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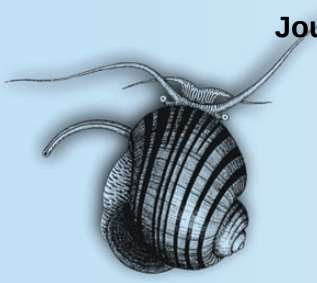

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RESEARCH NOTE

High-resolution melting (HRM) analysis: a highly sensitive alternative for the identification of commercially important *Crassostrea* oysters

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Oysters are distributed around the world along the shores of all continents (except Antarctica) and some oceanic islands. They are important species in the shellfish industry; the world production of oysters was 4.4 million tons in 2011, accounting for more than 30% of the total marine molluscan yield (FAO, 2013). Due to their economic value and significant role in nearshore ecosystems (Ruesink, Lenihan & Trimble, 2005), oysters have been extensively studied. All commercially important oysters that are common along the coast of China belong to the genus *Crassostrea*, including *C. gigas* (Thunberg, 1793), *C. angulata* (Lamarck, 1819), *C. ariakensis* (Fujita, 1913), *C. hongkongensis* (Lam & Morton, 2003) and *C. sikamea* (Amemiya, 1928) (Liu *et al*., 2011). They are commonly found on rocks, concrete and other hard substrates in almost all intertidal zones and harbours. Because of their phenotypically plastic shell morphology, oysters cannot be reliably identified using morphological characteristics. The use of morphological characteristics has led to numerous errors and confusion in oyster classification (Harry, 1985; Li & Qi, 1994; Wang, Zhang & Zhang, 2004), with negative effects on culture efficiency and conservation. Hence, alternative identification techniques that are efficient and accurate are needed for oyster research and aquaculture.

In some previous studies, DNA markers have been used for the identification of oyster species (Klinbunga *et al*., 2003, 2005; Cordes, Stubbs & Reece, 2005; Wang & Guo, 2008b). In particular, mtDNA data have been useful to identify oyster species by means of restriction-fragment length polymorphisms (Pie *et al*., 2006), denaturing-gradient gel electrophoresis (Yu & Li, 2008), nucleotide sequencing (Reece *et al*., 2008; Yu *et al*., 2003) or specific multiplex PCR (Wang & Guo, 2008a). However, these methods are mostly complicated and labour intensive, hindering their high-throughput applicability.

High-resolution melting (HRM) analysis is a highly sensitive molecular method, which distinguishes specimens on the basis of single-nucleotide polymorphisms (SNPs) or small deletions in amplified DNA sequences (Reed, Kent & Wittwer, 2007; Vandersteen *et al*., 2007). All the processes of PCR amplification during HRM take place in the same tube during 2 h, the product is denatured and the changes in sample fluorescence with temperature are monitored. This technique has already been used to screen for genetic variation in human disease (Vossen *et al*., 2009), mammals (Berry & Sarre, 2007), insects (Malewski *et al*., 2010; David & Cheolho, 2013) and fish (Haynes *et al*., 2009; McGlauflin *et al*., 2010). Thus, it has been considered to be a rapid, sensitive, reliable and cost-effective method (Camila & Mauricio, 2011). In oysters, HRM has been successfully used for identification of *Crassostrea* species and for detection of hybrids between *C. sikamea* and *C. angulata*, based on mitochondrial cytochrome *c* oxidase subunit I (COI) sequences (Wang *et al*., 2014; Xu *et al*., 2014). As the rate of molecular evolution of COI is about three times greater than that of the mitochondrial large ribosomal subunit (16S rDNA) (Knowlton & Weigt, 1998), there is usually some difficulty in amplification using universal primers. Therefore, in this study we tested the utility of relatively conserved 16S rRNA sequences in HRM analysis, and show that this provides a fast and effective method to differentiate five commercially important species of *Crassostrea* oysters.

In total, 95 oysters were collected from typical areas where they occur. *Crassostrea gigas* ($n = 20$) was obtained from the rocks of coastal waters around Rushan, Shandong Province; *C. angulata* ($n = 19$) was collected from Putian, Fujian Province; *C. ariakensis* ($n = 19$) was sampled from the Sea of Ariake, Japan; *C. hongkongensis* ($n = 19$) was collected from Beihai, Guangxi Province; and *Crassostrea sikamea* ($n = 18$) was collected from Haimen, Jiangshu Province. Genomic DNA was extracted from adductor muscle by standard proteinase-K digestion and phenol-chloroform extraction. The purity of the samples was measured on a NanoDrop-2000 spectrophotometer (NanoDrop Technologies) at 260/280 nm, adjusted to the same level. The DNA extracts were stored at −20 °C until used.

Mitochondrial 16S rRNA sequences of all known *Crassostrea* species were downloaded from GenBank (accession numbers: AF280611, KC170322, FJ743507, HQ660978, JF808181). A 300-bp 16S rRNA sequence alignment that contained variation unique to each of the five species was examined for variation between species. Primers were designed using the Primer Premier v. 5.0 program (PREMIER Biosoft International). Design criteria included a similar annealing temperature at 60 ± 2 °C and target amplicon size of 60–150 bp. A primer set (F: 5′-AGATTTTTAGGTGGGGCG-3′, R: 5′-GCGTAAC TTCTCCTATGATCG-3′) was created based on consensus regions between the different *Crassostrea* species and a 83 bp amplicon with unique form-specific melting curves, to ensure maximum resolution in HRM analysis.

All DNA extractions were diluted to 10 ng/μl. PCR amplification and HRM analysis were performed on a LightCycler®480 real-time PCR instrument (Roche Diagnostics). The reaction

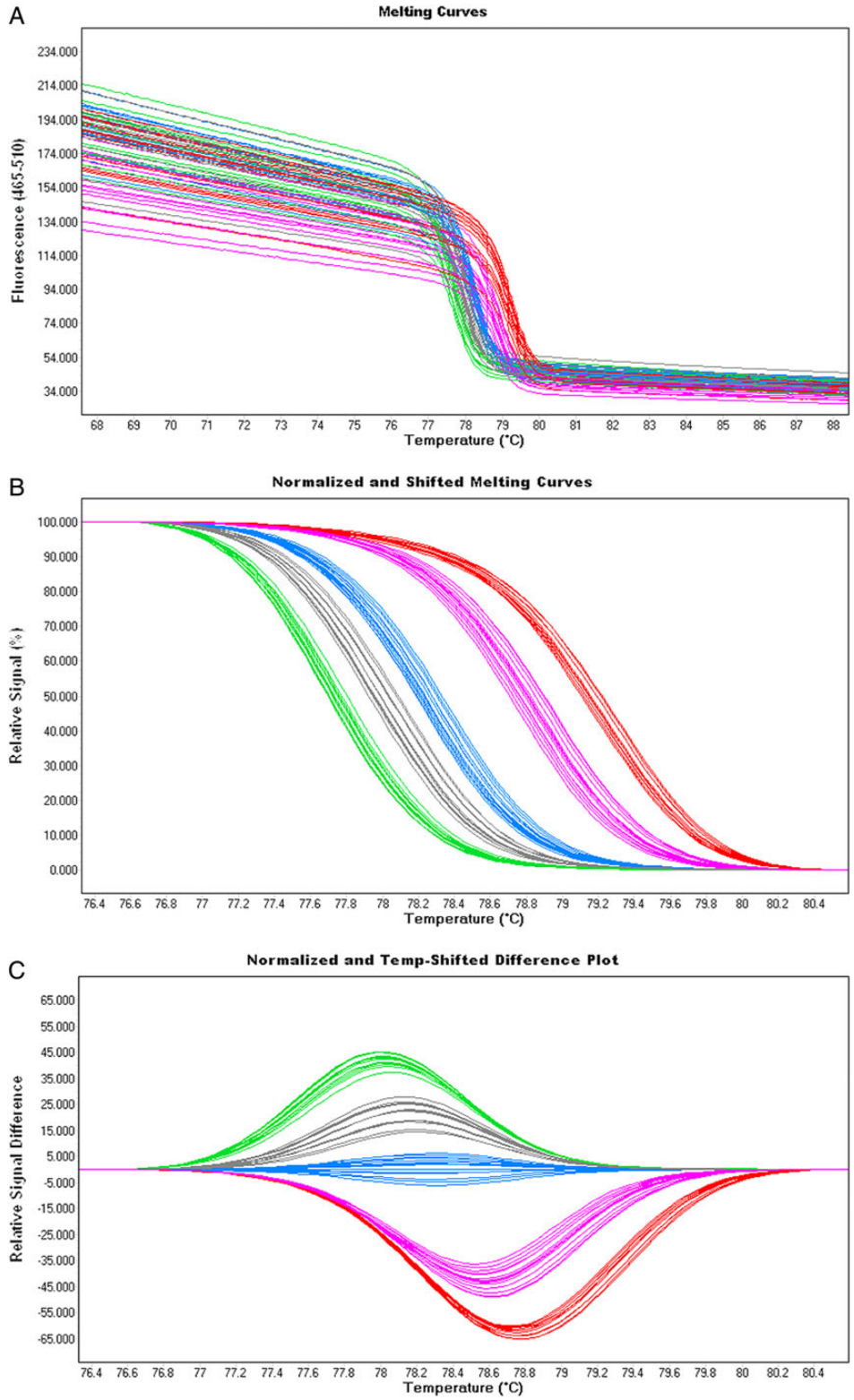

Figure 1. Discriminations of single-nucleotide polymorphisms (SNPs) among five *Crassostrea* species using high-resolution melting analysis. **A.** Raw melting profile of real-time quantitative PCR showing dissociation of double-stranded DNA into single strands as temperature is incrementally increased. **B.** Normalization of fluorescence from the raw melting data for further discrimination in a difference plot. **C.** The difference plot amplifies minute differences between the melting curves, thus genotype identification is dependent on similarities in melting behaviour at different temperatures rather than overlapping melt profiles. *Crassostrea angulata* (blue) is reference, *C. ariakensis* is represented by green, *C. hongkongensis* by grey, *C. angulata* by blue, *C. gigas* by purple and *C. sikamea* by red.

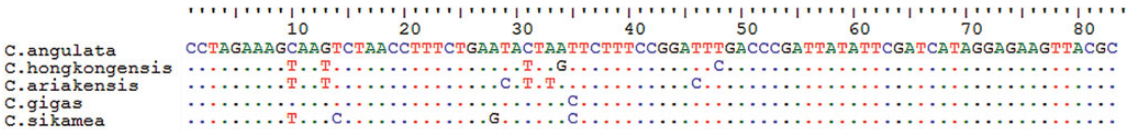

Figure 2. Sequence alignment showing single-nucleotide polymorphisms (SNPs) within the target region across the five *Crassostrea* species.

mixture contained 1 μl template, 1 × LightCycler®480 HRM Master Mix with ResoLight® dye (Roche Diagnostics), 0.2 μM of each primer and adjusted with RNase-free water to a final volume of 10 μl. A negative control of RNase-free water was added during each run of the PCR to check for contamination. The amplification was achieved by a touchdown PCR protocol: first denaturation at 95 °C for 5 min, then 45 cycles of denaturation at 95 °C for 40 s, annealing and extension for 40 s at 62 °C for the first cycle and thereafter at 0.5 °C decrease each for 10 cycles, and a final extension at 72 °C for 40 s. Following amplification, the products were denatured at 95°C for 1 min, and then annealed at 40°C for 1 min to form DNA duplexes randomly. Melting curves were generated by heating samples from 60 to 90 °C with 25 data acquisitions per degree. Data were analysed using the LightCycler®480 Gene Scanning Software v. 1.5 (Roche Diagnostics). Genotypes were identified by examining normalized melting curves, difference and derivative plots of the melting data.

Two samples of each representative melt profile were randomly selected for sequencing. Fragments amplified from mitochondrial 16S rRNA were sequenced to confirm expected SNPs or reveal new SNPs in their amplicons and flanking regions. PCR products were sequenced in both directions on an ABI PRISM 3730 automatic sequencer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using CLUSTALW in the MEGALIGN DNASTAR software (Lasergene).

HRM profiles were successfully obtained for 96 samples (Fig. 1A). HRM is capable of detecting subtle difference as small as 0.2 °C in melting temperature. Analysis of the melting profiles showed five that were clearly different, and species-specific genotypes for the five oyster species (Fig. 1B). Characteristic melting profiles were found to be consistent between all five species. Furthermore, by selecting *C. angulata* as initial reference in the difference plot module, HRM profiles clearly exhibited unique melting temperatures (Tm) for each sample with nonoverlapping, separate inflection points for each *Crassostrea* species between 78 and 79 °C (Fig. 1C). This supports our claim that HRM is a sensitive technique for distinguishing these five species.

Sequencing analysis was performed in order to confirm the species-specific melting curves detected by HRM. Figure 2 shows the alignment of sequences of the five haplotypes detected among the ten samples. Each melting profile exhibited only one haplotype. In total, 11 SNP sites were detected from the 83-bp amplicons of the target region to distinguish the species and no insertion or deletion sites were found. Transitions were observed at nine sites and transversions at two sites. The amplicons contained only one SNP (T/C) between *C. gigas* and *C. angulata*; this difference was still distinguished by HRM (Fig. 1B, C), which demonstrates that the HRM analysis is both sensitive and robust.

This study shows that HRM analysis allows precise differentiation of five commercially important species of *Crassostrea* oysters with only one pair of primers. Small amplicon size minimizes the influence of undesired variation within an amplicon. This method does not require multiplexing reactions by electrophoresis, or prior/post-PCR procedures, suggesting that HRM may be a more simple and efficient identification tool than current methods. In other taxa, HRM analysis has offered a quick and cost-effective method for identifying species of mosquitoes (David & Cheolho, 2013) and Antarctic fish (Fitzcharles, 2012) and *Symbiodinium* strains (Camila & Mauricio, 2011), which are difficult to distinguish using classic morphological markers. Although sequencing is still considered the most accurate method for genotyping, its procedure is complicated, time-consuming and expensive. Overall, there are several advantages of HRM, making it an attractive technique. First, it is a closed-tube technique that reduces cross-contamination and does not require the handling of hazardous materials, such as acrylamide, formamide and ethidium bromide. Second, it is time-effective; as many as 96 (or even 383) samples can be distinguished in 2 h, which facilitates rapid turnover. Third, the differences in DNA are clearly distinguished by changes in the melting curves, which are easily visualized (Wang *et al.*, 2014). Recent advances in reaction chemistries, instrument sensitivity and assay design have all led to improvements in the sensitivity and robustness of HRM technique. Therefore, it is now possible to use HRM as a routine genetic tool.

Compared with traditional PCR, HRM analysis is particularly suitable for the analysis of SNPs or any other mutation sites. Similarly, although DNA barcoding of oysters remains a good reference for comparative identification purposes (Liu *et al.*, 2011), the time, cost and expertise it requires can be prohibitive for routine identifications. In contrast, the experimental design of HRM analysis is simple, and provides a rapid, sensitive and cost-effective alternative for species identification. Nevertheless, some limitations and/or factors such as the presence of other nucleotide variations in the amplicon among individuals of different regions, and the quality of DNA, could influence HRM optimization and applicability. In this study, an 83-bp segment of mitochondrial 16S rRNA was found to be an appropriate locus for the discrimination of five commercially important species of *Crassostrea* oysters. These species are closely related and probably diverged over a short period of evolutionary time. Hence, the results show that HRM analysis has great potential for species identification.

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REFERENCES

BERRY, O. & SARRE, S.D. 2007. Gel-free species identification using melt-curve analysis. *Molecular Ecology Notes*, **7**: 1–4.

CAMILA, G.C. & MAURICIO, R.L. 2011. The use of high-resolution melting analysis for genotyping *Symbiodinium* strains: a sensitive and fast approach. *Molecular Ecology Resources*, **11**: 394–399.

CORDES, J.F., STUBBS, J.B. & REECE, K.S. 2005. Phylogenetics and species identification of *Crassostrea* oysters based on sequences and PCR-RFLP analyses of ITS-1 and COI markers. *Journal of Shellfish Research*, **24**: 647.

DAVID, K. & CHEOLHO, S. 2013. Identification of *Culex* complex species using SNP markers based on high-resolution melting analysis. *Molecular Ecology Resources*, **13**: 369–376.

FAO. 2013. World aquaculture production of fish, crustaceans, molluscs, etc., by principal species. http://www.fao.org/figis/servlet/TabSelector.

FITZCHARLES, E.M. 2012. Rapid discrimination between four Antarctic fish species, genus *Macrourus*, using HRM analysis. *Fisheries Research*, **127–128**: 166–170.

HARRY, H.W. 1985. Synopsis of the supraspecific classification of living oysters (Bivalvia: Gryphaeidae and Ostreidae). *Veliger*, **28**: 121–158.

HAYNES, G.D., GONGORA, J., NICHOLAS, F.W. & ZENGER, K.R. 2009. Rapid identification of maternal lineages in common carp (*Cyprinus carpio* L.) using real-time PCR and high resolution melt-curve analysis. *Aquaculture*, **287**: 59–66.

KLINBUNGA, S., KHAMNAMTONG, B., PUANGLARP, N., JARAYABHAND, P., YOOSUKH, W. & MENASVETA, P. 2005. Molecular taxonomy of cupped oysters (*Crassostrea*, *Saccostrea*, and *Striostrea*) in Thailand based on COI, 16S and 18S rDNA Polymorphism. *Marine Biotechnology*, **7**: 306–317.

KLINBUNGA, S., KHAMNAMTONG, N., TASSANAKAJON, A., PUANGLARP, N., JARAYABHAND, P. & YOOSUKH, W. 2003. Molecular genetic identification tools for three commercially cultured oysters (*Crassostrea belcheri*, *Crassostrea iredalei*, and *Saccostrea cucullata*) in Thailand. *Marine Biotechnology*, **5**: 27–36.

KNOWLTON, N. & WEIGT, L.A. 1998. New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society of London*, *Series B*, **265**: 2257–2263.

LI, X. & QI, Z. 1994. Studies on the comparative anatomy, systematic classification and evolution of Chinese oysters. *Studia Marina Sinica*, **35**: 143–173.

LIU, J., LI, Q., KONG, L.F., YU, H. & ZHENG, X.D. 2011. Identifying the true oysters (Bivalvia: Ostreidae) with mitochondrial phylogeny and distance-based DNA barcoding. *Molecular Ecology Resources*, **11**: 820–830.

MALEWSKI, T., DRABER-MONKO, A., POMORSKI, J., LOS, M. & BOGDANOWICZ, W. 2010. Identification of forensically important blowfly species (Diptera: Calliphoridae) by high-resolution melting PCR analysis. *International Journal of Legal Medicine*, **124**: 277–285.

McGLAUFLIN, M.T., SMITH, M.J., WANG, J.T., YOUNG, S.F., CHEN, N., LEE, Y.C., PASCAL, C., SEEB, L.W., STEVENS, J. & SEEB, J.E. 2010. High-resolution melt analysis for the discovery of novel single-nucleotide polymorphisms in rainbow and cutthroat trout for species identification. *Transactions of the American Fisheries Society*, **139**: 676–684.

PIE, M.R., RIBEIRO, R.O., BOEGER, W.A., OSTRENSKY, A., FALLEIROS, R.M. & ANGELO, L. 2006. A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana*, *C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in Southern Brazil. *Aquaculture Research*, **37**: 1598–1600.

REECE, K.S., CORDES, J.F., STUBBS, J.B., HUDSON, K.L. & FRANCIS, E.A. 2008. Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. *Marine Biology*, **153**: 709–721.

REED, G.H., KENT, J.O. & WITTWER, C.T. 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*, **8**: 597–608.

RUESINK, J.L., LENIHAN, H.S. & TRIMBLE, A.C. 2005. Introduction of non-native oysters: ecosystem effects and restoration implications. *Annual Review of Ecology, Evolution, and Systematics*, **36**: 643–689.

VANDERSTEEN, J.G., BAYRAK-TOYDEMIR, P., PALAIS, R.A. & WITTWER, C.T. 2007. Identifying common genetic variants by high-resolution melting. *Clinical Chemistry*, **53**: 1191–1198.

VOSSEN, R.H., ATEN, E., ROOS, A. & DEN DUNNEN, J.T. 2009. High resolution melting analysis (HRMA)-more than just sequence variant screening. *Human Mutation*, **30**: 860–866.

WANG, H.Y. & GUO, X.M. 2008a. Identification of *Crassostrea ariakensis* and related oysters by multiplex species-specific PCR. *Journal of Shellfish Research*, **27**: 481–487.

WANG, H.Y. & GUO, X.M. 2008b. ITS length polymorphism in oysters and its potential use in species identification. *Journal of Shellfish Research*, **27**: 489–493.

WANG, H.Y., ZHANG, G.F. & ZHANG, F.S. 2004. Classification of jinjiang oysters *Crassostrea rivularis* (Gould, 1861) from China, based on morphology and phylogenetic analysis. *Aquaculture*, **242**: 137–155.

WANG, J.F., XU, F., LI, L. & ZHANG, G.F. 2014. A new identification method for five species of oysters in genus *Crassostrea* from China based on high-resolution melting analysis. *Chinese Journal of Oceanology and Limnology*, **32**: 419–425.

XU, F., LI, L., WANG, J.F. & ZHANG, G.F. 2014. Use of high-resolution melting analysis for detecting hybrids between the oysters *Crassostrea sikamea* and *Crassostrea angulate* reveals bidirectional gametic compatibility. *Journal of Molluscan Studies*, doi: 10.1093/mollus/eyu040.

YU, H. & LI, Q. 2008. Denaturing gradient gel electrophoresis as a tool for the identification of commercially important *Crassostrea* oysters in China. *Fisheries Science*, **74**: 1354–1356.

YU, Z.N., KONG, X.Y., ZHANG, L.S., GUO, X.M. & XIANG, J.H. 2003. Taxonomic status of four *Crassostrea* oysters from China as inferred from mitochondrial DNA sequences. *Journal of Shellfish Research*, **22**: 31–38.