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RESEARCH ARTICLE

Assessment of high-resolution melting (HRM) profiles as predictors of microsatellite variation: an example in Providence Petrel (*Pterodroma solandri*)

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Abstract High-resolution melting (HRM) analysis is an emerging technology to screen microsatellites for polymorphism. A potential issue surrounding this method is that amplicon sizes for HRM should typically be short (80–100 bp) for highest sensitivity to reveal polymorphism via the presence of two peaks in the curve of the derivative of fluorescence over temperature (dF/dT). In contrast, microsatellite amplicons are typically 100–400 bp. Therefore, we compared HRM analysis melting temperature range (ΔT_m) and multiple dF/dT peaks for predicting microsatellite polymorphism. We assessed polymorphism at 27 microsatellite loci, with estimated lengths of 122–321 bp, in Providence Petrel (*Pterodroma solandri*). We validated HRM assessment using traditional capillary electrophoresis (CE). While 100 % of loci exhibiting multiple peaks in the dF/dT curve were confirmed as polymorphic by CE, 16 % improvement in sensitivity (83 vs. 67 %) was achieved by using ΔT_m, and 25 % (92 vs. 67 %) by using ΔT_m in addition to multiple dF/dT peaks. We suggest HRM melting temperature range as new predictor of polymorphism that can be used to rapidly assess microsatellites polymorphism.

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Introduction

Microsatellites or simple sequence repeats (SSRs) have been popular markers in population genetics for the last two decades due to their allelic variability, codominance and high reproducibility of scoring (Distefano et al. 2012). However, in many studies about half of candidate loci are rejected for use as a result of insufficient PCR amplification, monomorphism, or multicopy status. While developments in DNA sequencing technology have greatly expedited the discovery of microsatellites (Gardner et al. 2011), screening of loci for PCR amplification success and polymorphism remains a costly and time-consuming step (Arthofer et al. 2011; Guichoux et al. 2011). Recent advances in high-resolution melting (HRM) real-time PCR analysis can potentially expedite this process, reducing both time and monetary costs in comparison to traditional screening involving the use of labeled PCR primers and capillary electrophoresis (CE).

HRM is a closed-tube method based on PCR amplification in the presence of a saturating dye, e.g., EvaGreen, followed by a high-resolution melting step (Liew et al. 2004; Reed and Wittwer 2004; Wittwer et al. 2003). During the melting step, changes in the strength of fluorescence signal are recorded as the double-stranded DNA disassociates. This transition is a function of amplicon length and nucleotide composition (i.e., % of GC content), and is represented by peaks in the curve of the derivative of fluorescence over temperature (dF/dT) (Tindall et al. 2009). In theory, heterozygous individuals will produce a

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dF/dT curve containing two peaks; heteroduplex molecules will have a lower melting temperature (T_m), reflecting nucleotide mispairing in the double stranded molecule, resulting in an early peak in the dF/dT curve relative to the two possible homoduplex molecules, that are usually indistinguishable from each other (Fig. 1). More than two dF/dT peaks within an individual may represent multicopy loci, multiple mutated sites, or amplification of non-specific bands, while the absence of dF/dT peaks indicates insufficient amplification (Arthofer et al. 2011).

A potential issue surrounding the use of HRM to screen polymorphism of microsatellites is that amplicon sizes for HRM should typically be short (80–100 bp) for highest sensitivity (Gundry et al. 2008; Herrmann et al. 2006; Liew et al. 2004; Reed and Wittwer 2004). However, for microsatellite screening most PCR products will exceed this size owing to optimal placement of primers as inferred from primer design algorithms (e.g., Primer3) (Rozen and Skaletsky 2000), and constraints based on the length and frequency of the repeat motif (e.g., $[AAAG]_{15}$). Furthermore, in downstream analyses there is usually the desire to multiplex loci during CE, and therefore screen loci with a range of sizes (100–400 bp) (Guichoux et al. 2011). In those cases, HRM polymorphism detection decreases and a single dF/dT peak can appear in polymorphic loci, leading to a reduction of sensitivity (1-false negatives; %) of HRM analysis.

Previous study on HRM analysis shows that loci with a very narrow melting temperature ranges (ΔT_m) are less likely to be polymorphic (Arthofer et al. 2011). Here, we investigate whether variation in ΔT_m, rather than multiple peaks in dF/dT curves, can better predict polymorphism. Given the negative relationship between ΔT_m and fragment length (Arthofer et al. 2011; Gundry et al. 2008; Liew et al. 2004; Muleo et al. 2009; Smith et al. 2010), we also examined the effect of fragment length on polymorphism to verify that any relationship between ΔT_m and polymorphism does not simply reflect spurious correlation owing to a relationship between repeat number (and therefore fragment length) and polymorphism (Bachtrog et al. 2000; Brinkmann et al. 1998).

To examine these hypotheses we assessed polymorphism of 27 microsatellites using HRM in Providence Petrel (*Pterodroma solandri*) (Table 1), a pelagic seabird that is IUCN listed as Vulnerable due to a breeding range restricted to two islands (IUCN 2011). The loci screened have been developed from other members of Procellariiform (Table 1), and both amplification success and polymorphism are less likely in Providence Petrel in comparison to loci characterized directly from the target species (Crawford et al. 1998). In addition, there is no sequence information from Providence Petrel with which to redesign primers and reduce amplicon size in an effort to increase HRM sensitivity. Under these circumstances a rapid and low-cost (no requirement of labeled primers or electrophoresis) method to assess polymorphism is particularly desirable.

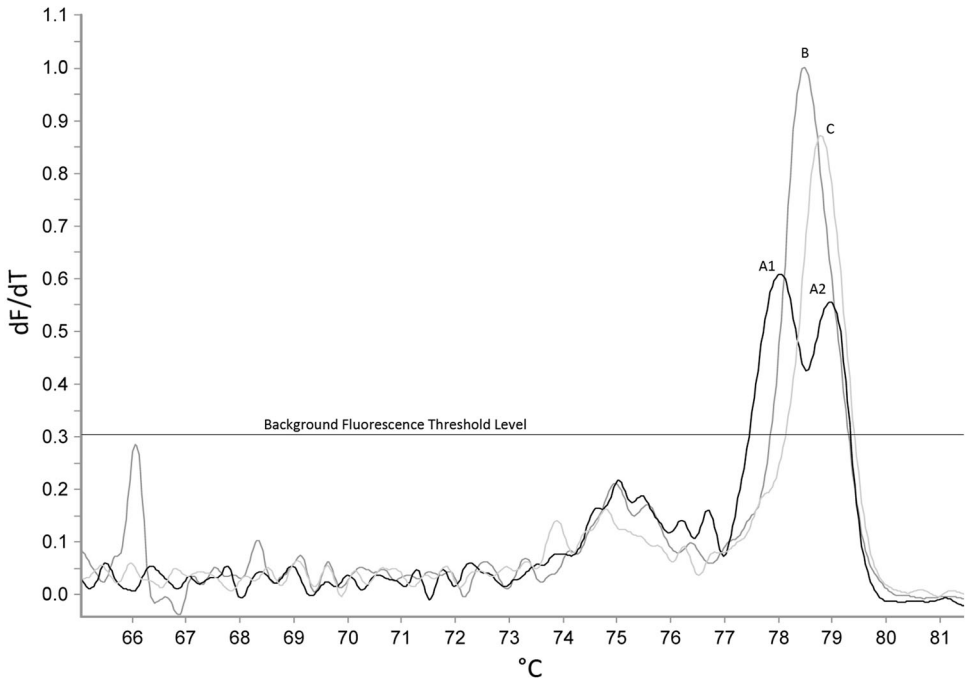

Fig. 1 dF/dT curve of locus *Ptero09* showing a heterozygous individual (*black*) exhibiting typical double peaks (*black*, *A1*, *A2*) and homozygous individuals (*grey*, *B*, *C*) showing single peaks. Threshold is adjusted above background levels

Table 1 Results of high-resolution melt analysis and capillary electrophoresis at 27 microsatellite loci in 10 *Pterodroma solandri* individuals (five individuals from Phillip Island and five individuals from Lord Howe Island)

Microsatellite loci background information			HRM screening		CE	
Loci	Published size range	Originally reported repeat motif	Max dF/dT peaks	ΔT_m (°C)	Size (bp)	Number of alleles
10C5[a]	160–163	$(GA)_{11}(GC)_2GT(GC)_2$	1	1.55	175	1
12H8[a]	185–191	$(GT)_7(AT)_7$	>2	–	–	–
Calex01[b]	224–232	$(GT)_2GA(GT)_3GC$	2	2.07	237–255	7
De11[c]	164–170	$(AC)_{9.5}(TA)_7(CA)_4(CG)_5$.	1	0.68	186	1
OC84[d]	317–321	$(AG)_9$	1	1.25	315	1
Oc87B[d]	280–286	$(GA)_{12}$	>2	–	–	–
Paequ3[e]	228–262	$(GA)_{19}$	2	0.98	222–232	5
Paequ10[e]	152–154	$(CA)_8$	1	0.48	204	1
Paequ13[e]	136–142	$(GT)_9$	2	1.47	146–148	2
Parm01[f]	201–235	$(CA)_{13}$	1	0.61	159–230	6
Parm02[f]	179–200	$(CA)_6TA(CA)_9$	2	2.13	192–198	3
Parm03[f]	174–192	$(CA)_5TA(CA)_{11}$	1	1.30	177–181	3
Parm04[f]	207	$(CA)_5(GACA)_2$	>2	–	–	–
Parm05[f]	122	$(CA)_{11}$	1	0.67	144	1
Parm06[f]	160	$(CA)_6GA(CA)_2TACA$	1	0.80	182	1
Ptero01[g]	163–167	$(CA)_7$	1	1.47	187	1
Ptero02[g]	131–141	$(CA)_9$	1	0.38	136	1
Ptero03[g]	130–142	$(CA)_9$	1	0.66	157	1
Ptero04[g]	146–160	$(CA)_{13}$	2	5.40	150–168	5
Ptero05[g]	206–215	$(AAG)_2AGG(AAG)_3$	1	1.00	235	1
Ptero06[g]	145–177	$(AAGG)_{13}$	1	1.85	141–149	3
Ptero07[g]	253–289	$(AAAG)_8$	2	1.93	264–344	16
Ptero08[g]	162–226	$(AAGG)_8$	>2	–	–	–
Ptero09[g]	212–236	$(AAGG)_8$	2	1.35	187–235	10
Ptero10[g]	205–290	$(TAGGA)_9…(TAGGA)_7$	>2	–	–	–
RBG18[h]	145–159	$(GT)_{11}$	1	1.07	196–199	2
RBG29[h]	155–163	$(GT)_{13}$	2	1.15	124–136	5

[a] Dubois et al. (2005), [b] Kupper et al. (2007), [c] Burg (1999), [d] Sun et al. (2009), [e] Techow and O'Ryan (2004), [f] Brown and Jordan (2009), [g] Welch and Fleischer (2011), [h] Given et al. (2002)

Materials and methods

PCR amplification and high resolution melting (HRM) analysis

High resolution melting (HRM) was performed on a Rotorgene Q (Qiagen, Valencia, CA USA) using five individuals from both of the two known *P. solandri* populations (Lord Howe Island, 31°30′S, 159°05′E; Phillip Island, 29°12′S, 167°95′E). Reactions consisted of 1X Type-it HRM PCR kit (Qiagen), 0.175 µM forward and reverse primers, and 0.8 sµL template DNA (ca. 10 ng/µL) in 10 µL total reaction volume. Cycling conditions were 3 min initial hot start at 95 °C followed by 70 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s, with amplification success quantified by fluorescence acquired after each elongation step. Cycling was followed by a 1 min hold at 95 °C, and then rapid cooling to a 40 °C hold for 1 min, to ensure complete renaturation of products and to maximize heteroduplex formation (Smith et al. 2010). HRM consisted of a 65 °C hold for 1 min, followed by ramping to 90 °C in 0.1 °C increments with fluorescence acquired after holding for 2 s at each increment. Data analyses were performed using the Rotor-Gene Q Series Software v2.0.2 (Qiagen). After manual adjustment of the dF/dT threshold above background levels, the number of peaks in the first derivative melting curve was recorded and a ΔT_m was calculated (Table 1). We excluded loci showing complex melting patterns (>2 dF/dT peaks in an individual) given these primers may be amplifying multiple loci, although they could also represent single loci with multiple melt regions and multiple SNPs.

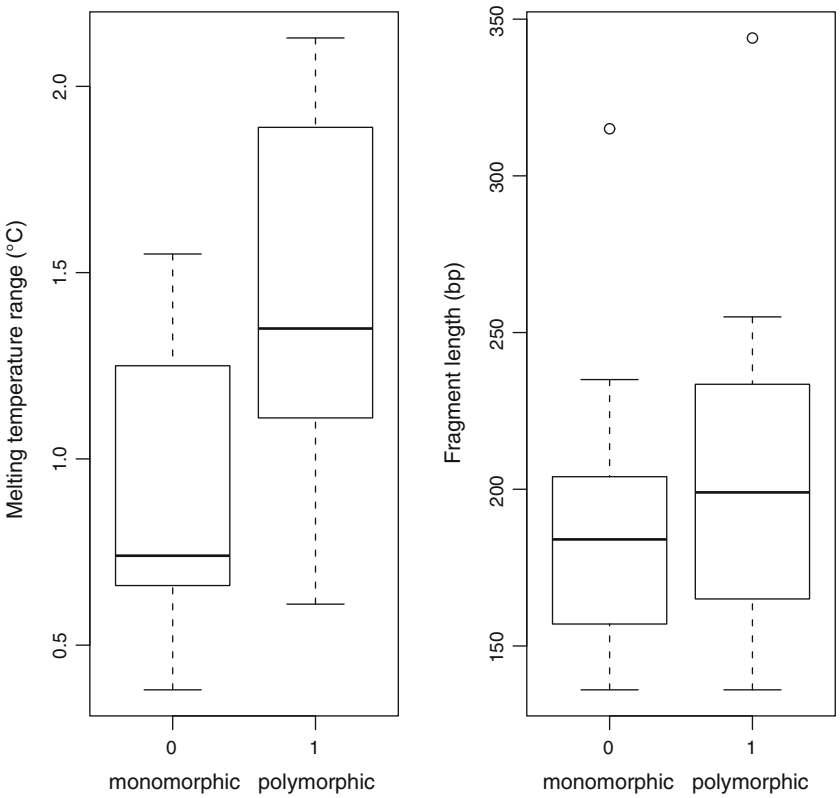

Fig. 2 Boxplots describing melting temperature range (ΔT_m) and fragment length with respect to polymorphism in 22 microsatellite loci from *Pterodroma solandri*. *Left* means of ΔT_m with respect to monomorphic (0.84 °C) and polymorphic (1.40 °C) loci. *Right* means of fragment length with respect to monomorphic (192 bp) and polymorphic (209 bp) loci

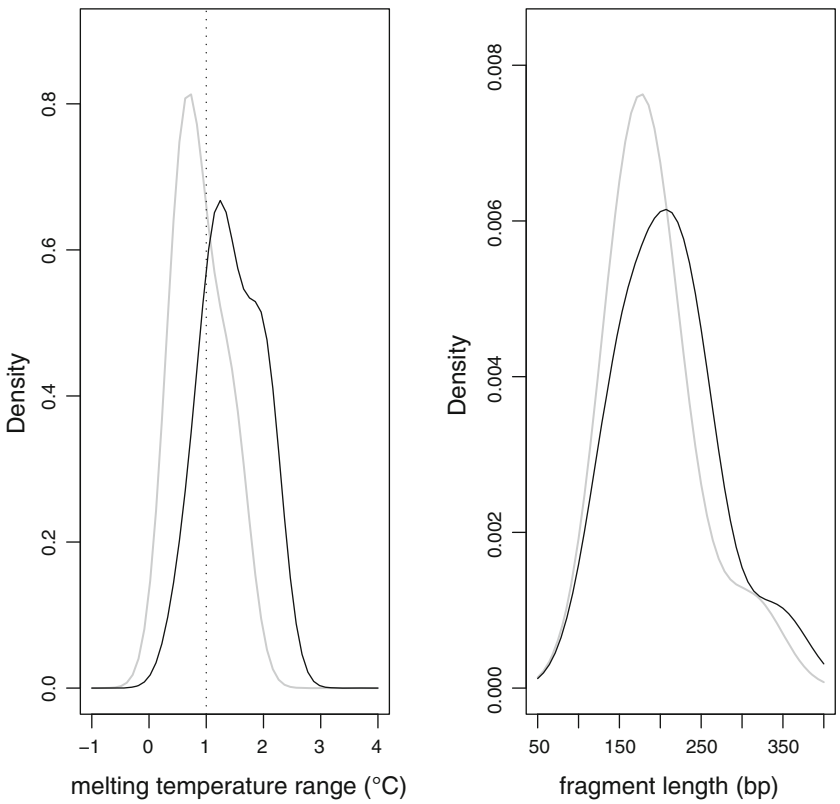

Fig. 3 Kernel density estimations showing the probability density functions of high resolution melt temperature range (ΔT_m) and fragment length based on their repartition with respect to polymorphism. *Left* ΔT_m. *Right* fragment length. *Grey lines* represent probability density functions of monomorphic loci; *black lines* represent probability density functions of polymorphic loci. *Dotted lines* represent an assessment of the junction between monomorphic and polymorphic probability density functions

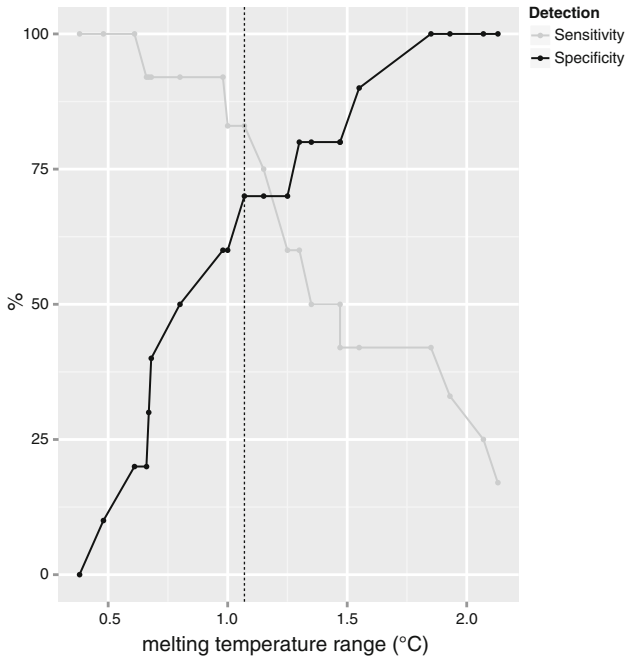

Fig. 4 Variation of high resolution melt detection (%) according to melting temperature range (ΔT_m). *Grey line* represents sensitivity (1-false negatives; %); *black line* represents specificity (1-false positives; %). *Dotted line* represents the optimal detection threshold ΔT_m

Capillary electrophoresis (CE)

All amplicons from loci showing one or two peaks in the dF/dT curve were subjected to CE. Forward primers were dye labeled with either NED, PET, VIC, or FAM and amplified in three multiplex reactions where each label type was represented for only a single locus, employing the MyTaq HS (Bioline) protocol. Products were then separated by CE on a AB3730*xl* DNA Analyzer (Applied Biosystems Inc.) using the LIZ600 size standard.

Statistical analyses

We compared means of ΔT_m and fragment length for polymorphic and monomorphic loci using a one-way between groups ANOVA (parametric distribution) and a Wilcox-test (non-parametric distribution), respectively. We constructed kernel density estimation of both ΔT_m and fragment length with respect to polymorphism using the package *sm* in R version 3.1.2, and assessed the junction of their distributions. We analyzed the variation in sensitivity (1-false negatives, %) versus specificity (1-false positives, %) of HRM analysis according to ΔT_m with the package *ggplot2* in R. We detected a threshold maximizing both specificity and sensitivity of HRM analysis and we compared these results to those obtained when using the presence of two peaks in the dF/dT curve to assess polymorphism.

Results

Five loci exhibiting more than two peaks in the dF/dT curve for an individual were discarded. Eight loci exhibiting two dF/dT peaks for at least one individual were confirmed as polymorphic with CE, displaying between two and 16 alleles. Four loci failed to be detected as polymorphic, showing only a single dF/dT peak (sensitivity = 67 %). All monomorphic loci exhibited single dF/dT peaks (specificity = 100 %) (Table 1). ΔT_m for *Ptero04* deviated substantially from the distribution of other loci, and was excluded as an outlier for statistical analysis (Table 1). The difference between means of ΔT_m for polymorphic and monomorphic loci was significant (ANOVA, $F = 7.45$, $p = 0.013$), but the difference between means of sequence lengths for polymorphic and monomorphic loci was not (Wilcox-test, $p = 0.459$) (Fig. 2). For this reason, we pursued analyses of ΔT_m. The junction between the two probability density functions of ΔT_m with respect to polymorphism was estimated as $\sim$1.0 °C (Fig. 3). We then detected 1.07 °C as the optimal ΔT_m to maximize both sensitivity (83 %) and specificity (70 %) (Fig. 4; Table 2). A significant improvement in sensitivity (83 vs. 67 %) was achieved by using ΔT_m (threshold 1.07 °C) to assess polymorphism relative to multiple peaks in the dF/dT curve for an individual (Table 2). A larger improvement in sensitivity (92 vs. 67 %) was achieved by using this ΔT_m threshold in addition to multiple peaks in the dF/dT curve to assess polymorphism (Table 2).

Discussion

Sensitivity and specificity of HRM analysis depend mainly on instrument resolution and amplicon size (Herrmann et al. 2006). Here, we assessed polymorphism at 27 microsatellite loci, with lengths of 122–321 bp, and we showed that ΔT_m better predicted polymorphism than fragment length or multiple dF/dT peaks for an individual. We also detected a threshold value of ΔT_m to assess polymorphism that can be generalized to further studies. Nevertheless, as reaction conditions can widely vary between PCR products, the reproducibility of T_m measurements can be compromised (e.g., T_m standard deviation of 0.03–0.39 °C attributable to DNA extractions already observed). To partly correct resolution limitations imposed by the instrument and solution chemistry between samples, internal temperature controls (or complementary unlabeled oligonucleotides) can be included, allowing subsequent temperature correction of the melting profile, leading to a reduction of T_m standard deviation (Seipp et al.

Table 2 Results of high resolution melt detection of polymorphism at 22 microsatellite loci in 10 *Pterodroma solandri* individuals using different predictors: two dF/dT peaks within an individual, melting temperature range (ΔT_m), or two dF/dT peaks and ΔT_m. Sensitivity (1-false negatives) and specificity (1-false positives) are represented as percentages

Loci	Alleles	Size (bp)	ΔT_m	Poly. Y/N	HRM inference of polymorphism		
					dF/dT peaks	ΔT_m (>1.07)	dF/dT peaks + ΔT_m
Ptero02	1	136	0.38	N	Y	Y	Y
Paequ10	1	204	0.48	N	Y	Y	Y
Parm01	6	230	0.61	Y	N	N	N
Ptero03	1	157	0.66	N	Y	Y	Y
Parm05	1	144	0.67	N	Y	Y	Y
De11	1	186	0.68	N	Y	Y	Y
Parm06	1	182	0.8	N	Y	Y	Y
Paequ3	5	232	0.98	Y	Y	N	Y
Ptero05	1	235	1	N	Y	Y	Y
RBG18	2	199	1.07	Y	N	Y	Y
RBG29	5	136	1.15	Y	Y	Y	Y
Oc84	1	315	1.25	N	Y	N	N
Parm03	3	181	1.3	Y	N	Y	Y
Ptero09	10	235	1.35	Y	Y	Y	Y
Ptero01	1	187	1.47	N	Y	N	N
Paequ13	2	148	1.47	Y	Y	Y	Y
10C5	1	175	1.55	N	Y	N	N
Ptero06	3	149	1.85	Y	N	Y	Y
Ptero07	16	344	1.93	Y	Y	Y	Y
Calex01	7	255	2.07	Y	Y	Y	Y
Parm02	3	198	2.13	Y	Y	Y	Y
Ptero04	5	168	5.40	Y	Y	Y	Y
Sensitivity (%)					67	83	92
Specificity (%)					100	70	70

2007). This will not control for concentration of amplified DNA, but since different PCR reactions tend to plateau at the same product concentration, these variations represent a minor concern (Gundry et al. 2003).

Regardless of the decrease of specificity (increase in false positives) observed by using HRM ΔT_m to assess polymorphism relative to multiple dF/dT curves, we suggest ΔT_m as an additional predictor that can detect more polymorphic loci for subsequent analysis. HRM is substantially cheaper and faster than CE, even when employing cost-efficient methods of dye label incorporation (Schuelke 2000), as the HRM approach does not have electrophoresis costs. Other approaches to screen polymorphism that avoid labeled primers, such as high-resolution agarose (Hughes and Queller 1993) or non-denaturing polyacrylamide electrophoresis (Lessa and Applebaum 1993) also require electrophoresis relative to HRM, and likewise exhibit low sensitivity relative to denaturing CE (Andersen et al. 2003). Furthermore, in contrast to CE, HRM thermal cyclers will also become increasingly available within laboratories as their ability to perform real-time genotyping of single loci become increasingly realized (Mackay et al. 2008; Muleo et al. 2009; Smith et al. 2010; Xiao et al. 2012).

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Author contributions Anicee Lombal and Theodore Wenner constructed the manuscript and collected the molecular data. Anicee Lombal performed statistical analyses. Christopher Burridge designed the study and contributed to the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest regarding this study.

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