have been attributed to: (i) existence of multiple copies of microsatellite sequences in the genome (Nève & Meglécz 2000); (ii) similarity of the flanking regions, which in some cases are almost identical (Meglécz et al. 2004); (iii) low frequency of microsatellite loci in Lepidoptera genomes (Ji et al. 2003; Zhang 2004); and (iv) a very high frequency of null alleles (Meglécz et al. 2007), probably the cause of loci departure from Hardy–Weinberg equilibrium. Using the ‘web of science’ search engine, we looked for Lepidoptera articles published in Molecular Ecology Notes between 2002 and 2008. We found 33 published papers, and 30 of them mentioned the complications discussed earlier regarding the isolation of microsatellite loci in Lepidoptera. We encountered the same difficulties, nevertheless, the technical approach used by us permitted to develop nine highly polymorphic microsatellites with no null alleles. These markers are expected to be useful tools for detecting bottleneck events and to estimate their severity.

Acknowledgements

The authors wish to thank Sabrina Baudry, Amandine Fossoud and Isabelle Merlin for technical assistance, Florence Mougel, Lisa Pope (Molecular Ecology Notes Subject Editor), and Michele Schiffer for their helpful comments and suggestions for improving the manuscript, and Yannery Gomez and Jean-Louis Zeddam for providing samples from Costa Rica and Guatemala.

References


doi: 10.1111/j.1755-0998.2009.02656.x

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Isolation and characterization of polymorphic microsatellite loci for the three-toed box turtle (Terrapene carolina triunguis) and cross-amplification in other Terrapene species

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Abstract

We isolated and characterized eight polymorphic microsatellite loci for a Texas population of three-toed box turtle, Terrapene carolina triunguis, using a refined hybridization capture

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North American box turtles (those having the ability to close their shell completely; genus Terrapene) typically are found in mesic woodlands, grasslands, and semi-arid deserts throughout the USA and tropical Mexico (Dodd 2001). Although the four acknowledged species (T. carolina, T. ornata, T. nelsoni, and T. coahuila) are thought of as ‘common’, Dodd (2001) cautions that even protected Terrapene populations have waned. Due to habitat fragmentation, commercial trade, and human-based mortality, North American box turtles are of significant conservation concern (Dodd 2001; Ceballos & Fitzgerald 2004; Smith 2004). As a result, several states have elevated particular Terrapene species to an endangered or threatened status. Herein, we describe the isolation and characterization of microsatellites for the three-toed box turtle (T. carolina), with successful cross-amplification in the ornate box turtle (T. ornata).

Whole blood extracted from the caudal femoral vein of two T. c. triunguis individuals (collected by Lutterschmidt et al. 2007, Walker County, Texas), was the source of genomic DNA. DNA was extracted using a DNeasy Kit (QIAGEN). A source genomic library was constructed by enriching extracted DNA for AG, TG, AAC, AAG, AAT, ACT, and ATC repeats following Glenn & Schable’s (2005) protocol. DNA was digested with Rsal and Xmnl, ligated to double-stranded SuperSNX linkers (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′ and 5′-GATCTGCTAGCTAGGCTAGCTGCTTAAAACAAAA-3′) and enriched for the above repeats via incubation with 3′-biotinylated oligonucleotides. Target DNA was captured by streptavidin-coated Dynabeads (Dynal) and recovered by polymerase chain reaction (PCR) using the SuperSNX Forward primer. Enriched DNA was ligated into pCR 2.1-TOPO TA plasmids (Invitrogen) and transformed into TOP 10 electrocompetent Escherichia coli cells (Invitrogen). Plasmids were isolated from white colonies with QIAprep Spin Minipreps (QIAGEN). Presence of inserts was verified by digestion with EcoRI and visualization on 0.8% agarose gels. Plasmids containing target-size inserts (500–1200 bp) were sequenced with M13 forward and reverse primers by Macrogen. Sequences were exported into msatcommander (Faircloth 2007) to automatically search for and design primers for microsatellite repeats.

Of 30 primer pairs designed, the eight pairs that produced consistent, scorable products were tested for polymorphism and are reported here. Genomic DNA was extracted from blood of 39 T. c. triunguis individuals collected in Walker County, Texas, using a modified proteinase K method (USDS Hendrick Laboratory; http://hedricklab.ucsd.edu/Protocol/TailPreps.html). One primer from each pair was made with a 5′-CAG tail (5′-CAGTCGGGCCGTCACTCA-3′) to use in a three-primer fluorescent labelling system described by Glenn (2001) and Boutin-Ganache et al. (2001). Primer sequences, specific repeats amplified, product size, and optimum annealing temperatures are listed in Table 1. PCR (modified from Glenn 2001) for 20-μL reactions was conducted on a Bio-Rad (model MyCycler thermocycler) as follows: 1× PCR buffer (Promega), 25 μg/mL BSA, 0.15 mM dNTPs, 1.5 mM MgCl₂, 0.5 μM regular untagged primer, 0.5 μM universal fluorescent primer, 0.05 μM regular tagged primer, 0.5 U Taq polymerase (Promega). Reactions were amplified for 15 cycles at specific optimal annealing temperatures (Table 1), then for an additional 40 cycles at 62 °C after the addition of a universal CAG primer with a 5′D4 WellRED fluorescent dye (Proligo, Sigma-Aldrich, Inc.). Amplified products (1 μL) were electrophoresed on a Beckman Coulter CEQ 8000 sequencer and allele sizes were determined using Beckman Coulter CEQ 8000 Genetic Analysis software (DNA size standard ~ 600).

Of the 39 DNA accessions assayed, 28 resulted in scorable products for more than 50% (5 of 8) of loci; therefore, only these 28 were included in analyses. All loci were amplified and ambiguous alleles were scored as ‘?’ rather than as null alleles. Summary characteristics are presented in Table 1. Linkage disequilibrium (LD), deviations from Hardy–Weinberg equilibrium (HWE), and null allele frequency (NA) were estimated with GenePop 4.0 (Raymond & Rousset 1995). The EM method was used to estimate NA and 95% confidence intervals. LD was not significant for any pair of alleles, even without the more conservative Bonferroni-corrected alpha (P < 0.01). Significant deviations from HWE were found for six loci (TCTB2, TCTG7, TCTL12, TCT011, TCTP11, and TCTS2). These results likely were due to inbreeding as indicated by correspondingly high FIS values for those loci (FIS: 0.2086–0.4475). It is unlikely that the presence of null alleles made contributions to HWE deviation detected at any locus, as none were significant (P > 0.05).

Overall genetic diversity was quantified by measuring number of alleles per locus (Nn), allele size range (bp), observed heterozygosity (Hs), expected heterozygosity (He), and inbreeding relative to subpopulation/fragment (FIS). Effective number of alleles (Ne) was estimated using the formula Ne = 1/Σ xi2, where xi is the frequency of the jth
allele at locus x (Kimura & Crow 1964). Individual inbreeding relative to subpopulation \( F_{\text{IStotal}} \), gene diversity intraindividuals \( (1 - Q_{\text{INTRA}}) \), gene diversity interindividual intrapopulation \( (1 - Q_{\text{INTER}}) \), allelic diversity \( (A) \), and mean \( n_e \) across all loci were estimated. Number of alleles ranged from 4 to 12, with allelic diversity of 7.750. All loci analyzed were polymorphic, with observed \( (H_o) \) and expected \( (H_e) \) heterozygosities ranging from 0.1852 to 0.6667 and from 0.2718 to 0.9094, respectively. The \( F_{\text{IStotal}} \) was moderate (0.2907), suggesting relatively low levels of inbreeding overall. Intra- and interindividual gene diversities (0.4247 and 0.5988, respectively) were robust, indicating that the sampled individuals did not exhibit significant genetic similarity.

In addition, all eight primer pairs amplified successfully at all loci in seven ornate box turtles \( (T. o. ornata) \) collected in Texas, showing cross-amplification. Linkage disequilibrium was detected for one pair of loci in \( T. o. ornata \) (TCTG7 and TCTP11, \( P = 0.0221 \)), and departures from HWE were found for two loci (TCTG7 and TCTP11). The number of alleles ranged from 2 to 7, with an allelic diversity of 5 and \( H_e \) from 0 to 0.7143 and from 0.2226 to 0.8901, respectively; \( F_{\text{IStotal}} \) was moderate (0.2462).

This study provides researchers with eight microsatellite primers for two species of threatened box turtles. These loci may be used for evaluating baseline population genetic structure and variation in the conservation efforts for box turtles \( (T. carolina \) and \( T. ornata) \), and can most likely be applied to other Terrapene species.

### Table 1

Characterization of eight variable microsatellite loci in the three-toed box turtle \( (T. carolina triunguia) \) sampled from 28 Texas individuals: locus with GenBank Accession numbers, primer sequence, repeat motif, optimal annealing temperature \( (T_{\text{A}}) \), number of alleles per locus \( (n_e) \), size range (bp), observed heterozygosity \( (H_o) \), expected heterozygosity \( (H_e) \), inbreeding relative to subpopulation/fragment \( (F_{\text{IS}}) \), effective number of alleles \( (n_e) \), and null allele frequency. Also reported are overall inbreeding relative to subpopulation/fragment \( (F_{\text{IStotal}}) \), gene diversity intraindividuals \( (1 - Q_{\text{INTRA}}) \), gene diversity inter-individuals intra-population \( (1 - Q_{\text{INTER}}) \), allelic diversity \( (A) \), and mean \( n_e \) across all loci.

<table>
<thead>
<tr>
<th>Locus (Accession no.)</th>
<th>Primer sequence 5'–3'</th>
<th>Repeat motif</th>
<th>( T_{\text{A}} ) (°C)</th>
<th>( N_e )</th>
<th>Size range (bp)</th>
<th>( H_o )</th>
<th>( H_e )</th>
<th>( F_{\text{IS}} )</th>
<th>( n_e )</th>
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<tbody>
<tr>
<td>TCTB2 (FJ150411) F:</td>
<td>AAACTTTCCTTGTGCGGACCACTT*</td>
<td>(GA)(10 \ldots (GA)(3 GT)_3</td>
<td>58</td>
<td>5</td>
<td>255–263</td>
<td>0.1852</td>
<td>0.2718</td>
<td>0.3229</td>
<td>1.364</td>
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<tr>
<td>R:</td>
<td>GGTCTTACATTTGCTGGTC</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>TCTG7 (FJ150415) F:</td>
<td>GAAAGCGGCTGGGTTGCTG</td>
<td>(CT)(_5 GT)_7</td>
<td>58</td>
<td>12</td>
<td>248–274</td>
<td>0.6667</td>
<td>0.9094</td>
<td>0.2718</td>
<td>8.909</td>
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<td>R:</td>
<td>AGTGGACAGATTTGTGCTGC</td>
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<tr>
<td>TCTL12 (FJ150416) F:</td>
<td>AGAGCGACCTCAAGTACACC*</td>
<td>(AC)(_5 \ldots (CT))_8</td>
<td>59</td>
<td>8</td>
<td>203–235</td>
<td>0.5000</td>
<td>0.6193</td>
<td>0.2815</td>
<td>3.080</td>
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<tr>
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<tr>
<td>TCTO11 (FJ150417) F:</td>
<td>TAAAGTGTCGCAAGGACCC*</td>
<td>(GA)(<em>4 )</em></td>
<td>60</td>
<td>4</td>
<td>245–255</td>
<td>0.3333</td>
<td>0.4193</td>
<td>0.2086</td>
<td>1.697</td>
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<tr>
<td>R:</td>
<td>CCCCCAAAAGCCTTGGACC</td>
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<td></td>
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<tr>
<td>TCTP11 (FJ150418) F:</td>
<td>GAAGATGCCGCTTCGGACCA</td>
<td>(CA)(_8 \ldots (CT))_8</td>
<td>58</td>
<td>4</td>
<td>241–247</td>
<td>0.4000</td>
<td>0.6936</td>
<td>0.4296</td>
<td>3.088</td>
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<tr>
<td>R:</td>
<td>TGGGAGCAGTATTGGACACC</td>
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<td>TCTQ7 (FJ150419) F:</td>
<td>ACTGGCCACTCCACTCATC</td>
<td>(TG)(_4 GA)_4</td>
<td>58</td>
<td>16</td>
<td>127–165</td>
<td>0.4762</td>
<td>0.8525</td>
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<td>GGGGCTGGCTTCCTTCTTC</td>
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<td>TCTR7 (FJ150420) F:</td>
<td>TCTGGGCCCTTTCCCTCCG*</td>
<td>(ATTT)(<em>6 )</em></td>
<td>57</td>
<td>6</td>
<td>183–211</td>
<td>0.2952</td>
<td>0.2962</td>
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<tr>
<td>TCTS2 (FJ150421) F:</td>
<td>GGAGGACATGATTGGACACC*</td>
<td>(TC)(_5 TG(TA))_3</td>
<td>58</td>
<td>11</td>
<td>202–230</td>
<td>0.6667</td>
<td>0.7917</td>
<td>0.1608</td>
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</table>

\( F_{\text{IStotal}} = 0.2907 \)

1 – \( Q_{\text{INTRA}} = 0.4247 \)

1 – \( Q_{\text{INTER}} = 0.5988 \)

\( A = 7.750 \)

\( n_e = 3.726 \)

*indicates primers labelled with a 5’-CAG tag.

### References


Kimura M, Crow JF (1964) The number of alleles that can be maintained in a finite population. Genetics, 39, 725–738.


doi: 10.1111/j.1755-0998.2009.02593.x

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