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[Technical Note]

A Rapid, High-Throughput Technique for Detecting Tanner Crabs *Chionoecetes bairdi* Illegally Taken in Alaska's Snow Crab Fishery

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Abstract.—Tanner crabs *Chionoecetes bairdi* and snow crabs *C. opilio* are both important commercial species for Alaskan fisheries. The geographical ranges of Tanner and snow crabs overlap in the southeastern Bering Sea where a commercial fishery targets the more abundant snow crabs. Morphological identification of these species and of hybrids has proven difficult and impedes monitoring of the harvests. Techniques for the genetic identification of these species have been developed, but it may not be possible to process the desired number of samples because of throughput limitations and sample requirement constraints. Here we present a rapid, high-throughput assay for identifying these species based on single nucleotide polymorphisms (SNPs) in the nuclear rRNA internal transcribed spacer 1 region and the mitochondrial DNA 16S rRNA gene. These SNPs can also be used to infer the direction of hybridization (i.e., the species of each gender).

The geographical ranges of Tanner crabs *Chionoecetes bairdi* and snow crabs *C. opilio* overlap in the southeastern Bering Sea, where a commercial fishery worth US$44.2 million targets the more abundant snow crabs. Tanner and snow crabs are morphologically similar but can generally be distinguished by eyestalk color (red in Tanner crabs and green in snow crabs) and by the shape of the epistome (M-shaped or curved in Tanner crabs, but straight in snow crabs). Tanner crabs are larger than snow crabs at maturity, but size varies among individuals and among geographical areas, so that size is not a reliable guide to species identification. These species produce hybrid individuals (Karinen and Hoopes 1971), which often possess a mixture of characters that make identification based on morphology difficult. For fishery management purposes, hybrids are considered a legal part of the snow crab catch. The fact that hybrids are generally larger than snow crabs provides incentive for fishermen to target these individuals. Catches are routinely inspected by fishery observers at sea or by dockside inspectors upon arrival in port. A catch containing one or two percent Tanner crabs might result in a warning, and a catch containing more than that could result in confiscation of the catch (often worth over $0.25 million) and possibly the ship and fishing rights as well (J. D. Urban, Alaska Department of Fish and Game, personal communication).

Allozyme markers used to identify species of *Chionoecetes* and their hybrids (Johnson 1976; Grant et al. 1978) have further shown that most hybrids represent first generation crosses, but that F_2 and later generations are also present (Merkouris et al. 1998). Urban et al. (2002) observed that biologists specializing in the identification of *Chionoecetes* identified unknown samples based on morphological traits consistently with each other and with identifications based on allozyme data. Fishery observer trainees, however, produced identifications that were inconsistent with allozyme data up to 8% of the time for Tanner crabs, 42% of the time for snow crabs, and 45% of the time for hybrids. Given this error rate in morphological identification, allozyme markers have played an important role in confirming species identifications made in the field; however, because considerable care must be taken in handling and shipping these samples, DNA-based markers are more commonly used to samplecatcher–processor catches. Furthermore, the time and labor involved in allozyme analyses limit the number of individuals that can be analyzed from any given fishery sample and the number of samples that can be analyzed within a season. The objective of our study was to develop DNA-based markers that could rapidly distinguish Tanner crabs from snow crabs and hybrids in large sample sizes. ?1

Methods

A DNeasy Tissue Kit (QIAGEN) was used to extract DNA from 10 each of Tanner, snow, and ?2

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TABLE 1.—Oligonucleotide PCR primers and probes used in the 16S and internal transcribed spacer, single nucleotide polymorphism genotyping assays. All probes had minor groove binders and nonfluorescent quenchers attached to their 3′ ends.

Locus	PCR Primers (5′-3′)	Probes (5′-3′)
16S	CGGACAAAGGAAAAGCTGTCTTTA	VIC-CCCCTAAAGAATCTAA
	GCCCCAACAAAACACATTAATTAGAT	6FAM-CCCCTAAAAAATC
ITS	GCGGAAGGATCATTAACGAATT	VIC-TGCTGTTGAGCGGC
	CACCCAGAAGTTTTGGTTTAGTTTC	6FAM-TGCTGTTCAGCGGC

their hybrids (i.e., Tanner × snow crabs and reciprocals). The polymerase chain reaction (PCR) primers 18 d and 28z (Hillis and Dixon 1991) were used to amplify the internal transcribed spacer (ITS) separating the 18S and 5.8S rRNA genes in each crab. Amplifications were done in 60-μL volume, which included 1 μL template DNA and 1 μL (5 units) DNA polymerase (Promega). Reactions included 100 pmol each primer, 80 μM dNTPs, 20 mM tris (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 0.1% Triton X-100, 10 mM $(NH_4)SO_4$, and 0.1 mg/mL bovine serum albumin. Cycling was carried out in a Tetrad thermal cycler (MJ Research) as follows: an initial denaturation of 10 min at 96°C, followed by 30 cycles of 94°C for 10 s, 65°C for 15 s, and at 72°C for 30 s. Amplification products were cleaned with QIAquick columns (QIAGEN), sequenced using the Applied Biosystems Inc. (ABI) BigDye 3.1 cycle sequencing kit, purified with DyeEx 2.0 columns (QIAGEN), and electrophoresed on an ABI 377 DNA sequencer.

Using Lasergene software (DNASTAR), we aligned 225 nucleotide bases of the nuclear ITS region (NCBI numbers: AY234856, AY234857) and 464 nucleotide bases of the mitochondrial 16S rRNA gene (NCBI numbers AY227445 and AY227446). No intraspecific variation was observed in either of these loci. Between the two species, however, two nucleotide positions differed in the ITS region and five positions differed in the 16S gene. The hybrid individuals that we sequenced were heterozygous for both of the single nucleotide polymorphisms (SNPs) in the ITS region.

To rapidly genotype fishery samples for the ITS region and the 16S gene SNPs, we employed the 5′-nuclease assay (Lee et al. 1993) as follows. Oligonucleotide primers and probes for genotyping one 16S SNP and one ITS SNP were designed and synthesized using ABI's Assays by Design service (Table 1). Genotyping assays for the ITS and 16S SNPs were run on an ABI7900HT real-time sequence detection system in 384-well reaction plates, two wells in each plate serving as negative controls (no-template). Each well contained a 2.5-μL 2X TaqMan PCR cocktail (ABI), 900-nM PCR primers and 200-nM probes for one of the two SNP genotyping assays, and a 0.15-μL template DNA in a total reaction volume of 5 μL. An initial denaturation of 10 min at 95°C was followed by 42 cycles of 92°C for 15 s and 60°C for 1 min. Allele scoring was performed using Sequence Detection Software 2.0 (ABI). This software presented endpoint analysis in the form of a scatter-plot for each of the 384 wells per plate of samples (Figure 1). Scoring of the entire plate of individuals generally took less than 5 min. Optimal separation of alleles was observed using ROX as a passive reference for the ITS assay and no passive reference for the 16S assay.

Results

The SNP genotyping assays were tested on 51 snow crabs, 56 Tanner crabs, and 57 hybrids that had previously been identified using allozyme and polymerase chain reaction−restriction fragment length polymorphism (PCR-RFLP) assays. Individuals scored as homozygous for the nuclear SNP and as having the mtDNA haplotype for the same species were labeled as that species. Individuals exhibiting heterozygosity for the nuclear SNP and those individuals whose nuclear genotypes and mtDNA haplotypes were from different species were labeled as hybrids. No differences were observed between species identifications based on allozymes, PCR-RFLP analysis, or the SNP genotyping assays.

In February 2003, 500 crabs were collected from commercial fishing vessels in Dutch Harbor, Alaska. Crab biologists experienced in morphological variation in *Chionoecetes* identified each crab to species on site and sent a sample of muscle tissue (approximately 0.5 cm^3 from each crab in 95% ethyl alcohol at ambient temperature) to the Gene Conservation Laboratory in Anchorage. The SNP genotyping assays were applied to these samples, and the results were compared to morphological identifications.

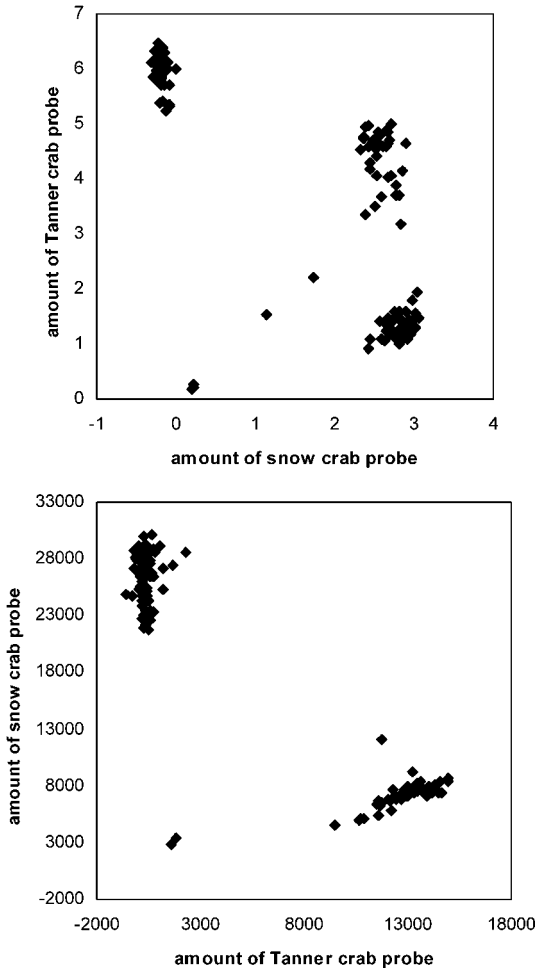

FIGURE 1.—Example of the data produced by (top) the ITS and (bottom) 16S single nucleotide polymorphism genotyping assays for Tanner and snow crabs. The *x* and *y* axes on each plot are the amounts of each of the two allele-specific probe signals observed in each reaction (absolute in the case of 16S and relative in the case of ITS). Each point on the scatter plot represents an individual crab whose genotype is determined by its location relative to the two axes. In the 16S scatter plot, the two clusters of points represent the two possible haplotypes. In the ITS scatter plot, a hybrid cluster is observed between the two species clusters. Dots in the lower left corner of each plot are negative controls (no template DNA added to polymerase chain reaction [PCR]). The two points in the ITS plot that are not part of any of the clusters represent failed PCRs.

Morphological identifications of the 2003 samples were 451 snow crabs, 2 (illegal) Tanner crabs, and 47 hybrids. The SNP genotyping assays provided exact concordance for snow and Tanner crab samples. Of the 47 crabs identified as morphological hybrids, however, the SNP assay identified 5 as snow crabs. This discrepancy may be due to the fact that only a single nuclear locus and the agreement between that locus and the mtDNA haplotype were examined (assuming these individuals were F2 or later generation hybrids), or it may reflect difficulties inherit in identifying hybrids based on the variable morphological characters (Urban et al. 2002). Data from the mtDNA SNP suggested that, as expected, most (40/42) of the hybrids were products of male Tanner crab × female snow crab crosses. These results were provided to fishery managers less than 24 h after our laboratory received the tissue samples.

Discussion

For our technique (or any genetic assays) to be ?5
provide accurate input on this management issue required assuming that F_2 or later generation hybrids compose a relatively small part of the population. The observation in both morphological and genetic surveys that *Chionoecetes* hybrids of any generation are rare (<10%) suggests that second and later generation hybrids will be very rare and, thus, lends some rationale for making this assumption. The observed concordance between the genetic and morphological data presented here, as well the findings by Merkouris et al. (1998) and Urban et al. (2002), suggest that both techniques provide reliable identifications of Tanner crabs, snow crabs, and their hybrids. A better understanding of hybridization between Tanner and snow crabs or of how each of these species vary throughout parts of their respective geographic ranges beyond the Bering Sea, however, will require the development and examination of additional nuclear markers and collection of samples covering a broader range.

Reagents required for the genotyping assays we presented here are comparable to those required for other DNA-based genetic markers and more expensive than reagents required for allozyme analysis. Because of the lack of an electrophoretic component and the ease with which alleles are scored, the throughput rate of these SNP genotyping assays is relatively high: 380 individuals four times per day, multiplied by the number of thermal cycler blocks available. The technician cost for performing SNP genotyping relative to that required for performing PCR-RFLP or allozyme analyses will therefore be reduced quickly as the number of samples to be processed increases. Because of this, the relative cost of applying 5′-nuclease SNP genotyping will depend on the balance be-

tween technician and chemical costs within any laboratory and upon the number of samples to be processed. A disadvantage of the 5′ -nuclease assay relative to PCR-RFLP or allozyme analyses is the cost of the hardware required to perform the former. The cost of real-time PCR instruments is decreasing, however, as technology improves and the number of vendors increases. For the specific management application presented here, SNP markers are a compelling choice due to (1) their high throughput rate and concomitant small requirement of laboratory staff time for processing large numbers of samples, and (2) their applicability, as PCR-based markers, to a broad range of sample formats.

The 5′-nuclease assay enables in-season estimates of catch composition and, thus, real-time monitoring of the snow crab fishery based on a larger number of samples and a broader range of samples than was previously feasible. In providing the ability to quickly survey large numbers of *Chionoecetes* individuals for Tanner crabs, this assay represents a useful management and conservation tool.

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