Research Article

Microsatellite evidence for multiple paternity in non-native populations of *Pomacea canaliculata* (Caenogastropoda: Ampullariidae) in China

Qian-Qian Yang[a], Su-Wen Liu[a], Jia-Nan Li, Dao Wang and Xiao-Ping Yu*

Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, College of Life Science, China Jiliang University, Hangzhou 310018, China

Author e-mails: *yqq@cjlu.edu.cn (QQY), suwenliu1234@163.com (SWL), 673977033@qq.com (JNL), 745043845@qq.com (DW), yxp@cjlu.edu.cn (XPY)*

[a]*These authors contributed equally to this work*

Corresponding author

Abstract

Reproductive success is critical for the establishment and spread of non-native species. The apple snail, *Pomacea canaliculata,* is a dioecious and internally fertilizing freshwater gastropod that causes serious economic losses in its non-native ranges annually. Previous studies on *P. canaliculata* using laboratory-controlled experiments indicated that mating with a second male displaces sperm from the first one, suggesting that multiple paternity occurs in this snail. To investigate the occurrence and frequency of multiple paternity in natural populations, we provide results from two polymorphic microsatellite loci in eight egg clutches collected from four localities in China. By genotyping the hatchlings from each population, we found at least one to three siring fathers per clutch. The paternal contributions of the multiply sired clutches were strongly skewed in *P. canaliculata*. The total population genetic diversity in the hatchlings exhibited higher observed heterozygosity than that in the adult snails. Increased offspring heterozygosity through multiple paternity is a potential mechanism that may aid *P. canaliculata* in overcoming genetic bottlenecks, and enhance the ability to adapt to new habitats as an invasive species.

Key words: apple snails, parentage analysis, GERUD, microsatellites, genetic variation

Introduction

Polyandry is a universal phenomenon observed in a wide variety of animal and plant taxa (McCullough et al. 2017; Noble et al. 2013; Rhodes et al. 2017). Multiple males contributing to a single brood of offspring, which is called multiple paternity, is a straightforward result in many polygamous species (Taylor et al. 2014). Studies of parentage and sperm utilization patterns in marine, land and freshwater snails have shown that multiple paternity commonly occurs in gastropods (Brante et al. 2011; Dupont et al. 2006; Kupfernagel et al. 2010; Nakadera et al. 2016). Despite the cost of mating, hypotheses predict that multiply mated females and their offspring will exhibit an increase in the mean variance in fitness compared with those in monandrous mating systems (Holman and Kokko 2013). The

elevated offspring performance in multiple-paternity broods is assumed to result from direct benefits, such as obtaining nuptial feedings (Burela and Martín 2011, 2014), and indirect benefits, such as "good genes" benefits (Neff and Pitcher 2005), genetic compatibility (Zeh and Zeh 1996), or increased genetic diversity (Brown 1997). For example, multiple-paternity strategies in the European invasive gastropod *Crepidula fornicata* enhance sex reversal and promote reproductive insurance (Dupont et al. 2006); a high level of multiple paternity in the freshwater snail *Chilina dombeiana* is considered an efficient strategy to avoid inbreeding and to increase the hetozygosity of their offspring (Bórquez and Brante 2017).

Successful reproduction is a critical trait for establishing new populations of introduced non-native species (McFarland et al. 2016; Rossi Lafferriere et al. 2016; Gibbs et al. 2017). Originating from South America, *Pomacea canaliculata* has been introduced to, and become established in many areas of Asia, North America, Europe, and Oceania since the 1980s (Hayes et al. 2008). Since that time, the snail has continuously colonized new habitats and spread rapidly in its introduced ranges, causing serious threats to local ecology and agriculture (Mochida 1991; Cowie 2002; Yang et al. 2018a). Studies on mating systems and breeding strategies of *P. canaliculata* have provided knowledge knowledge for understanding the mechanisms underlying its successful invasion, and also for effective control of the invasive populations (Estebenet and Cazzaniga 1993; Lach et al. 2000; Tamburi and Martín 2011).

Pomacea canaliculata is a dioecious and internally fertilizing freshwater snail (Cowie 2002; Winik et al. 2009) that deposits calcareous eggs above the waterline with an average of ~ 260 eggs per clutch (Hayes et al. 2012). Both females and males of *P. canaliculata* are frequently observed mating with different partners (Albrecht et al. 1996; Burela and Martín 2011). The female tract of *P. canaliculata* has a seminal receptacle for sperm storage and fertilization (Andrews 1964; Hayes et al. 2015). The stored sperm are able to fertilize for up to 140 days after copulation, laying up to 3,000 viable eggs during this period (Estebenet and Cazzaniga 1993; Estebenet and Martín 2002). Sperm storage provides an opportunity for multiple paternity in *P. canaliculata*. Moreover, previous studies of both shell color polymorphism and genetic variation in offspring have indicated that mating with a second male displaces sperm from the first one in *P. canaliculata*, suggesting that multiple paternity occurs in this snail (Yamamoto et al. 2018; Yusa 2004).

Microsatellite loci are highly polymorphic markers and have proven effective in parental relationship analyses in a wide range of animal groups (Calsbeek et al. 2007; Paterson et al. 2001; Thonhauser et al. 2014). Microsatellite loci have been tested in assessing the paternity of *P. canaliculata* in laboratory-controlled experiments in which a female mates with two males (Yamamoto et al. 2018). However, the paternity patterns in wild populations of the invasive apple snails are largely unknown. In this study,

we collected egg clutches and adults of *P. canaliculata* from natural populations in China. By genotyping the hatchlings of egg clutches and adults using microsatellite loci, we aimed to determine the paternity construction of *P. canaliculata* and illustrate the genetic effects of multiple paternity on its successful invasion.

Materials and methods

Specimen collection and DNA extraction

During November 2016–September 2017, we collected egg clutches of apple snails from four geographical populations in China. There were three populations from Zhejiang Province, the Xihu District (HZXH) and Jianggan District (HZJG) of Hangzhou City, and Wenling (TZWL) of Taizhou City, and one population from Guangdong Province, the Tianhe District of Guangzhou City (GZTH) (Table S1). The establishment of apple snails in the four localities occurred in different years. The HZXH and HZJG populations were reported no more than ten years ago (Yang et al. 2016); the TZWL population was introduced nearly 20 years ago (Yu et al. 2001); the GZTH population was among the first introduced populations in China over 35 years ago (Yang et al. 2010).

Two egg clutches from each population were employed in the analyses. Eggs were permitted to hatch in a greenhouse at China Jiliang University at 25 ± 1 °C under a relative humidity of 65 ± 5% and a 16:8 h light:dark cycle. After calculating the egg numbers and hatching rates of each clutch, we preserved the hatchlings in 95% ethanol and stored them at −20 °C. We sampled 100 hatchlings from each HZXH clutch, 96 hatchlings from each HZJG clutch, and 48 hatchlings from each of the TZWL and GZTH clutches. The sampling proportions were 8.9–39.5% of total hatchings from each clutch depending on the size and hatching rates. We also collected 26, 37, 25, and 14 snails from the HZXH, HZJG, TZWL, and GZTH populations, respectively.

Genomic DNA was extracted from whole hatchlings or the foot tissue of adult snails using a DNeasy Blood and Tissue Extraction Kit (Tiangen, China) following the manufacturer's protocol. DNA obtained from each sample was eluted in 100 μL of sterile deionized water and stored at −20 °C.

Species identification

The maternally inherited mitochondrial COI gene was used to confirm species identities. The COI barcode region of two hatchlings per clutch and each adult was amplified with the primers LCO1490 and HCO2198 (Folmer et al. 1994) using the method described by Yang et al. (2018b).

Microsatellite loci and genotyping

We tested microsatellite loci from five previously published papers (Chen et al. 2011; Guo et al. 2013; Matsukura et al. 2016; Sun et al. 2012; Yamamoto

Table 1. Characterization of two microsatellite loci used in paternity analysis. HEX and ROX are names of fluorescent dyes.

Locus	Sequence (5'-3')	Repeat motif	Size range (bp)	Tm (°C)	References
PcN4	F-HEX: CCAACAAGCTAACATACAAT R: TTCACTGCCTGACCTGTA	CAA	129–151	50	(Yamamoto et al. 2018)
PcPm06	F-ROX: CAATATTCAGATTAACACCG R: AGCAAACATCACAGATCAGC	ATG	112–181	53	(Matsukura et al. 2016)

et al. 2018). The loci were screened for amplification in 25 μL reactions containing 0.625 U of TaKaRa *Ex* Taq, 1× *Ex* Taq buffer, 0.2 mM of each dNTP, 0.08–0.16 μM of each primer, and 1 μL of genomic DNA. The following PCR conditions were used: one cycle of 95 °C for 5 min, 48–51 °C for 1 min, and 72 °C for 1 min, followed by 34 cycles of 95 °C for 60 s, 50–53 °C for 20 s, and 72 °C for 20 s. A final extension at 72 °C for 30 min and an incubation period of 4 °C for 10 min were used to terminate each reaction. The amplification of each locus was optimized under different annealing temperatures. PCR products were visualized in 1% agarose gels.

Four loci, i.e., Pc76 (Guo et al. 2013), Pc82 (Chen et al. 2011), PcN4 (Yamamoto et al. 2018), and PcPm06 (Matsukura et al. 2016), amplified single and clear PCR products of the expected size, which were subsequently tested for polymorphism in 100 individuals from HZXH-C1 before being applied to other clutches. Of these loci, the genotyping results showed that Pc82 produced stutters and Pc76 was invariant in most individuals tested. Thus, we chose PcN4 and PcPm06 for further analyses and labeled their forward primers with fluorescent dyes (Table 1). Fluorescently labeled PCR products were mixed in one reaction tube with a total volume of no more than 15 μL and then electrophoresed on an ABI 3730 XL DNA Analyzer with GS-500 standard (Qingke Biotechnology, China). The volume of different fluorescently labeled PCR products was calculated by analyzing gel electrophoresis images, using ImageJ 1.8.0 (http://rsbweb.nih.gov/ij/) to balance the amount of each product in one reaction.

Allele sizes were determined using Geneious 9.0 (Kearse et al. 2012) with the microsatellite plugin. Each profile was verified individually, and only samples with unambiguous and strong peaks were included in the analyses. The number of alleles, size range of alleles, and observed and expected heterozygosities were calculated using Popgene 1.2 (Raymond and Rousset 2007). Null alleles were determined using Micro-Checker 2.2.3 with four methods, i.e., the Oosterhout, Chakraborty, Brookfield 1, and Brookfield 2 methods (Van Oosterhout et al. 2010). Deviations from Hardy-Weinberg equilibrium at each locus and genotypic linkage disequilibrium were estimated using Genepop 4.2 (Raymond and Rousset 2007).

Paternity analyses

Parental inference analyses were conducted the minimum set of parents using an exhaustive search algorithm implemented in GERUD 2.0 (Jones 2005) without the requirement of the genotype of either parent. GERUD 2.0

Table 2. Hardy-Weinberg exact tests for the two microsatellite loci used in paternity analysis.

Locus	P_p	P_{HD}	P_{HE}	P_{HD} (Global)	P_{HE} (Global)
PcN4	0	0	1		
PcPm06	0	0.0004	0.9996	0.022	0.981

P_p, Hardy-Weinberg probability test; P_{HD}, Heterozygote deficiency test; P_{HE}, Heterozygote excess test; P_{HD} (Global), Heterozygote deficiency test across all loci using Fisher's method; P_{HE} (Global), P-value for the heterozygote excess test across all loci using Fisher's method.

also estimates the number of offspring sired by each father. When multiple minimum-parent solutions are possible for a given progeny array, this program ranks the solutions and chooses the most likely one based on patterns of Mendelian segregation and expected genotypic frequencies in the population (Jones 2005).

Results

COI sequences and species identification

The COI sequences obtained from the hatchlings and adult snails were deposited in GenBank under accession numbers MH211400–MH211415 and MK189323–MK189424. Based on the phylogenetic analysis of COI sequences, all egg clutches used in the analyses were molecularly identified as *P. canaliculata*. There were 24, 14, 21, and 14 snails from the HZXH, HZJG, TZWL, and GZTH populations identified as *P. canaliculata* and included in genotyping, respectively.

Characterization of microsatellite loci

The numbers of alleles of PcN4 detected in the egg clutches were two in the HZXH population and four in the HZJG, TZWL, and GZTH populations, with an average number of 3.5. The numbers of alleles of PcPm06 detected in the egg clutches were five in the HZXH population, four in both the HZJG and TZWL populations, and three in the GZTH population, with an average number of four (Table S2). The observed heterozygosity of the hatchlings ranged from 0.147 to 0.552 for PcN4 and from 0.158 to 0.885 for PcPm06 (Table S2).

The overall observed heterozygosity rates of PcN4 were higher in the hatchlings than in the adults in all four populations; the observed heterozygosity rates of PcPm06 were higher in the hatchlings than in the adults in the HZXH and GZTT populations, but the reverse was observed in the HZJG and TZWL populations (Tables S2, S3). The overall observed heterozygosity rates of both loci were higher in the hatchlings than in the adults (Tables S2, S3). Both the PcN4 and PcPm06 loci showed significant ($p < 0.05$) deviations from Hardy-Weinberg equilibrium expectations and exhibited a heterozygote deficiency (Table 2). The predicted null allele frequencies were 0.0044–0.0384 for PcN4 and 0.1758–0.2398 for PcPm06 among the four methods.
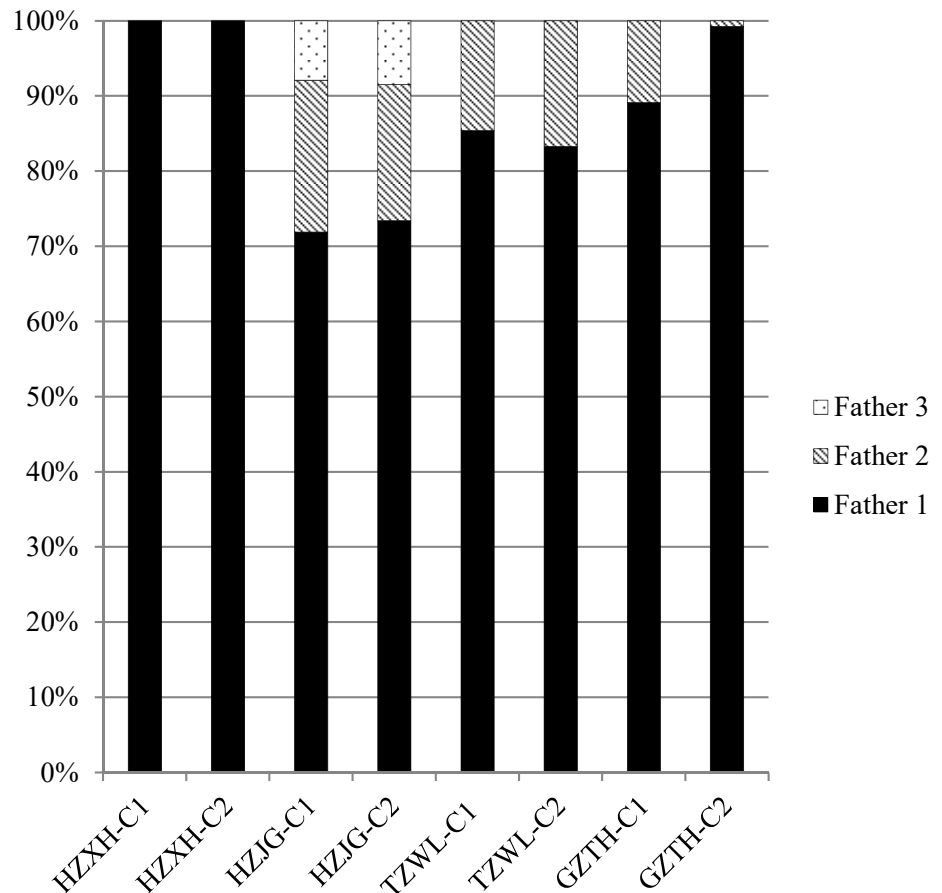
Figure 1. Relative contribution of fathers inferred by GERUD 2.0 to clutches from non-native Chinese populations of *Pomacea canaliculata*.

Paternity analysis

From the analyses of the PcPm06 and PcN4 microsatellite markers, we detected one to three siring fathers per egg clutch among the four populations (Figure 1). The siring numbers were the same in the two clutches of each population. One siring father was detected per clutch from the HZXH population, with sampling 10.4% and 31.2% of the total hatchlings from the two clutches, respectively. Three siring fathers were detected per clutch in the HZJG population, with sampling 27.6% and 39.5% of the total hatchlings per clutch, while two siring fathers were detected for each clutch in the TZWL and GZTH populations, with sampling 8.9–9.9% and 23.3–25.2% of the total hatchlings per clutch, respectively. Among the multiple-paternity clutches in the three populations, paternity was obviously biased to one father, accounting for 71.9–93.3% of the paternity in different clutches.

Discussion

The present study provides the first molecular evidence of the occurrence and frequency of multiple paternity in wild non-native populations of *P. canaliculata*. Our findings, based on microsatellite assessments of 544 hatchlings from eight clutches, indicated a moderate level of multiple

paternity (at least one to three fathers per clutch) in the sampled invasive populations of *P. canaliculata*. Based on 41 microsatellite loci obtained from both the literature, only two screened loci were effective in our samples. Both loci deviated from Hardy-Weinberg equilibrium and exhibited a heterozygote deficiency, which was probably due to insufficiently large populations, inbreeding, and null alleles. Despite the different sampling percentages, the two clutches from each population exhibited the same number of fathers. Our samples covered populations established for different numbers of years in China, with the assumption that the longer-established populations would have accumulated more detectable variation than the recently established ones; however, we did not find obvious differences between the geographical locations and the siring levels.

Our results on the number of siring fathers per clutch were consistent with the mating frequency from laboratory observations, in which egg laying occurred when females mated twice on average in isolated pairs of *P. canaliculata* (Albrecht et al. 1996). However, we expected that the siring level in wild populations would be higher for the following reasons. First, since only a proportion of each clutch was analyzed, the estimated number of siring fathers contributing to a single clutch may be lower than the true number. Second, our analyses employed only two loci, with some populations having fewer alleles in the adults than in the hatchlings, which may have also resulted in underestimation of the numbers of sires. Although the exact number of microsatellite loci necessary to estimate a certain level of parentage has not been determined (Xue et al. 2014), it has been reported that the accuracy of parentage determination increases with the number of microsatellite loci (Harrison et al. 2013). Moreover, the levels of polymorphism of the markers are a very important factor for obtaining accurate assignments (Sefc and Koblmüller 2009; Harrison et al. 2013). Therefore, markers with high polymorphism, such as single nucleotide polymorphisms (SNPs), could provide more conclusive results on the level of multiple paternity and may reveal comprehensive insights into this strategy.

We found that the siring contribution was strongly skewed to one of the inferred fathers in the multiple-sire clutches of *P. canaliculata*, leading to an unequal proportion of offspring among fathers, with one male fathering > 70% of the progeny. Skewed paternity patterns have been detected in other species with multiple paternity, such as the marine gastropod *Rapana venosa* (Xue et al. 2014) and the invasive crayfish *Procambarus clarkii* (Yue et al. 2010). A laboratory experiment on *P. canaliculata* in which two males were mated with one female revealed that the fathers contributing to the progeny were strongly skewed but with a higher proportion of offspring sired by the second male than by the first male (Yamamoto et al. 2018). The skewed contribution of sires indicates that sperm competition and/or cryptic female choice might have occurred, leading to post-copulatory paternity bias in *P. canaliculata*. However, for a better understanding of

the post-copulatory selection strategy in *P. canaliculata*, it would be necessary to track sperm donors of the offspring by analyzing parentage in controlled experiments.

In *P. canaliculata*, females often obtain nuptial gifts secreted by males during copulation (Burela and Martín 2011, 2014), which indicates that females may gain direct benefits through multiple mating. Additionally, our data showing higher genetic heterozygosity in the hatchlings than in the adults suggest that females may also receive indirect benefits from multiple paternity, which contribute to the long-term maintenance of genetic diversity in offspring. Non-native species often lose their genetic diversity when only small numbers of propagules are introduced to new regions, known as founder effect, and bottleneck effects may occur in established populations (Lombaert et al. 2010). Previous population genetic studies have shown that Chinese populations of *P. canaliculata* have a very limited number of haplotypes, which indicates low genetic diversity, suggesting that these non-native populations have undergone genetic drift caused by founder and bottleneck effects (Lv et al. 2013; Yang et al. 2018a). An increase in genetic diversity is often thought to increase the invasion success of non-native species (Ellstrand and Schierenbeck 2000; Milne and Abbott 2004). Previous studies have reported that hybridizing with other species (i.e., *Pomacea maculata*) is one way to increase the genetic diversity of offspring in *P. canaliculata* (Hayes et al. 2012; Matsukura et al. 2013). Multiple paternity is probably another way to help optimize genetic diversity and increase the invasion success of *P. canaliculata*.

In summary, this study has revealed the occurrence and frequency of multiple paternity in non-native populations of *P. canaliculata*. Multiple paternity probably serves as a potential strategy for *P. canaliculata* to overcome the loss of genetic diversity caused by genetic drift and bottlenecks. We found that paternity was strongly skewed, while previous studies indicated that mating with a second male displaces sperm from the first one in *P. canaliculata* (Yamamoto et al. 2018; Yusa 2004). However, questions on whether sperm competition and/or postcopulatory female choice happened, or how multiple paternity strategies affect the fitness of offspring in *P. canaliculata*, still require further exploration. Nevertheless, our study is informative for furthering the understanding of invasion success and evolution of apple snails.

Acknowledgements

References

Albrecht EA, Carreño NB, Castro-Vazquez A (1996) A quantitative study of copulation and spawning in the South American apple-snail, *Pomacea canaliculata* (Prosobranchia: Ampullariidae). *The Veliger* 39: 142–147

Andrews EB (1964) the functional anatomy and histology of the reproductive system of some pilid gastropod molluscs. *Journal of Molluscan Studies* 36: 121–140, https://doi.org/10.1093/oxfordjournals.mollus.a064939

Bórquez J, Brante A (2017) Paternity outcomes in the freshwater gastropod, *Chilina dombeiana* in the Biobío River, Chile. *PLoS ONE* 12: e0169574, https://doi.org/10.1371/journal.pone.0169574

Brante A, Fernández M, Viard F (2011) Microsatellite evidence for sperm storage and multiple paternity in the marine gastropod *Crepidula coquimbensis*. *Journal of Experimental Marine Biology and Ecology* 396: 83–88, https://doi.org/10.1016/j.jembe.2010.10.001

Brown JL (1997) A theory of mate choice based on heterozygosity. *Behavioral Ecology* 8: 60–65, https://doi.org/10.1093/beheco/8.1.60

Burela S, Martín PR (2011) Evolutionary and functional significance of lengthy copulations in a promiscuous apple snail, *Pomacea canaliculata* (Caenogastropoda: Ampullariidae). *Journal of Molluscan Studies* 77: 54–64, https://doi.org/10.1093/mollus/eyq035

Burela S, Martín PR (2014) Nuptial gifts in *Pomacea canaliculata* (Ampullariidae, Caenogastropoda): experimental and field evidence about their function. *Malacologia* 57: 319–327, https://doi.org/10.4002/040.057.0205

Calsbeek R, Bonneaud C, Prabhu S, Manoukis N, Smith TB (2007) Multiple paternity and sperm storage lead to increased genetic diversity in Anolis lizards. *Evolutionary Ecology Research* 9: 495–503, https://doi.org/10.1007/s10682-006-9004-4

Chen L, Xu H, Li H, Wu J, Ding H, Liu Y (2011) Isolation and characterization of sixteen polymorphic microsatellite loci in the golden apple snail *Pomacea canaliculata*. *International Journal of Molecular Sciences* 12: 5993–5998, https://doi.org/10.3390/ijms12095993

Cowie RH (2002) Apple snails (Ampullariidae) as agricultural pests: their biology, impacts and management. In: Barker G (ed), Molluscs as Crop Pests. CAB-International, Wallingford, UK, pp 145–192, https://doi.org/10.1079/9780851993201.0145

Dupont L, Richard J, Paulet Y, Thouzeau G, Viard F (2006) Gregariousness and protandry promote reproductive insurance in the invasive gastropod *Crepidula fornicata*: evidence from assignment of larval paternity. *Molecular Ecology* 15: 3009–3021, https://doi.org/10.1111/j.1365-294x.2006.02988.x

Ellstrand NC, Schierenbeck KA (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences of the United States of America* 97: 7043–7050, https://doi.org/10.1073/pnas.97.13.7043

Estebenet AL, Cazzaniga NJ (1993) Egg variability and the reproductive strategy of *Pomacea canaliculata* (Gastropoda: Ampullariidae). *APEX* 8: 129–138

Estebenet AL, Martín PR (2002) *Pomacea canaliculata* (Gastropoda: Ampullariidae): life-history traits and their plasticity. *Biocell* 26: 83–89

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299, https://doi.org/10.4028/www.scientific.net/DDF.7.460

Gibbs M, Watson P, Johnson-Sapp K, Lind C (2017) Reproduction revisited - a decade of changes in the reproductive strategies of an invasive catfish, *Pterygoplichthys disjunctivus* (Weber, 1991), in Volusia Blue Spring, Florida. *Aquatic Invasions* 12: 225–239, https://doi.org/10.3391/ai.2017.12.2.10

Guo J, Chen L, Xu H, Ding H, Wu J, Liu Y, Chen B (2013) Identification of twelve polymorphic microsatellite loci in the golden apple snail *Pomacea canaliculata*. *Journal of Genetics* 92: 1–4, https://doi.org/10.1007/s12041-013-0289-0

Harrison H, Saenz-Agudelo P, Planes S, Jones G, Berumen M (2013) Relative accuracy of three common methods of parentage analysis in natural populations. *Molecular Ecology* 22: 1158–1170, https://doi.org/10.1111/mec.12138

Hayes KA, Joshi RC, Thiengo SC, Cowie RH (2008) Out of South America: multiple origins of non-native apple snails in Asia. *Diversity and Distributions* 14: 701–712, https://doi.org/10.1111/j.1472-4642.2008.00483.x

Hayes KA, Cowie RH, Thiengo SC, Strong EE (2012) Comparing apples with apples: clarifying the identities of two highly invasive Neotropical Ampullariidae (Caenogastropoda). *Zoological Journal of the Linnean Society* 166: 723–753, https://doi.org/10.1111/j.1096-3642.2012.00867.x

Hayes KA, Burks RL, Castro-Vazquez A, Darby PC, Heras H, Martín PR, Qiu JW, Thiengo SC, Vega IA, Wada T (2015) Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). *Malacologia* 58: 245–302, https://doi.org/10.4002/040.058.0209

Holman L, Kokko H (2013) The consequences of polyandry for population viability, extinction risk and conservation. *Philosophical Transaction of the Royal Society B* 368: 20120053, https://doi.org/10.1098/rstb.2012.0053

Jones AG (2005) GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. *Molecular Ecology Notes* 5: 708–711, https://doi.org/10.1111/j.1471-8286.2005.01029.x

Kearse M, Moir R, Wilson A, Stoneshavas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649, https://doi.org/10.1093/bioinformatics/bts199

Kupfernagel S, Rusterholz HP, Baur B (2010) Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphrodite land snail. *Biological Journal of the Linnean Society* 99: 350–361, https://doi.org/10.1111/j.1095-8312.2009.01361.x

Lach L, Britton DK, Rundell RJ, Cowie RH (2000) Food preference and reproductive plasticity in an invasive freshwater snail. *Biological Invasions* 2: 279–288, https://doi.org/10.1023/a:1011461029986

Lombaert E, Guillemaud T, Cornuet J, Malausa T, Facon B, Estoup A (2010) Bridgehead effect in the worldwide invasion of the biocontrol Harlequin ladybird. *PLoS ONE* 5: e9743, https://doi.org/10.1371/journal.pone.0009743

Lv S, Zhang Y, Liu H, Hu L, Liu Q, Wei F, Guo Y, Steinmann P, Hu W, Zhou X (2013) Phylogenetic evidence for multiple and secondary introductions of invasive snails: *Pomacea* species in the People's Republic of China. *Diversity and Distributions* 19: 147–156, https://doi.org/10.1111/j.1472-4642.2012.00924.x

Matsukura K, Okuda M, Cazzaniga NJ, Wada T (2013) Genetic exchange between two freshwater apple snails, *Pomacea canaliculata* and *Pomacea maculata* invading East and Southeast Asia. *Biological Invasions* 15: 2039–2048, https://doi.org/10.1007/s10530-013-0431-1

Matsukura K, Hayes KA, Cowie RH (2016) Eleven microsatellite loci for two invasive freshwater apple snails, *Pomacea canaliculata* and *P. maculata* (Ampullariidae). *Biological Invasions* 18: 1–4, https://doi.org/10.1007/s10530-016-1237-8

McCullough EL, Buzatto BA, Simmons LW (2017) Benefits of polyandry: molecular evidence from field-caught dung beetles. *Molecular Ecology* 26: 3546–3555, https://doi.org/10.1111/mec.14127

McFarland K, Soudant P, Jean F, Volety AK (2016) Reproductive strategy of the invasive green mussel may result in increased competition with native fauna in the southeastern united states. *Aquatic Invasions* 11: 411–423, https://doi.org/10.3391/ai.2016.11.4.06

Milne RI, Abbott RJ (2004) Geographic origin and taxonomic status of the invasive Privet, *Ligustrum robustum* (Oleaceae), in the Mascarene Islands, determined by chloroplast DNA and RAPDs. *Heredity* 92: 78–87, https://doi.org/10.1038/sj.hdy.6800385

Mochida O (1991) Spread of freshwater *Pomacea* snails (Pilidae, Mollusca) from Argentina to Asia. *Micronesica* Supplement 3: 51–62

Nakadera Y, Mariën J, Straalen NMV, Koene JM (2016) Multiple mating in natural populations of a simultaneous hermaphrodite, *Lymnaea stagnalis*. *Journal of Molluscan Studies* 83: 56–62, https://doi.org/10.1093/mollus/eyw043

Neff BD, Pitcher TE (2005) Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. *Molecular Ecology* 14: 19–38, https://doi.org/10.1111/j.1365-294x.2004.02395.x

Noble DWA, Keogh JS, Whiting MJ (2013) Multiple mating in a lizard increases fecundity but provides no evidence for genetic benefits. *Behavioral Ecology* 24: 1128–1137, https://doi.org/10.1093/beheco/art040

Paterson IG, Partridge V, Buckland-Nicks J (2001) Multiple paternity in *Littorina obtusata* (Gastropoda, Littorinidae) revealed by microsatellite analyses. *Biological Bulletin* 200: 261–267, https://doi.org/10.2307/1543508

Raymond M, Rousset F (2007) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249, https://doi.org/10.1093/oxfordjournals.jhered.a111573

Rhodes MK, Fant JB, Skogen KA (2017) Pollinator identity and spatial isolation influence multiple paternity in an annual plant. *Molecular Ecology* 26: 4296–4308, https://doi.org/10.1111/mec.14115

Rossi Lafferriere NA, Antelo R, Alda F, Mårtensson D, Hailer F, Castroviejo-Fisher S, Ayarzagüena J, Ginsberg JR, Castroviejo J, Doadrio I, Vilá C, Amato G (2016) Multiple paternity in a reintroduced population of the Orinoco Crocodile (*Crocodylus intermedius*) at the El Frío biological station, Venezuela. *PLoS ONE* 11: e0150245, https://doi.org/10.1371/journal.pone.0150245

Sefc KM, Koblmüller S (2009) Assessing parent numbers from offspring genotypes: the importance of marker polymorphism. *Journal of Heredity* 100: 197–205, https://doi.org/10.1093/jhered/esn095

Sun J, Wang M, Wang H, Zhang H, Zhang X, Thiyagarajan V, Qian PY, Qiu JW (2012) De novo assembly of the transcriptome of an invasive snail and its multiple ecological

applications. *Molecular Ecology Resources* 12: 1133–1144, https://doi.org/10.1111/1755-0998.12014

Tamburi NE, Martín PR (2011) Effects of food availability on reproductive output, offspring quality and reproductive efficiency in the apple snail *Pomacea canaliculata*. *Biological Invasions* 13: 2351–2360, https://doi.org/10.1007/s10530-011-0047-2

Taylor ML, Price TAR, Wedell N (2014) Polyandry in nature: a global analysis. *Trends in Ecology & Evolution* 29: 376–383, https://doi.org/10.1016/j.tree.2014.04.005

Thonhauser KE, Thoã M, Musolf K, Klaus T, Penn DJ (2014) Multiple paternity in wild house mice (*Mus musculus musculus*): effects on offspring genetic diversity and body mass. *Ecology and Evolution* 4: 200–209, https://doi.org/10.1002/ece3.920

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2010) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Resources* 4: 535–538, https://doi.org/10.1111/j.1471-8286.2004.00684.x

Winik BC, Catalán M, Gamarra-Luques C, Castro-Vazquez A (2009) Ectaquasperm-like parasperm in an internally fertilizing gastropod. *Invertebrate Biology* 128: 223–231, https://doi.org/10.1111/j.1744-7410.2009.00158.x

Xue D, Zhang T, Liu JX (2014) Microsatellite evidence for high frequency of multiple paternity in the marine gastropod *Rapana venosa*. *PLoS ONE* 9: e86508, https://doi.org/10.1371/journal.pone.0086508

Yamamoto S, Komasu H, Kitaura J, Aoyama T, Iwaguchi SI, Nakamura M, Kawane M, Collins TM, Yusa Y (2018) Development of 11 microsatellite markers and paternity analysis in the invasive apple snail *Pomacea canaliculata*. *Venus* 76: 79–84

Yang Q, Liu S, Ru W, Liu G, Yu X (2016) Molecular identification of invasive golden apple snails in Zhejiang Province based on DNA barcoding. *Biodiversity Science* 24: 341–350

Yang Q, Liu S, He C, Yu X (2018a) Distribution and the origin of invasive apple snails, *Pomacea canaliculata* and *P. maculata* (Gastropoda: Ampullariidae) in China. *Scientific Reports* 8: 1185, https://doi.org/10.1038/s41598-017-19000-7

Yang Q, Liu S, He C, Cowie RH, Yu X, Hayes KA (2018b) Invisible apple snail invasions: importance of continued vigilance and rigorous taxonomic assessments. *Pest Management Science* 75: 1277–1286, https://doi.org/10.1002/ps.5241

Yang Y, Hu Y, Li X, Wang X, Mu X, Song H, Wang P, Liu C, Luo J (2010) Historical invasion, expansion process and harm investigation of *Pomacea canaliculata* in China. *Chinese Agricultural Science Bulletin* 26: 245–250

Yu X, Wada T, Zhong L, Zhong L, Sun L, Zhu Y, Chen J, Zhen X, Xu H (2001) Occurrence of golden apple snail, *Pomacea canaliculata* (Lamarck), in paddy fields and its management. *Acta Agriculturae Zhejiangensis* 13: 247–252

Yue G, Li J, Wang C, Xia J, Wang G, Feng J (2010) High prevalence of multiple paternity in the invasive crayfish species, *Procambarus clarkii*. *International Journal of Biological Sciences* 6: 107–115, https://doi.org/10.7150/ijbs.6.107

Yusa Y (2004) Inheritance of colour polymorphism and the pattern of sperm competition in the apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Journal of Molluscan Studies* 70: 43–48, https://doi.org/10.1093/mollus/70.1.43

Zeh JA, Zeh DW (1996) The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 263: 1711–1717, https://doi.org/10.1098/rspb.1996.0250

Supplementary material

The following supplementary material is available for this article:

Table S1. Information for egg clutches of the four non-native populations of *Pomacea canaliculata* used in paternity analysis. *N*: total number of eggs per clutch.

Table S2. Polymorphism of the two microsatellite loci in hatchlings of the four non-native Chinese populations of *Pomacea canaliculata*. *n,* number of individuals analyzed; *A*, number of alleles; Ho, mean observed heterozygosity; He, mean expected heterozygosity.

Table S3. Polymorphism of the two microsatellite loci in adult snails of the four non-native Chinese populations of *Pomacea canaliculata*. *n,* number of individuals analyzed, the numbers before and after "/" are the number of individuals analyzed for PcN4 and PcPm06, respectively; *A*, number of alleles; Ho, mean observed heterozygosity; He, mean expected heterozygosity.

This material is available as part of online article from:

http://www.reabic.net/aquaticinvasions/2019/Supplements/AI_2019_Yang_etal_SupplementaryTables.xlsx