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# Species-specific oligonucleotides and multiplex PCR for forensic discrimination of two species of scallops, *Placopecten magellanicus* and *Chlamys islandica*

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**Abstract**

Characterization of DNA that remains in seafood products after skin, scales, and shells are removed is widely used in forensic species identification, however, ordinary methods may be prohibitively expensive or time-consuming if large sample series need to be discriminated. Forensic discrimination of two species of bivalves commercially harvested from the North Atlantic, sea scallops (*Placopecten magellanicus*) and Icelandic scallops (*Chlamys islandica*), was made by means of species-specific oligonucleotides (SSOs) in a multiplex polymerase chain reaction (PCR). The test is a simultaneous in vitro amplification of a portion of the mitochondrial Cytochrome Oxidase I locus with a PCR anchor primer for a sequence identical in both species, and two alternative SSOs that selectively amplify either a 619-bp in *Placopecten* or a 459-bp DNA fragment in *Chlamys*. Fragment size and thus species identity are determined directly by gel electrophoresis. In the forensic application, analysis of more than 900 scallops from a series of samples seized from two fishing vessels showed significantly variable proportions of the species from the closed and open fisheries (*Placopecten* versus *Chlamys*, respectively). The multiplex SSO test provides a direct means of forensic identification of large population sample series, without the necessity of secondary DNA sequencing, RFLP mapping, or fingerprinting, and can be adapted to other loci and species.
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*Keywords:* Forensic genetics; *Placopecten magellanicus*; *Chlamys islandica*; Scallops; Species-specific oligonucleotides; Seafood identification; Multiplex PCR

## 1. Introduction

Identification of species when external identifying marks are missing or have been removed remains a leading problem of forensic science [1,2]. Forensic issues raised by non- or misidentification of seafood products include enforcement of fishing regulations [3], threats to endangered species [4], and compromise of the integrity of commercial products [5–13]. In such cases, DNA testing has found wide application in forensic determination of species identity. In particular, the polymerase chain reaction (PCR) has been used to recover trace amounts of DNA remaining in seafood tissues used as food [14].

Here, we are concerned with a question of possession and retention of a scallop species (*Placopecten*: Pectinidae) from a closed fishery as bycatch in a directed fishery for a second species (*Chlamys*). The question is whether the observed proportion of a prohibited species exceeds some critical value set by regulatory agencies, within certain statistical limits. For such purposes, it is desirable to have a rapid, cost-effective molecular procedure that can identify to species a sufficiently large number of individuals so as to estimate those limits precisely. A limitation of conventional forensic DNA techniques is the necessary time, effort, and cost to characterize PCR products by secondary experimental methods, such as sequencing, endonuclease digestion, fingerprinting, RAPD analysis, and so on. Analysis of these results requires further investment of time, for example, RFLP mapping, sequence editing, fingerprint alignment, interpretations of banding patterns, and database comparison. What is required in such cases is a method in which the product of the PCR reaction can be interpreted directly.

Allele-specific oligonucleotides (ASOs) detect the presence or absence of particular alleles at a given locus that differ by one

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or a few nucleotide substitutions [15]. An extension of this concept is the species-specific oligonucleotide (SSO), which relies on a pattern of genetic variation in which interspecific sequence differences are sufficient to define unique priming sites in each of the species of interest, yet intraspecific variation is small enough that most or all most conspecific individuals share such sites. Such tests can be multiplexed, that is, two or more PCR reactions can be run simultaneously in the same tube.

We describe here a rapid, cost-effective method for discrimination of two commercial species of shellfish, based on multiplex SSO amplification of species-specific PCR fragments of different sizes. This test is described for one particular mitochondrial DNA (mtDNA) locus and two particular species of scallops, however, the experimental design is easily adapted to forensic discrimination of other species for which locus-specific DNA sequence data are available.

## 2. Materials and methods

### 2.1. Sample collection

Scallops were seized from two commercial fishing vessel by officers from the Enforcement Division of the Department of Fisheries and Oceans, Marystown, Placentia Bay, Nfld. Samples of scallops were taken from the top, middle, and bottom of each storage compartment on each vessel. The sample series from the first vessel comprised 27 sample bags taken from nine compartments, where each bag contained an approximately equal volume (a two-cup measure). The series from the second vessel comprised 16 bags. Prior to freezing, each sample bag was weighed and the number of scallops counted.

Sample bags were brought to the laboratory, thawed in cold water, and a sample of ca. 100 μg was removed from each scallop with a new razor blade. DNA was extracted from each sample by a protease-based method with a QIAamp® DNA Mini Kit (Qiagen Inc.), according to the manufacturer's instructions.

### 2.2. Design of species-specific oligonucleotides

The GenBank database includes the complete mtDNA genome sequence for a sea scallop (*Placopecten magellanicus*) (NC07234) and a partial sequence for the mitochondrial DNA (mtDNA) Cytochrome Oxidase I locus (hereinafter COI) for Icelandic scallop (*Chlamys islandica*) (GenBank accession AB033665) [16]. To design species-specific oligonucleotides (SSOs), we looked for two sorts of regions in the two DNA sequences, those with not more than one mismatch between the two species over 25–30 bases, and those that contained $\gtrsim 10$ mismatches over a similar sequence length (Fig. 1). A match at the 3′-most base is considered most critical for proper primer annealing, all other factors being equal [17]. Two regions of the first sort and four of the latter were identified; preliminary experiments indicated that two forward SSOs designated *PmaCOI-F1* and *CisCOI-F2* together with a common reverse primer *Scallop-R2* (Fig. 1) gave the best combination of desirable characteristics (see Section 4):

- *PmaCOI-F1*:
  5′ GTAGTGTCTTCTTCTTTTATGGACGGGCTA 3′
- *CisCOI-F2*:
  5′ TGGTTACTTTTTTAAACATACGCGGAAAGTC 3′
- *Scallop-R2*:
  5′ CACCMGTGGGCACAGCAATTATATAG 3′

The expected size of the DNA fragment produced by the *PmaCOI-F1*/*Scallop-R2* primer combination is 619 bp in *Placopecten*, and the expected size of the *CisCOI-F2*/*Scallop-R2* product is 459 bp in *Chlamys*.

### 2.3. Multiplex polymerase chain reaction

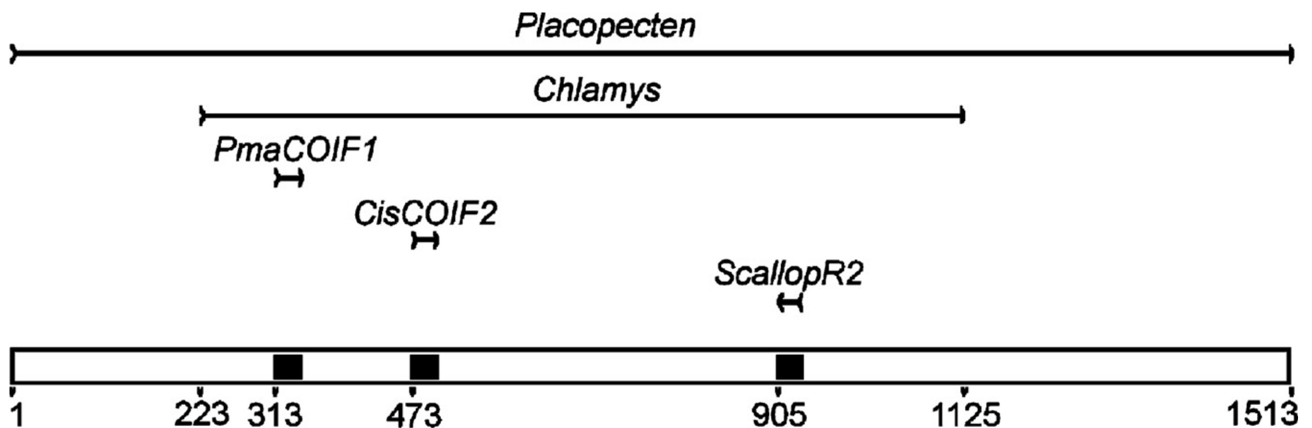
DNA from each scallop sample was amplified in a single multiplex reaction with both species-specific forward primers and the common reverse primer. We prepared 25-μl PCR reactions containing 1X PCR reaction buffer and 1 U of HotStar *Taq* polymerase (Qiagen Inc.), 400 nM of each of the three primers (Operon Inc.), 50 μM of each deoxynucleoside triphosphate (dNTP; Amersham Pharmacia Biotech), and 3 μl of template DNA. Following an initial incubation at 95 °C for 15 min to activate the polymerase, reactions were taken through 40 cycles, each comprising denaturation at 93 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. The last cycle was followed by a final extension at 72 °C for 10 min. All thermal manipulations were performed in an Eppendorf Mastercycler gradient thermal cycler. PCR product sizes were determined against a ΦX-174/*Hae*III size standard by electrophoresis of 4 μl of the product through 1.5% agarose in 1× TBE buffer containing ethidium bromide for 45 min. Gels were photographed with a ChemiImage™ Ready 4400 v5.5 photodocumentation system.

### 2.4. Statistical methods

Chi-square tests for proportions were calculated by the method of Snedecor and Cochran [18]; these are equivalent to a calculation of the homogeneity of binomial variances. Product-moment correlations and multiple range tests (sum of squares simultaneous test procedure) were calculated with the program BIOMstat 3.30 m of Sokal and Rohlf [19]. Variances of the binomial distribution of *Placopecten* were calculated as $P(1-P)/2N$, where $P$ is the fraction of *Placopecten* and $N$ is the total sample size. Critical values for extreme values of the chi-square distribution were calculated with the library function of Minitab, as supplied by D. Schneider (personal communication).

## 3. Results

A total of 967 scallops were examined. Amplicon bands of one or the other of the two expected sizes were obtained in all but one instance (from which no PCR product was obtained)



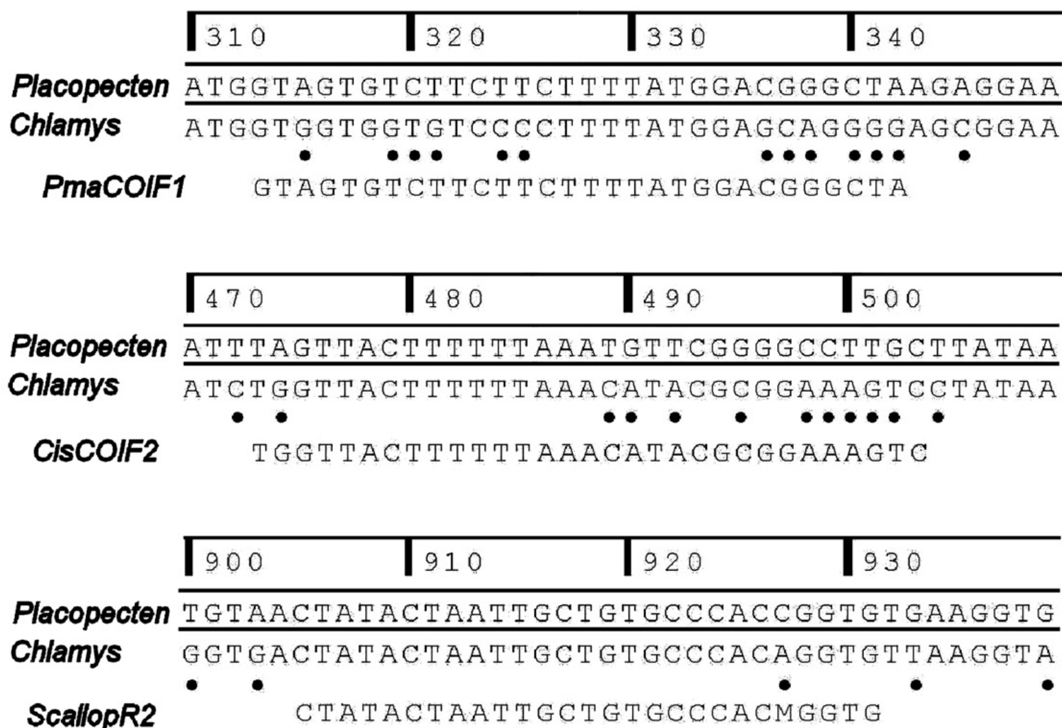




Fig. 1. Primer design in the scallop mitochondrial Cytochrome Oxidase I (COI) gene. The complete locus is 1513 bp in *Placopecten*; the sequence of a 903-bp segment is known for *Chlamys*. The 5′ ends of three primer regions (*PmaCOI-F1*, *CisCOI-F2*, and *Scallop-R2*) are located as indicated. Primer sequences and miss-matches between them are given: the *Scallop-R2* primer is a reverse primer and its sequence (see text) is the reverse complement of that indicated here. The *PmaCOI-F1* + *Scallop-R2* combination amplifies a 619-bp fragment in *Placopecten*, the *CisCOI-F2* + *Scallop-R2* combination amplifies a 459 bp fragment in *Chlamys*.

(Fig. 2). DNA sequencing of several amplicons of either size showed them in all cases to have the expected species-specific DNA sequence (results not shown).

The first vessel seizure comprised 617 scallops in nine series of three bags, one each taken from the top, middle, and bottom of separate storage compartments in the vessel (Table 1). The 27 sample bags had a mean weight of 16.6 oz (range 12.2–22.4), and contained 21.9 scallops (range 11–33) per pound. Across all 27 samples, 64.9% were *Placopecten* (range 23–100%): six samples contained a minority of *Placopecten*, and seven were entirely *Placopecten*. The variation is significantly heterogeneous ($\chi^2 = 166.63$, $p < 0.001$ for a one-tailed test). Sorted by the nine sample series, the proportion of *Placopecten* varied from 43.4% to 76.7%. The variances of the binomial distributions are significantly heterogeneous ($\chi^2 = 37.36$, $p < 0.001$ for a one-tailed test). This is strongly influenced by the one sample in which *Placopecten* is in the minority, otherwise $\chi^2 = 16.74$, $p < 0.05$. A multiple range test (sum of squares simultaneous test procedure) with $\alpha = 0.001$ indicates four non-significant ranges, which are ##7, 9, (8, 2, 4), and (3, 5,

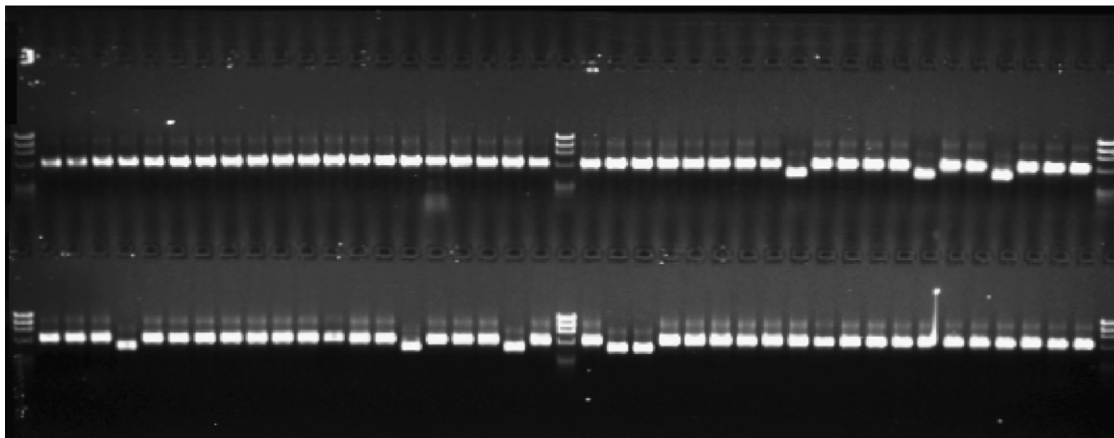


Fig. 2. PCR products from amplification of scallop DNA with species-specific oligonucleotides (SSOs). The molecular size standards in the outside and centermost lanes are ΦX-174 DNA digested with *Hae*III; the fourth band from the top is 603 bp. Two diagnostic size classes are seen, a 0.62 kbp amplicon for *Placopecten*, and a 0.46 kbp amplicon for *Chlamys*. Among 80 amplicons run as four sets of 20, a total of eight (0, 3, 2, and 3, in each set, clockwise from upper left) are identified as *Chlamys* and the remaining 72 as *Placopecten*.

6, 1), with 43.4%, 53.0%, 65.3%, and 75.7% *Placopecten*, respectively. Sorted by the three positions (top, middle, or bottom), the proportions of *Placopecten* were 60.6%, 70.3%, and 64.8%, respectively; the heterogeneity is not significant ($\chi^2 = 5.01$, $0.10 > p > 0.05$).

The proportion of *Placopecten* shows a strong negative correlation with the number of scallops in the sample bag ($r = -0.82$, $F_{[1,25]} = 49.63$, $p \ll 0.001$), that is, bags with more scallops tended to include a disproportionately greater number of *Chlamys* (Fig. 2). When the scallop count is corrected by the weight of the sample bag, the correlation between proportion of *Placopecten* and the number of scallops per pound is enhanced ($r = -0.87$, $F_{[1,25]} = 80.04$, $p \ll 0.001$).

The seizure from the second vessel comprised 350 scallops in 16 bags, of which all but four (three in one sample bag, one in another) were identified as *Placopecten* (98.9%) (results not shown).

## 4. Discussion

### 4.1. Technical considerations for SSO design

The design of appropriate SSOs must satisfy several requirements. The first is to identify at least two regions of a target gene sequence that differ substantially between the two species, and at least one that is highly conserved. Likely target loci for seafood species are found in the mitochondrial genome, including the cytochrome *b* and COI loci, for both of which there is an extensive database of reference sequences [16]. In the present case, the aligned sequence regions of the COI genes of two scallop species were more than 80% similar: only a few regions could be located that contained at least 6–8 contiguous or nearly contiguous mismatched nucleotides. SSOs can then be designed in which those positions are placed at the 3′ end to ensure annealing only to the DNA of the intended species. Regions with almost perfect sequence identity between species [no more than one mismatch in 25–30 bp] can serve as anchor sites for common reverse primers. The actual length of each SSO should be adjusted so that the polymerase chain reaction for each forward and reverse combination will proceed at the same optimal annealing temperature ($T_A$); this can be calculated with programs such as the OLIGO 4.1 Primer Analysis Software [20,21]. This prevents preferential amplification of one product over the other according to the choice of annealing temperature. Finally, the amplicons produced by the SSOs should be of such sizes as are readily resolvable by brief electrophoresis in a small-scale gel assembly, approximately 0.2–0.7 kbp.

We identified two forward regions and two reverse regions that satisfied these criteria. For the large-scale assay described here, we selected the SSO set in which the *Placopecten* amplicon was larger than the *Chlamys* amplicon. This avoided any possible bias in favour of detection of the prohibited species (*Placopecten*), if smaller fragments were amplified preferentially [17]. This combination also maximized the amplicon size for both species, so as to maximize the information content of their DNA sequences in the event that a subsequent sequencing study were indicated or desirable.

### 4.2. Detectability of scallop poaching

The instant investigation took place under Section 33 of the Fisheries Act (Possession of Fish caught contrary to the Act and/or Regulations), in this case possession of sea scallops when that fishery was closed. Incidental catch of scallops from a closed fishery during a directed fishery for an alternate species is to be avoided, and live scallops are to be returned to the water from where they are caught in a manner that causes the least harm. Although intact sea scallops and Icelandic scallops are readily distinguishable by shell morphology [22], Canadian Department of Fisheries and Oceans enforcement practice recognizes that possession of some small fraction of a regulated species as bycatch along with a species from the directed fishery is possible, despite due diligence of fishers. A tolerance of approximately 5% would establish a one-tailed statistical test criterion for which the critical values of $\chi^2$ with one degree of

Table 1
Distribution of *Placopecten* (*Pla*) and *Chlamys* (*Chl*) among sample bags and series

| Bag | # *Pla* | # *Chl* | Total | f(*Pla*) | # *Pla* | # *Chl* | Total | f(*Pla*) | $\sigma_{(Pla)}^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 14 | 1 | 15 | 0.933 | | | | | |
| 2 | 14 | 8 | 22 | 0.636 | | | | | |
| 3 | 18 | 5 | 23 | 0.783 | 46 | 14 | 60 | 0.767 | 0.00149 |
| 4 | 11 | 0 | 11 | 1.000 | | | | | |
| 5 | 12 | 0 | 12 | 1.000 | | | | | |
| 6 | 8 | 17 | 25 | 0.320 | 31 | 17 | 48 | 0.646 | 0.00238 |
| 7 | 16 | 3 | 19 | 0.842 | | | | | |
| 8 | 16 | 10 | 26 | 0.615 | | | | | |
| 9 | 16 | 3 | 19 | 0.842 | 48 | 16 | 64 | 0.750 | 0.00146 |
| 10 | 19 | 3 | 22 | 0.864 | | | | | |
| 11 | 19 | 0 | 19 | 1.000 | | | | | |
| 12 | 12 | 21 | 33 | 0.364 | 50 | 24 | 74 | 0.676 | 0.00148 |
| 13 | 9 | 15 | 24 | 0.375 | | | | | |
| 14 | 15 | 0 | 15 | 1.000 | | | | | |
| 15 | 21 | 0 | 21 | 1.000 | 45 | 15 | 60 | 0.750 | 0.00156 |
| 16 | 20 | 0 | 20 | 1.000 | | | | | |
| 17 | 12 | 11 | 23 | 0.522 | | | | | |
| 18 | 22 | 6 | 28 | 0.786 | 54 | 17 | 71 | 0.761 | 0.00128 |
| 19 | 7 | 24 | 31 | 0.226 | | | | | |
| 20 | 16 | 7 | 23 | 0.696 | | | | | |
| 21 | 13 | 16 | 29 | 0.448 | 36 | 47 | 83 | 0.434 | 0.00148 |
| 22 | 14 | 13 | 27 | 0.519 | | | | | |
| 23 | 15 | 14 | 29 | 0.517 | | | | | |
| 24 | 18 | 0 | 18 | 1.000 | 47 | 27 | 74 | 0.635 | 0.00157 |
| 25 | 19 | 11 | 30 | 0.633 | | | | | |
| 26 | 16 | 7 | 23 | 0.696 | | | | | |
| 27 | 9 | 21 | 30 | 0.300 | 44 | 39 | 83 | 0.530 | 0.00150 |
| | **401** | **216** | **617** | **0.650** | | | | | **0.00018** |

The 27 bags were sampled from nine different compartments, and three samples were taken from each compartment, from the top, middle, and bottom. Each block shows the sample series' distribution from one compartment. The binomial variance $\sigma^2_{(Pla)}$ of the fraction of *Placopecten* in each compartment is $P(1-P)/2N$, where $P$ is the fraction of *Placopecten* and $N$ is the total sample size.

freedom are 6.64 for $p < 0.01$, 10.03 for $p < 0.001$, and 23.93 for $p < 10^{-6}$. The seized samples comprised 65% and 99%, respectively, of the species from the closed fishery: the probability that either is a chance excess is negligible. With a sample of 617 scallops from the first vessel, possession of 7.6% scallops from the closed fishery would exceed the 5% criterion at $p < 0.001$ ($\chi^2 = 8.44$). In general, possession of 11% of a prohibited species in a sample of 100, or 6.8% in a sample of 1000, would exceed the 5% criterion at $p < 0.001$ ($\chi^2 = 7.58$ and 6.82). Note that these represent 120% or 36% of the tolerated limit, such that the smaller sample could not rule out as much as 10% bycatch retention.

The heterogeneity of the variance in the proportion of *Placopecten* among sample series from the first vessel (43–77%) indicates that the products in different compartments represent distinct samples, all of which individually exceed a

5% limit. The multiple range test identifies four significant ranges, differentiated by about 10% each, which might be expected if each represents a catch taken at a different place with different proportions of *Placopecten*. Variation of the proportion of *Placopecten* sorted by position within compartments was not statistically significant. A lower proportion of the prohibited species in the topmost layer might have been expected if the legal species were ''salted'' to cover the prohibited species.

Each two-cup scallop sample weighs roughly 16 oz and contains an average of 22 scallops, and thus corresponds to a commercial ''20/30 count'' product. The observation that the proportion of *Placopecten* is negatively correlated with the scallop count (Fig. 3) or count corrected for sample weight, may provide a useful ''rule of thumb'' for on-site estimation of species composition of mixed products. If scallop count is treated as an independent variable, the regression equation [*Placopecten* (%) = $-3.60 \times$ (number of scallops/bag) + 152.3] predicts approximately 43 scallops in a *Chlamys*-only sample, versus approximately 15 scallops in a *Placopecten*-only sample. Fig. 3 suggests a ''30/30 rule,'' that a two-cup, 16 oz sample containing 30 or fewer scallops is likely to contain more than 30% *Placopecten*.

### 4.3. Applicability to other species

With respect to bivalve molluscs, forensic PCR methods have been used to distinguish four species of canned scallops (*Pecten maximus*, *Mimachlamys varia*, *Aequipecten opercularis*, and *Chlamys distorta*) by characterization of RFLP patterns in the nuclear 5.8S rDNA gene and its flanking regions [23]. Most recently, the first two of these species as well as *A. opercularis* were distinguished via combinations of species-specific primers for the mitochondrial 16S rDNA and nuclear 18S rDNA loci, in order to discriminate larval species in the Irish bivalve fishery [24]. Inter alia, a pair of 16S mitochondrial rDNA primers was designed to amplify the king scallop (*Placopecten maximus*), and used to discriminate this species from black scallop (*M. varia*) and queen scallop (*A. opercularis*). King and black scallops were distinguished by 382 bp versus 398 bp amplicons; the last species did not consistently amplify.

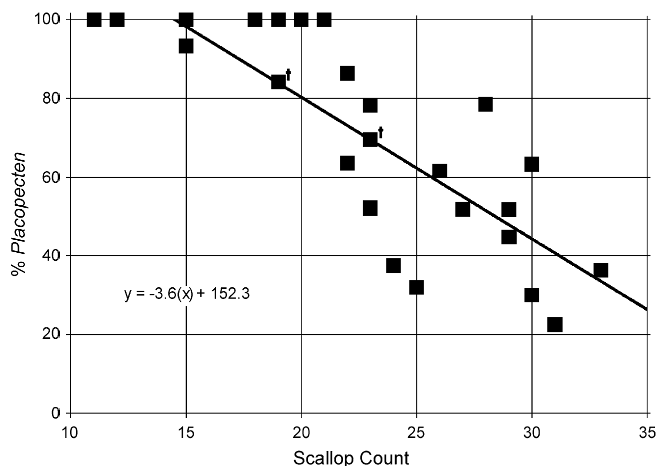



Fig. 3. Scallop count vs. *Placopecten* (%). Two pairs of samples, with identical counts and proportions of *Placopecten*, are flagged (†). The count of scallops per sample bag is significantly negatively correlated with the proportion of *Placopecten* as determined by molecular assay ($r = -0.82$, $p < 0.001$). The same result is obtained when the count is corrected for weight. This reflects the smaller average size of *Chlamys*.

The strategy offered here offers several advantages for discrimination of pectinid species. When reference sequences are available in GenBank, SSO primers can be designed specifically for the species of interest. Expected size differences among species' amplicons are then part of the multiplex experimental design, rather than a fortuitous consequence of interspecific indel variation. This also allows the diagnostic differences among amplicon sizes used as species assays to be deliberately maximized (169 bp versus 17 bp), so that reliable discrimination after brief electrophoresis (45 min versus 180 min) is possible.

The multiplex SSO design can be extended to other species. Among pectinid scallops, a phylogenetic study of Asian scallops has provided COI sequences for 17 species in 15 genera [25], including *Chlamys*, *Mimachlamys*, and *Pecten* spp. Each of these genera was assigned to a separate taxonomic tribe. Phylogenetic analysis of *Placopecten* (results not shown) indicates that it is more closely related to *Chlamys* than to any of these other genera, as expected given its high degree of sequence similarity in the *Scallop-R2* primer region. Pairwise comparison of the *Scallop-R2* primer sequence with the COI sequences of *Mimachlamys* and *Pecten* spp. shows 3–4 mismatches, which suggests that with appropriate modification this region would serve as a suitable anchor primer for these species as well. Of course, the GenBank database [16] provides a wealth of sequence data for the extension of the SSO method described here to other species of invertebrates and vertebrates of interest to forensic science.

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