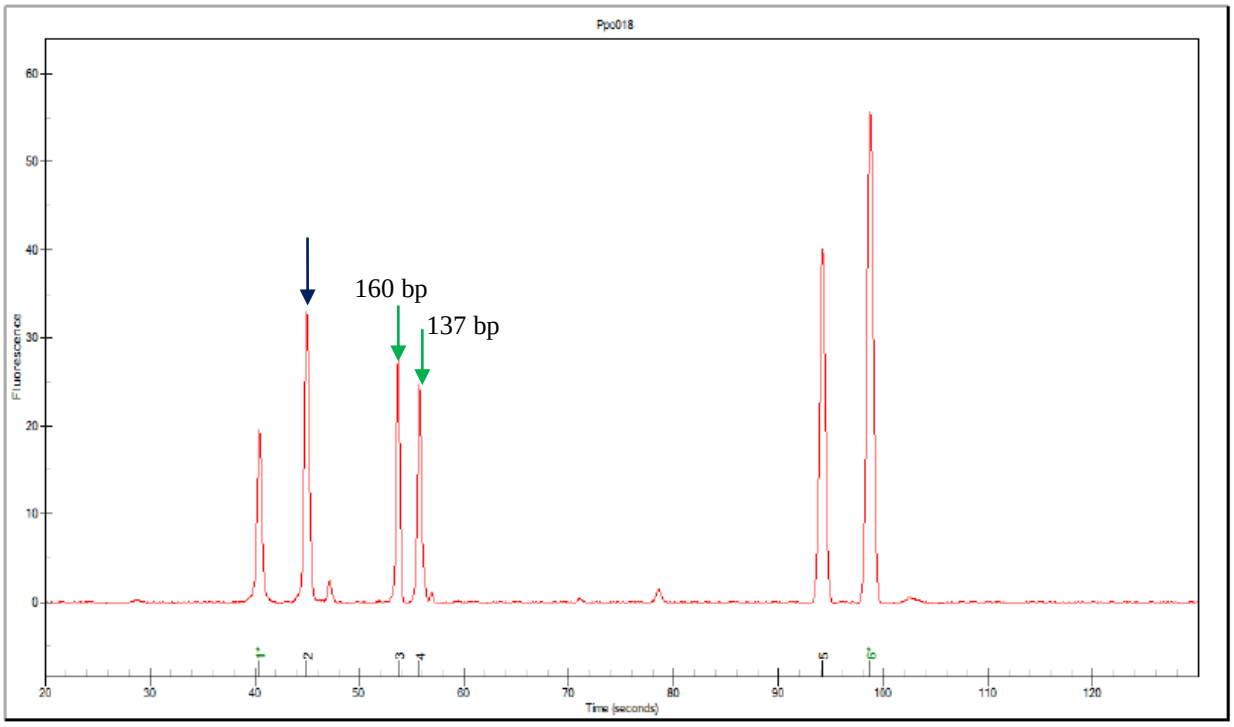

Figure 12 Experion™ capillary electrophoresis results for a heterozygous Ppo018 microsatellite locus. Green arrows indicate alleles of interest of sizes 137 (bp) and 160 (bp). Blue arrow indicates probable primer dimer. These alleles were isolated from specimen D-0044.

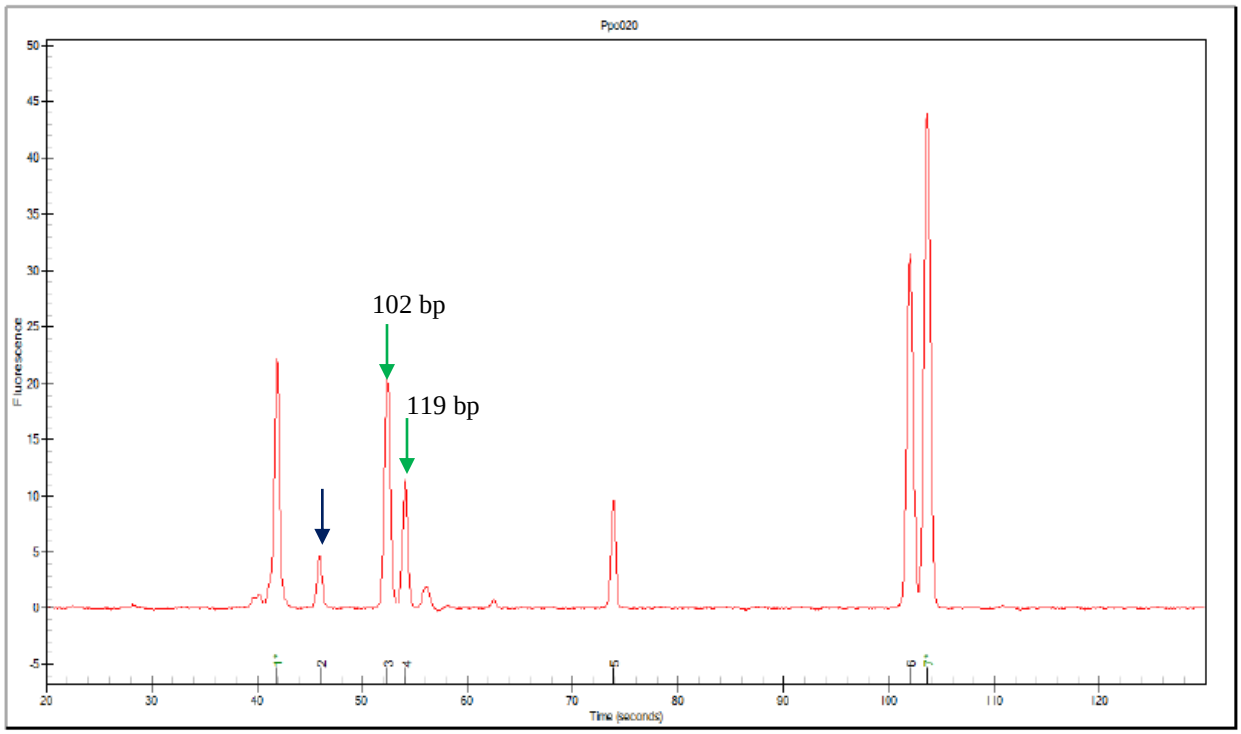

Figure 13 Experion™ capillary electrophoresis results for a heterozygous Ppo020 microsatellite locus. Green arrows indicate alleles of interest of sizes 102 and 119 (bp). Blue arrow indicates probable primer dimer. These alleles were isolated from specimen D-0044.

DISCUSSION

In this study, we managed to identify microsatellite sequences and their respective flanking regions using next-generation, shotgun sequencing technology. We were able to design primers that can successfully isolate microsatellite loci for an endemic species of freshwater mussels. Moreover, we were able to optimize the PCR conditions and protocols for these primers. These microsatellite loci have been analyzed by capillary electrophoresis and have exhibited polymorphism, and three of them exhibited potential heterozygosity (Table 3; Fig. 10; Fig. 11; Fig. 12; Fig. 13).

Once a large enough sample size is obtained, these 4 microsatellites will undergo a screening process to calculate expected heterozygosity, observed heterozygosity, null allele frequency, deviations from Hardy-Weinberg equilibrium, and linkage disequilibrium. The expected and observed heterozygosity should provide us insight into the population structure for a local *P. popeii* population. Testing for null allele frequency will allow us to determine if microsatellite loci are being misrepresented as a result of alleles not being amplified during PCR reaction runs. Testing for deviations from Hardy-Weinberg equilibrium for a microsatellite locus allows us to determine if the locus is under selection and can therefore be inherited in a Mendelian manner. Lastly, testing for linkage disequilibrium can ensure that microsatellite loci are being inherited independently from each other.

Once screened, these microsatellites can be used by researchers to conduct population genetic studies on *P. popeii*. The information currently available on populations of *P. popeii* is based on mark-and-recapture methods and timed searches (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007). Although these methods are useful in estimating populations size and species diversity, these approaches do not provide information on effective

population size, bottlenecks, migration patterns, gene flow, and genetic diversity (Burlakova & Karatayev, 2011; Burlakova & Karatayev, 2012; Carman, 2007). The use of microsatellites to conduct population genetic studies would allow for a more complete assessment on the status of populations of *P. popeii.*

Currently, the populations in the Black River in Eddy County, New Mexico, and in the Rio Grande in Webb County, Texas, are assumed to be properly reproducing based on juvenile mussel recruitment (Carmen, 2007; Karatayev *et al.*, 2012). Microsatellites analysis can substantiate the presumed reproductive health of these two populations by measuring their effective population size (Jehle & Arntzen, 2002; Selkoe & Toonen, 2006). These two populations are also believed to be stable and likely to survive in the near future (Burlakova & Karatayev, 2012; Carman, 2007). However, these assumptions are based on population size and the presence of multiple age classes in their populations and not on genetic information (Burlakova & Karatayev, 2012; Carmen 2007; Karatayev *et al.*, 2012). If these populations are lacking genetic diversity, it is likely that they are susceptible to a reduction in population fitness (Reed & Frankham, 2003). The use of microsatellites can provide researchers additional insight into the stability of these populations, which can be valuable information when establishing conservation priorities.

Populations in the Black River and those in the Rio Grande are believed to be isolated due to being hydrologically separated by large dams and reservoirs (USFWS, 2013). This can be verified by using microsatellites to test the occurrence of gene flow between these populations. If gene flow is occurring, that would suggest interbreeding is somehow taking place between these populations. For example, populations of mountain plover in the Rocky Mountains were previously believed to be isolated from each other, yet researchers demonstrated with the use of

microsatellites that gene flow was taking place among the populations (Oyler-McCance *et al.*, 2008). The authors concluded that the populations are interbreeding and were able to determine migration patterns (Oyler-McCance *et al.*, 2008). In the case of *P. popeii*, gene flow between the two populations would suggest host fish are able to displace glochidia over long distances. This knowledge would provide insight into host fish migration patterns and how they can influence the distribution of *P. popeii.*

Microsatellite studies of gene flow can also help identify unknown barriers among the known locations of *P. popeii* within the Rio Grande and the Devils River. A study on the red fox population in Hokkaido Island, Japan, identified a genetically isolated subpopulation despite the red fox's ability for wide dispersal and the lack of any outstanding geological barrier (Oishi *et al.*, 2011). This allowed the authors to propose possible ecological reasons for this isolation (Oishi *et al.*, 2011). In Texas, microsatellite analysis of *P. popeii* can help researchers identify subtle barriers that could be separating *P. popeii* populations, such as point sources of pollution or other types of anthropogenic effects. This information could be important when planning conservation management strategies and regulations.

The imperiled status of *P. popeii* reflects the threatened status of several mussel species throughout the world. This demonstrates an international need to survey and monitor many of these endangered species. The use of microsatellite analysis for population surveys and monitoring can provide additional information regarding the population structures of freshwater mussels that cannot be obtained through mark-and-recapture and tagging methods. Although designing microsatellite primers had been traditionally time consuming and expensive, the rise of next-generation sequencing has mitigated many of the costs and practical implications associated with the use of microsatellites (Csencsics *et al.*, 2010; Gardner *et al.*, 2011). As a result, the use

of microsatellites has become a more viable option for ecologists involved in conservation management research (Csencsics *et al.*, 2010; Gardner *et al.*, 2011).

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VITA

Name: Aldo Vazquez

Address: 508 Gale St. Apt. #7, Laredo, Texas, 78041

Email Address: aldovazquez@dusty.tamiu.edu

Education: B.S., Biology, Texas A&M International University

Awards:

Honor Roll: Fall, 2009, and Spring, 2012

Dean's List: Spring, 2010, Fall, 2011, Fall 2012, and Spring, 2013

TAMIU General Scholarship

University Honors Program: Fall 2010-Spring 2013

University Honors Scholarship

JAMP Scholarship: Fall, 2011-Fall 2012.

Completion of 2012 Summer Premedical Academy

Lamar Bruni Vergara (LBV) Assistantship Program: September 2013-December 2013.

Graduate Retention Enhancement at TAMIU (GREAT) Program: Current recipient since January 2014.

2nd Place in the LBV Conference Spring 2015

2nd Place in the Life Science Category in the TAMUS 12th Annual Pathways Student Research Symposium

2nd Place Overall Winner in the TAMUS 12th Annual Pathways Student Research Symposium