

Short Communication

Isolation and characterization of 17 polymorphic microsatellite loci for the widespread ascidian *Didemnum perlucidum* (Tunicata, Ascidiacea)

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Abstract

The colonial tunicate *Didemnum perlucidum* has been identified from numerous tropical and temperate locations worldwide, but its native and introduced ranges remain largely unknown. In Australia, *D. perlucidum* is listed as a target species under the introduced marine pest national monitoring network. In order to investigate the introduced status and potential routes of introduction and dispersal of *D. perlucidum*, we developed 17 new polymorphic microsatellite markers using 454 shotgun sequencing. Two to six alleles per locus were detected. No evidence of linkage disequilibrium between pairs of loci was identified and 12 of the 17 loci were in Hardy-Weinberg equilibrium.

Key words: Didemnidae, genetic markers, 454 sequencing, sea squirt, invasive, Western Australia

Introduction

The ascidian *Didemnum perlucidum* (Monniot, 1983) is a colonial tunicate with a reported worldwide distribution across the Atlantic, Pacific and Indian Oceans (Lambert 2002). Although *D. perlucidum* was first described from the island of Guadeloupe in the Caribbean (Monniot 1983), its native range is unknown. It is thought to have been introduced to many areas including Southern Brazil and Western Australia (WA) where it seems to have become established and has demonstrated invasive characteristics (da Rocha and Monniot 1995; Kremer et al. 2010; Munoz et al. 2015; Smale and Childs 2011). *Didemnum perlucidum* was first report in WA in the Swan River, Perth, in 2010 (Smale and Childs 2011). Since then, it has been found in numerous locations and has been listed as a target species under the introduced marine pest national monitoring network (Bridgwood et al. 2014).

Defining the status of a species as native or introduced is important as it can influence management decisions, but it is often challenging, particularly within communities which have been under the influence of human intervention (Carlton 1996). Molecular markers can be used to characterize species diversity at locations, providing important supporting evidence to identify native and introduced ranges, and investigate potential frequency and routes of colonization and dispersal (Rius et al. 2014; Stefaniak et al. 2012). In the present work we aimed to develop and characterise microsatellite markers to support invasion genetic studies on *D. perlucidum*.

Materials and methods

Next-generation sequencing

Genomic DNA (2.6 µg) was isolated from a 5 mg tissue sample from a *D. perlucidum* colony using

Table 1. Primer sequences, GenBank accession numbers, repeat motif, and levels of diversity at 17 microsatellite loci in the tunicate *Didemnum perlucidum*. Number of alleles (Na), polymorphic information content (PIC), observed heterozygosity (H_o), expected heterozygosity (H_E), probability value from a test for deviation from Hardy-Weinberg Equilibrium (P) and null allele frequency (F). F, N, V, and P indicate dyes FAM, NED, VIC, and PET respectively, followed by numbers 1, 2, 3, 4 and 5 indicating the five multiplexed sets of primers used.

| Locus | Primer Sequence (5'-3') | GenBank | Repeat Motif | Na | Size Range (bp) | PIC | H_o | H_E | P | F (Null) |
|--------------------|--|----------|---------------------|----|-----------------|------|-------|-------|-------|----------|
| Dp02 ^{V2} | F:AATATCGGAATGGACCACCA R:AGCTAGAAAGCGCCACAAAA | KT694049 | (TGAA) ₇ | 6 | 150-194 | 0.69 | 0.82 | 0.74 | 0.435 | -0.06 |
| Dp06 ^{F4} | F:TGACAGAGTGTAGCTCTACACCA R:GAGCGTGCTCTGTTTTGACA | KT694050 | (TAAA) ₅ | 3 | 150-170 | 0.30 | 0.15 | 0.33 | 0.000 | 0.37 |
| Dp07 ^{N1} | F:GAGCAGGACGACTATCTCGC R:CGCTATACAATTGCCGAAGC | KT694051 | (AAC) ₅ | 2 | 100-158 | 0.37 | 0.60 | 0.49 | 0.051 | -0.11 |
| Dp10 ^{V1} | F:AGCACAACATGACCACCCCT R:TTGCGCTACTTTTGGTTGAA | KT694052 | (ATC) ₅ | 4 | 100-132 | 0.46 | 0.54 | 0.54 | 0.218 | -0.02 |
| Dp13 ^{F2} | F:TCCTCGGTCAAGTTGTGTTG R:TGCTTGATCGTATTTGATGTTG | KT694053 | (ATT) ₅ | 4 | 200-222 | 0.46 | 0.59 | 0.53 | 0.427 | -0.06 |
| Dp14 ^{V1} | F:AAAATTTGGCAAACCTTAGCTATC R:TCCTGATATTTCCGTTTGGG | KT694054 | (AAT) ₁₅ | 5 | 200-226 | 0.62 | 0.74 | 0.68 | 0.258 | -0.04 |
| Dp15 ^{N2} | F:GTGGGATGTGAAACGCATTA R:CAACAACGCCAATTTGAACA | KT694055 | (CGTG) ₆ | 6 | 150-227 | 0.57 | 0.72 | 0.61 | 0.073 | -0.11 |
| Dp16 ^{P2} | F:CTTTCACGCAAGTAGGGCTC R:CGCTGCTACAAAATGACGAA | KT694056 | (AAAT) ₆ | 4 | 150-184 | 0.34 | 0.35 | 0.37 | 0.264 | 0.05 |
| Dp27 ^{V3} | F:TGCCTATACAACGATGACGG R:AGTAAAGTTTATGAAAGTAATGTTGGCA | KT694057 | (TAC) ₉ | 5 | 89-127 | 0.51 | 0.65 | 0.59 | 0.529 | -0.06 |
| Dp30 ^{V4} | F:ATTAGTGGTTTGTCTGACGG R:ACAGCAACAGCAACAACAGC | KT694058 | (TGG) ₆ | 5 | 200-267 | 0.49 | 0.26 | 0.57 | 0.00 | 0.38 |
| Dp42 ^{F3} | F:AGGCCTAAAGCAGGGGAGTA R:AAAATATGCATGTTTGGAGAA | KT694059 | (ATA) ₆ | 4 | 100-129 | 0.47 | 0.56 | 0.53 | 0.003 | -0.04 |
| Dp49 ^{V4} | F:CTTTCGTTTATTTCTCCGCC R:TCCAATACAGTCAAAACACAGCC | KT694060 | (GT) ₅ | 2 | 150-160 | 0.30 | 0.37 | 0.37 | 1.000 | -0.00 |
| Dp51 ^{P5} | F:CAATCCATCAGCAGAGCAA R:AGAGCACTCCACCATTTCAA | KT694061 | (TA) ₅ | 2 | 200-257 | 0.37 | 0.33 | 0.50 | 0.009 | 0.19 |
| Dp52 ^{N4} | F:CTTTCCAAACCATGCACCTT R:GCCCTCCTCAAATCTTTC | KT694062 | (AT) ₅ | 3 | 200-237 | 0.44 | 0.52 | 0.54 | 0.002 | 0.01 |
| Dp53 ^{F5} | F:GAAACCGATTAATTTCCGCA R:CTCAAGTTGATTTCGGAGGC | KT694063 | (TA) ₆ | 3 | 250-260 | 0.26 | 0.24 | 0.30 | 0.092 | 0.11 |
| Dp56 ^{N5} | F:GCTGCTTATAGCTGCTTGGTG R:GCCAAAGTAAATACCAACCACA | KT694064 | (AT) ₅ | 4 | 100-135 | 0.43 | 0.59 | 0.54 | 0.205 | -0.05 |
| Dp59 ^{F4} | F:CACCACAGCAAAGTCTACCG | KT694065 | (TA) ₅ | 2 | 100-110 | 0.03 | 0.03 | 0.03 | 1.000 | -0.00 |

a Fisher Biotec Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit. The DNA was sent to the Australian Genomic Research Facility for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX). The sample generated 133 198 individual sequences, with an average length of 399 bp, which were assembled into 2 292 contigs (806 of these > 500 bp). These contigs were scanned for Simple Sequence Repeats (SSRs) and a list of primer sequences and PCR conditions was generated for 247 perfect microsatellites using the open source QDD (v1.3) (Megléczy et al. 2010) and Primer3 (v2.3.3) (Rozen and Skaletsky 2000) software.

Primer testing

We selected 59 di-, tri-, tetra-, and penta-base repeat microsatellite loci with a PCR product of 80–480 bp for further development. These loci were trialled for amplification separately in 5 μ l

reactions containing 10 ng of DNA, 1 x MyTaq reaction buffer (containing 5 mM dNTP and 15 mM MgCl₂), 0.5 U MyTaq DNA polymerase (Bioline Reagents), and 0.2 μ M of each primer. The following PCR conditions were used: 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. PCR products were visualized on 3 % agarose gels stained with GelRed (Biotium Inc.) alongside a 100 base pair (bp) molecular weight marker (Axygen Biosciences) and visualised under UV light. Forty seven of the 59 targeted loci generated a product of the expected size. These loci were tested for polymorphism using DNA extracted from whole colonial tissue (with tunic) samples of seven distinct colonies collected by hand at Hillarys Boat Harbour (31°49'30.70"S, 115°44'07.71"E), Busselton (33°63'18.49"S, 115°39'31.81"E) and Dampier (20°66'30.50"S, 116°70'13.91"E), WA. From the 47 loci selected, 24 appeared polymorphic when

tested across the seven samples and were selected for fragment analysis.

Each of the forward primers for the 24 polymorphic loci selected for fragment analysis was labelled with a fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) and variation screened in 68 colonies of *D. perlucidum* collected by hand while snorkelling at Hillarys Boat Harbour, Perth, WA. PCR products (2.5 µl) were analysed on an ABI 3730 Sequencer, sized using the GeneScan-500 LIZ internal size standard and scored using GENEMARKER software (SoftGenetics).

We used CERVUS (Kalinowski et al. 2007) to calculate the number and range of allele sizes, polymorphic information content, observed and expected heterozygosity and the frequency of null alleles for each locus. To test for deviation from Hardy-Weinberg equilibrium and linkage disequilibrium between pairs of loci, we used the online version of GENEPOP 4.0 (Raymond and Rousset 1995). A sequential Bonferroni correction was applied to the tests for linkage disequilibrium (Rice 1989).

Results and discussion

Of the 47 loci initially screened, 24 (50%) produced PCR products with clear bands and appeared polymorphic after agarose gel electrophoresis. From these, 17 loci produced genotypes that were consistently scoreable. The number of alleles per locus ranged from two to six and the observed and expected heterozygosities ranged between 0.03 to 0.82, and 0.03 to 0.74 respectively (Table 1). Twelve of the 17 loci were in Hardy-Weinberg equilibrium, and there was no evidence of linkage disequilibrium between any pair of loci.

The markers developed here will prove useful in future studies investigating the origins of native and introduced populations of *D. perlucidum*, and potential high-risk routes of introduction. Such studies will support a science-based management approach to the future prevention, management and/or eradication of this important species.

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References

- Bridgwood SD, Munoz J, McDonald JI (2014) Catch me if you can! The story of a colonial ascidian's takeover bid in Western Australia. *BioInvasions Records* 3: 217–223, <http://dx.doi.org/10.3391/bir.2014.3.4.02>
- Carlton JT (1996) Biological invasions and cryptogenic species. *Ecology* 77: 1653–1655, <http://dx.doi.org/10.2307/2265767>
- Kalinowski S, Taper M, Marshall T (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1006–1099, <http://dx.doi.org/10.1111/j.1365-294X.2007.03089.x>
- Kremer LP, Rocha RM, Roper JJ (2010) An experimental test of colonization ability in the potentially invasive *Didemnum perlucidum* (Tunicata, Ascidiacea). *Biological Invasions* 12: 1581–1590, <http://dx.doi.org/10.1007/s10530-009-9571-8>
- Lambert G (2002) Nonindigenous Ascidiaceans in Tropical Waters. *Pacific Science* 56: 291–298, <http://dx.doi.org/10.1353/psc.2002.0026>
- Megléczy E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin JF (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403–404, <http://dx.doi.org/10.1093/bioinformatics/btp670>
- Monniot F (1983) Ascidiées littorales de Guadeloupe I. Didemnidae. *Bulletin du Musée d'Histoire Naturelle de Paris*, 4th series, 5: 5–49
- Munoz J, Page M, McDonald JI, Bridgwood SD (2015) Aspects of the growth and reproductive ecology of the introduced ascidian *Didemnum perlucidum* (Monniot 1983) in Western Australia. *Aquatic Invasions* 10: 265–274, <http://dx.doi.org/10.3391/ai.2015.10.3.02>
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249
- Rice WR (1989) Analysing tables of statistical tests. *Evolution* 43: 223–225, <http://dx.doi.org/10.2307/2409177>
- Rius M, Turon X, Bernardi G, Volckaert FAM, Viard F (2014) Marine invasion genetics: from spatio-temporal patterns to evolutionary outcomes. *Biological Invasions* 17: 869–885, <http://dx.doi.org/10.1007/s10530-014-0792-0>
- Rocha RM, Monniot F (1995) Taxonomic and ecological notes on some *Didemnum* species (Ascidiacea, Didemnidae) from Sao Sebastiao Channel, southeastern Brazil. *Revista Brasileira de Zoologia* 55(4): 639–649
- Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds), 'Bioinformatics Methods and Protocols: Methods in Molecular Biology'. Humana Press, Totowa, NJ, pp 365–386
- Smale DA, Childs S (2011) The occurrence of a widespread marine invader, *Didemnum perlucidum* (Tunicata, Ascidiacea) in Western Australia. *Biological Invasions* 14: 1325–1330, <http://dx.doi.org/10.1007/s10530-011-0167-8>
- Stefaniak L, Zhang H, Gittenberger A, Smith KF, Holsinger K, Lin S, Whitlatch RB (2012) Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002. *Journal of Experimental Marine Biology and Ecology* 422–423: 64–71, <http://dx.doi.org/10.1016/j.jembe.2012.04.012>