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ONLINE RESOURCES

Identification of twelve polymorphic microsatellite loci in the golden apple snail *Pomacea canaliculata*

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[Guo J., Chen L., Xu H., Ding H., Wu J., Liu Y. and Chen B. 2013 Identification of twelve polymorphic microsatellite loci in the golden apple snail *Pomacea canaliculata*. *J. Genet*. **92**, e99–e102. Online only: http://www.ias.ac.in/jgenet/OnlineResources/92/e99.pdf]

Introduction

Invasive species are recognized as the second largest threat to biodiversity after habitat loss (Lowe *et al*. 2000). Of South American origin (Argentina), the golden apple snail *Pomacea canaliculata* was introduced in Taiwan as human food around 1980 and subsequently in other East Asian and Southeast Asian countries (Hayes *et al*. 2008). However, commercial cultivation of the snail failed and it is now a serious pest of rice and other aquatic crops, especially in Southeast Asia (Cowie 2002), causing serious changes in the diversity and functioning of invaded natural wetlands (Carlsson *et al*. 2004; Fang *et al*. 2010). These snails are tolerant to variable temperatures, salinities and humidity, making them eminently suited for successful dispersal (Cowie 2002). In China, rapid spread of *P. canaliculata* has caused ecological problems and great economic losses in southern provinces. This species is nominated as one of the 100 worst invasive alien species in the world (Lowe *et al*. 2000) and one of the 16 invasive species reported in China by the State Environmental Protection Administration of China (http://www.mep.gov.cn/gkml/zj/wj/200910/t20091022_172155.htm).

Management strategies to control an invasive species can be improved through the knowledge of the reproductive behaviour, dispersal mode and population structure (Sakai *et al*. 2001). Many aspects of the biology, ecology and behaviour of *P. canaliculata* have been well studied, yet population genetic studies have lagged behind thus far (Rawlings *et al*. 2007; Hayes *et al*. 2008). A good way to study population genetic variation is through the use of specific molecular markers. Among them, microsatellites are the most useful due to their high abundance and polymorphisms, ease of genotyping and codominant inheritance. Microsatellites have been successfully applied to different invasive species to characterize their genetic structure, infer evolutionary aspects underlying their invasive processes and to identify the routes of their colonization (Lombaert *et al.* 2011; Guggisberg *et al.* 2012). Here, we characterize 12 microsatellite loci from *P. canaliculata* for ongoing population genetic studies.

Materials and methods

Polymorphic microsatellites were isolated from an enriched genomic DNA library constructed following the protocol of Gardner *et al*. (1999) with minor modifications. Genomic DNA was extracted from foot tissue of an individual captured at Shaoguan, Guangdong, southern China. Approximately 2 μg of the genomic DNA were digested with *Sau*3AI restriction enzyme (New England Biolabs, Ipswich, USA). The 300–800 bp fragments obtained were ligated to *Sau*3AI adaptors: oligo A: 5′-GGCCAGAGACCCCAAGCTTCG-3′ and oligo B: 5′-pGATCCGAAGCTTGGGGTCTCTGGCC-3′. The ligated fragments were hybridized and enriched with magnetic beads (Streptavidin magnesphere Paramagnetic Particles, Promega, Madison, USA) coated with 5′ biotinylated probe $(GA)_{10}$ and $(GACA)_6$. Nonspecifically bound and unbound DNA were removed by several nonstringent and stringent washes. These microsatellite-enriched DNA fragments were PCR-amplified again and then ligated into pGEM-T Easy vectors (Promega) and cloned into JM109 competent cells (Takara, Dalian, China). A combination of three primers, two vector primers (M13 forward (–20): 5′-GTAAAACGACGGCCAG-3′; M13 reverse: 5′-CAGGAAACAGCTATGAC-3′) and an oligonucleotide $5'(GA)_{10}3'$ or $5'(GACA)_6 3'$, depending on the biotinylated oligonuleotide was used to screen white colonies by polymerase chain reactions (PCR) for the presence of inserts with

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Keywords. golden apple snail; microsatellite; population genetics; *Pomacea canaliculata*.

Table 1. Characterization of the 12 microsatellite loci identified in *P. canaliculata.*

Locus	Primer sequence (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	N_a	H_O	H_E	GenBank acc. no.
Pc76	F: 5′ TAMRA-TCAAGTGTAGAGACAGGAA 3′ R: 5′ TGACATTAGGGATTAGGAT 3′	$(GA)_{26}$	227–273	55	11	0.792	0.849	KC206355
Pc504	F: 5′ 6-FAM-GATGATTTTCTCGTGCG 3′ R: 5′ GTGGTATCCTCGGTTTG 3′	$(GA)_{32}$	192–212	55	9	0.667	0.844	KC206356
Pc665	F: 5′ 6-FAM-CAGGCGTGAAGAAGGTGT 3′ R: 5′ GCGAGAAGCAGACGATTG 3′	$(GA)_{32}$	142–182	57	10	0.792	0.850	KC206357
Pc4169	F: 5′ 6-FAM-AGGGAAGCCTCCTCTCAA 3′ R: 5′ TACTCGCCCATCGCAAT 3′	$(GACA)_{14}$	173–185	55	4	0.500	0.529	KC206358
Pc4173	F: 5′ HEX-GCTCAAAGGCTGGTCTCC 3′ R: 5′ CGGGGTTCATTCCTGCTC 3′	$(GACG)_5(GACA)_5$	181–269	60	9	0.792	0.866	KC206359
Pc4244	F: 5′ TAMRA-ACACCAGTGGATGCCCTT 3′ R: 5′ TGTCCTCTCCGCCTCAAC 3′	$(AGGAC)_{12}$	184–264	57	5	0.500	0.748	KC206360
Pc4267	F: 5′ 6-FAM-GCGACCTGAGAAAAGCA 3′ R: 5′ TGTGTGCTGTGAGTGAGTCT 3′	$(CCAG)_{11}$	220–292	62	6	0.609	0.722	KC206361
Pc4288	F: 5′ 6-FAM-TGACTGAACACAGAAGAGACT 3′ R: 5′ TTCGCTCAAAGACATCG 3′	$(AACA)_{10}(GACA)_8$	196–272	55	7	0.125	0.777*	KC206362
Pc4343	F: 5′ HEX-CAAACGGCAAGAAATGA 3′ R: 5′ TGTGTGAAAGAGCAGCAG 3′	$(GACA)_{22}$	143–195	55	6	0.636	0.684	KC206363
Pc4347	F: 5′ 6-FAM-ATGAAGGACCAACCGAT 3′ R: 5′ AGTATGCCAAGATAACAAAT 3′	$(TC)_7A(GACA)_{23}$	193–261	55	6	0.542	0.675	KC206364
Pc4412	F: 5′ HEX-TGCGGGCACACACAGATAC 3′ R: 5′ TGCGAATGCGGAGGTTA 3′	$(GACA)_{10}GGCA(GACA)_5$	181–285	60	10	0.583	0.820	KC206365
Pc4444	F: 5′ 6-FAM-CCGCAGACATTTCCACAA 3′ R: 5′ TTTACGCTCCCTGGCACT 3′	$(GTCC)_7$	152–192	53	5	0.500	0.705	KC206366

T_a, annealing temperature; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *significant deviations from HWE after sequential Bonferroni correction.

microsatellite repeats. Clones giving two (or more) bands, after 2% agarose electrophoresis, were considered likely to contain a microsatellite and were amplified with the two vector primers. Positive clones were sequenced using an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, USA). For sequences containing microsatellites, primers were designed using Primer 3 (Rozen and Skaletsky 2000).

Variability was tested in 24 individuals of *P. canaliculata* collected in Shaoguan, southern China. PCR amplifications were carried out in 15-μL final reaction volumes containing about 20 ng of *P. canaliculata* genomic DNA, *Ex Taq* premix buffer 7.5 μL (Takara, Dalian, China) and 0.5 pmol of each of forward and reverse primers, forward primers were labelled with 6-FAM, HEX or TAMRA. PCR amplification cycles were as follows, an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 30 s at the locus specific optimal annealing temperature (see table 1) and 30 s at 72°C, followed by a final extension of 7 min at 72°C. Labelled fragments were then discriminated using capillary electrophoresis on an ABI PRISM 3730 xl DNA Analyzer (Applied Biosystems, Foster City, USA) and allele sizes were determined using GeneMapper ver. 4.0 (Applied Biosystems, Foster City, USA).

Calculations of expected and observed heterozygosity were done using the software Cervus 3.0 (Marshall *et al*. 1998). Linkage disequilibrium (LD) between pairs of loci and deviations from Hardy–Weinberg equilibrium (HWE) were examined using GenePop 4.0 (Rousset 2008). Results of tests for LD and HWE were corrected for multiple comparisons by applying sequential Bonferroni corrections (Rice 1989). MicroChecker 2.2.3 (Van Oosterhout *et al*. 2004) was used to identify possible null alleles, large allele dropout, scoring errors and typographic errors. The frequency of null alleles was calculated for each locus using FreeNA (Chapuis and Estoup 2007), with the maximum-likelihood estimation from Dempster *et al*. (1977).

Results and discussion

A total of 129 recombinant clones were sequenced and among which 44 successfully amplified primer pairs, 12 loci showed polymorphism in 24 individuals. The basic information of each microsatellite locus is shown in table 1. The number of alleles per locus ranged from 4 to 11, whereas the observed and expected heterozygosity ranged from 0.500 to 0.792 and from 0.529 to 0.866, respectively (table 1).

Only one of the 66 tests performed to detect LD was significant at $P < 0.05$, but it was not significant after applying the sequential Bonferroni correction, indicating that loci are not closely linked and that they can be treated as independent variables.

Deviations from HWE were observed for three loci at $P < 0.05$, only the locus Pc4288 was significant after Bonferroni correction, which may be due to the presence of null alleles as suggested by MicroChecker software. Results from MicroChecker analysis indicated the possible occurrence of null alleles at five of the microsatellites (Pc504, Pc4244, Pc4288, Pc4412 and Pc4444). However, there was no evidence for scoring error due to stuttering or large allele dropout. Except for locus Pc4288 (value = 0.364), the Dempster algorithm indicated that null alleles were present at intermediate frequency for loci Pc504 (value = 0.103), Pc4244 (value = 0.157), Pc4412 (value = 0.118) and Pc4444 (value = 0.126). Microsatellite null alleles have been found in a wide range of taxa (Dakin and Avise 2004), but seem to be particularly prevalent in mollusks (Li *et al*. 2003; Arias-Pérez *et al*. 2012). When null alleles are present, heterozygotes bearing null alleles will be mistyped as an excess of homozygotes leading to deviations from HWE. In addition, null alleles, lower apparent genetic variability, they may erroneously inflate levels of genetic differentiation and affect population genetic analyses that rely on HWE. The values of null allele frequency of all loci in this study were lower than 0.2 (except for Pc4288), indicating that null alleles are not expected to cause significant problems in the analysis (Chapuis and Estoup 2007). Therefore, only locus Pc4288 should be considered to be potentially problematic and corrected for allele frequency errors by using different procedures. Fortunately, some methods are now available to analyse microsatellite data in the presence of null alleles. For example, the FreeNA software allows computing unbiased F_{ST} estimates corrected for the presence of null alleles (Chapuis and Estoup 2007). These microsatellites described here will be useful for population genetic studies of *P. canaliculata*. Combined use of these markers and mtDNA to elucidate the population structure and spread of *P. canaliculata* in China are underway.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (NSFC) project (grant no. 31100274), Natural Science Foundation of Jiangsu Province (grant no. BK20131087), the National Key Technologies Research and Development Programme (grant no. 2012BAC01B01).We thank Li Ma and Yanqing Wu for their assistance and contribution in collecting samples.

References

Arias-Pérez A., Fernández-Tajes J., Gaspar M. B. and Méndez J. 2012 Isolation of microsatellite markers and analysis of genetic diversity among East Altantic populations of the Sword Razor Shell *Ensis siliqua*: A tool for population management. *Biochem. Genet*. **50**, 397–415.

Carlsson N. O. L., Brönmark C. and Hansson L. A. 2004 Invading herbivory: the golden apple nail alters ecosystem functioning in Asian wetland. *Ecology* **85**, 1575–1580.

Chapuis M. P. and Estoup A. 2007 Microsatellite null alleles and estimation of population differentiation. *Mol. Biol. Evol*. **24**, 621–631.

Cowie R. H. 2002 Apple snails (Ampullariidae) as agricultural pests: their biology, impacts and management. In *Molluscs as*

crop pests (ed. G. M. Barker), CABI publishing, Wallingford, Oxon, UK.

Dakin E. E. and Avise J. C. 2004 Microsatellite null alleles in parentage analysis. *Heredity* **93**, 504–509.

Dempster A. P., Laird N. M. and Rubin D. B. 1977 Maximum likelihood from incomplete data via the EM algorithm. *J. R. Stat. Soc., B* **39**, 1–38.

Fang L., Wong P. K., Li L., Lan C., Qiu J. W. 2010 Impact of invasive apple snails in Hong Kong on wetland macrophytes, nutrients, phytoplankton and filamentous algae. *Freshwater Biol*. **55**, 1191–1204.

Gardner M. G., Cooper S. J. B., Bull C. M. and Grant W. N. 1999 Isolation of microsatellite loci from a social lizard, *Egernia stokesii*, using a modified enrichment procedure. *J. Hered*. **90**, 301–304.

Guggisberg A., Welk E., Sforza R., Horvath D. P., Anderson J. V., Foley M. E. *et al*. 2012 Invasion history of North American Canada thistle, *Cirsium arvense*. *J. Biogeogr*. **39**, 1919–1931.

Hayes K. A., Joshi R. C., Thiengo S. C. and Cowie R. H. 2008 Out of South America: multiple origins of non-native apple snails in Asia. *Divers. Distrib*. **14**, 701–712.

Li G., Hubert S., Bucklin K., Ribes V. and Hedgecock D. 2003 Characterization of 79 microsatellite DNA markers in the Pacific oyster, *Crassostrea gigas*. *Mol. Ecol. Notes* **3**, 228–232.

Lombaert E., Guillemaud T., Thomas C. E., Lawson Handley L. J., Li J., Wang S. *et al*. 2011 Inferring the origin of populations introduced from a genetically structured native range by approximate Bayesian computation: case study of the invasive ladybird, *Harmonia axyridis*. *Mol. Ecol*. **20**, 4654–4670.

Lowe S., Browne M., Boudjelas S. and De Poorter M. 2000 *100 of the world's worst invasive alien species*. A selection from the global invasive species database. ISSG, Auckland, New Zealand.

Marshall T. C., Slate J., Kruuk L. E. B. and Pemberton J. M. 1998 Statistical confidence for likelihood—based paternity inference in natural populations. *Mol. Ecol*. **7**, 639–655.

Rawlings T. A., Hayes K. A., Cowie R. H. and Collins T. M. 2007 The identity, distribution, and impacts of non-native apple snails in the continental United States. *BMC Evol. Biol*. **7**, 97.

Rice W. R. 1989 Analyzing tables of statistical tests. *Evolution* **43**, 223–225.

Rousset F. 2008 GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour*. **8**, 103–106.

Rozen S. and Skaletsky H. 2000 PRIMER 3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol*. **132**, 365–386.

Sakai A. K., Allendorf F. W., Holt J. S., Lodge D. M., Molofsky J., With K. A. *et al*. 2001 The population biology of invasive species. *Annu. Rev. Ecol. Syst*. **32**, 305–332.

Van Oosterhout C., Hutchinson W. F. and Shipley P. 2004 MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538.

Received 10 May 2013; accepted 1 July 2013
Published on the Web: 28 November 2013