Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2011) **79**, 112–121
doi:10.1111/j.1095-8649.2011.02999.x, available online at wileyonlinelibrary.com

Identification of seagrasses in the gut of a marine herbivorous fish using DNA barcoding and visual inspection techniques

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(Received 24 May 2010, Accepted 1 April 2011)

Traditional visual diet analysis techniques were compared with DNA barcoding in juvenile herbivorous rabbitfish *Siganus fuscescens* collected in Moreton Bay, Australia, where at least six species of seagrass occur. The intergenic spacer *trn*H-*psb*A, suggested as the optimal gene for barcoding angiosperms, was used for the first time to identify the seagrass in fish guts. Four seagrass species and one alga were identified visually from gut contents; however, there was considerable uncertainty in visual identification with 38 of 40 fish having unidentifiable plant fragments in their gut. PCR and single-strand conformational polymorphism (SSCP) were able to discriminate three seagrass families from visually cryptic gut contents. While effective in identifying cryptic gut content to family level, this novel method is likely to be most efficient when paired with visual identification techniques.

Key words: intergenic spacer; *Siganus fuscescens*; single-strand conformational polymorphism (SSCP); *trn*H-*psb*A.

INTRODUCTION

Gut content analysis is an important tool that can provide insight into feeding ecology, food web interactions and nutrient flow between ecosystems (Lugendo *et al*., 2006; Barbour *et al*., 2010). Identification of ingested material to species level greatly facilitates the utility of such data, a process which is usually attempted visually and frequently with the caveat 'to the lowest possible taxon'. The effects of physical and chemical digestion on the ingested food may curtail ambitions concerning accurate identification leading to quite coarse taxonomic resolution (Jarman & Wilson, 2004; Smith *et al*., 2005). Small bite sizes and mechanical treatment in the oral or pharyngeal jaws, combined with the morphological similarity of some groups of aquatic plants, make herbivorous fishes among the most challenging targets for such studies (Cocheret de la Morinière *et al*., 2003).

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Molecular techniques, such as DNA barcoding, provide an alternative method of identifying biological gut contents that are digested or macerated beyond visual recognition. This technique uses short and unique sequences of DNA to identify unknown species of both plants and animals by comparing them to reference sequences of known species (Hebert *et al*., 2003). Indeed such approaches may be the only means of identifying specimens where prey morphology is so disrupted by processing; for example, by the pharyngeal mills of Hemiramphidae (Tibbetts & Carseldine, 2003) and Scaridae (Carr *et al*., 2006). While such methods have been successfully used to identify invertebrate prey species of larger invertebrates (Blankenship & Yayanos, 2005), insect prey of arthropod predators (Hosseini *et al*., 2008), plants in the diet of voles (Soininen *et al*., 2009) and insects (Matheson *et al*., 2008), microalgal prey of copepods (Nejstgaard *et al*., 2008), fish prey species of carnivorous fishes (Smith *et al*., 2005) and the prey of deepwater sharks (Dunn *et al*., 2010), no published studies have used such DNA detection methods for plant species consumed by fishes. A non-DNA approach, the detection of marker compounds following thermochemolysis, has however been used to distinguish food types in omnivorous fishes (McIntyre *et al*., 2007).

A wealth of DNA marker information exists that could be used for the DNA barcoding of seagrasses. Microsatellites (Reusch, 2006; Alberto *et al*., 2008), nuclear internal transcribed spacers (ITS; Les *et al*., 2002) and plastid (Les *et al*., 1997) genes have been used for seagrass population genetics, evolution and phylogenetic studies (Waycott *et al*., 2006). No published studies exist, however, that utilize genetic information specifically for the DNA barcoding of seagrass species. A key challenge for this approach is the lack of sufficient genetic differentiation of the mitochondrial genes among plant species (Kress *et al*., 2005). To address this problem, the use of an intergenic spacer has been suggested by Kress *et al*. (2005) as a suitable gene for DNA barcoding for all angiosperms, due to the higher sequence variability observed in non-coding regions. The efficacy of the proposed region, contained in the plastid *trn*H-*psb*A gene, as a barcode for different plant species has since been shown for several land plants (Kress & Erickson, 2007; Ragupathy *et al*., 2009).

A plastid *trn*H-*psb*A intergenic spacer DNA-barcoding method for subtropical Australian seagrass species was developed, using a fish model that typically takes food snips using its oral jaws and passes that material into the oesophagus using a non-macerative pharyngeal jaw, making possible the visual identification of gut contents. The model species in the current study was the mottled spinefoot *Siganus fuscescens* (Houttuyn, 1782), an herbivorous rabbitfish (Pillans *et al*., 2004) widely distributed in the Indo-Pacific Ocean (Woodland, 1990). It occurs in Moreton Bay where seagrass diversity is relatively high, with six known species (Waycott *et al*., 2004) providing a useful test of the technique in a natural context.

MATERIALS AND METHODS

STUDY SPECIES AND SAMPLE COLLECTION

Leaves of five seagrass species: *Halophila ovalis*, *Halophila spinulosa*, *Zostera muelleri*, *Cymodocea serrulata* and *Syringodium isoetifolium* ($n = 3$ for each species) were obtained from mixed seagrass beds in One Mile Harbour, North Stradbroke Island (27° 29′ 37·42″ S; 153° 24′ 01·82″ E) on the eastern coast of Australia. *Halodule uninervis*, the sixth seagrass

species found in the bay, was excluded from this study as it is uncommon and does not occur in measurable quantities within the collection area. Forty-two juvenile *S. fuscescens* were seined from mixed seagrass beds on two occasions in November 2008 and March 2009. Fish were euthanazed and frozen until gut contents were removed.

VISUAL IDENTIFICATION

Gut contents were removed from the *S. fuscescens* by dissection and identified using a taxonomic key (Waycott *et al*., 2004). Characteristic features were used to identify fragments, such as cross-veins, serrated edges or intra-marginal veins. A dissecting microscope (Olympus SZX7, $\times$12 magnification; www.olympus.com) was used for all identification, as most plant fragments were $\leq$1 mm. Following visual identification, samples were stored in 70% ethanol for subsequent DNA analysis.

DNA ISOLATION

Epiphyte-free blades of seagrass (1 cm^2) collected from the One Mile Harbour and gut content sub-samples (500 mg) were frozen in liquid nitrogen and subsequently homogenized into slurries. DNA was extracted from the slurry using the DNeasy Plant Mini Kit (www.qiagen.com) following the manufacturer's protocol.

PCR AMPLIFICATION

Approximately 300–450 bp of the plastid *trn*H-*psb*A intergenic spacer was amplified from DNA extracted from free-living seagrass and gut contents using angiosperm-specific primers (psbA forward 5′-GTTATGCATGAACGTAATGCTC-3′ and trnH reverse 5′-CGCGCATGGT GGATTCACAAATC-3′; Sang *et al*., 1997). All PCRs contained 1 ng of template DNA, 1 mM total deoxynucleoside triphosphate (dNTP), 10 pmol of each primer and 1·5 U of Taq DNA polymerase (Ampli-Taq, CinnaGen; http://www.cinnagen.com/) in a total volume of 50 µl. Amplification was performed using a Hybaid PCR Express Thermal Cycler (www.labequip.com) with the following thermal profile: 35 cycles of 1 min at 94° C, 1 min at 46° C, 45 s at 72° C. The PCR products were analysed by electrophoresis on 1·5% agarose gels (100 V, 40 mA), stained with ethidium bromide and visualized under UV.

SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS

Single-stranded conformational polymorphism (SSCP) analysis was performed on all samples, using 10 µl of PCR product mixed with an equal volume of loading buffer (95% formamide, 10 mM NaOH, 0·25% bromophenol blue and 0·25% xylene cyanol) and heat denatured for 5 min at 95° C. After denaturing, the mixed aliquots were immediately chilled on crushed ice and run on a polyacrylamide mutation detection electrophoresis (MDE) gel (FMC Bioproducts; http://www.cambrex.com/) in 0·6$\times$ tris-borate- EDTA (TBE) at 160 V for 10 h at room temperature. The SSCP patterns were stained with ethidium bromide and visualized with UV transillumination.

DNA SEQUENCING AND PHYLOGENETIC ANALYSIS

DNA sequencing and phylogenetic analysis of sequences were undertaken on seven gut contents with SSCP profiles that suggested single species and five free-living seagrass samples. This was to confirm whether the differences observed in the PCR products from the SSCP analysis were due to different sequences. DNA sequences were determined in both directions using the dye terminator automatic sequencing facility at the Australian Genome Research Facility at the University of Queensland, Australia. Sequences were manually edited and submitted to GenBank. Accession numbers for all derived sequences are listed in Table I. All

TABLE I. List of GenBank accession codes for sequenced plastid *trn*H-*psb*A intergenic spacer DNA of *Siganus fuscescens* and five of its dietary seagrass species

	GenBank accession code
Siganus fuscescens	
S6	GU906228
S15	GU906230
S27	GU906231
S34	GU906232
S39	GU906233
S41	GU906229
S42	FJ648794
Seagrass species	
Syringodium isoetifolium	FJ648793
Cymodocea serrulata	FJ648790
Zostera muelleri	GU906227
Halophila spinulosa	FJ648792
Halophila ovalis	FJ648791

sequences were aligned with plastid *psb*A (partial), *trn*H-*psb*A intergenic spacer (complete) and tRNA-His (*trn*H) gene (partial) DNA sequences of the seagrass species *Zostera marina* and the terrestrial angiosperm *Alocasia odora* (outgroup) (accession numbers DQ786516 and DQ786512, respectively). The alignment was conducted using CLUSTAL-X (Thompson *et al*., 1997).

Phylogenetic analyses of aligned sequences were undertaken using the maximum-likelihood method. The programme ModelTest version 3.7 (Posada & Crandall, 1998) was used to find the most appropriate substitution model for the data. Maximum-likelihood analysis was performed using the phylogenetic analysis using parsimony (PAUP) computer programme, PAUP beta version 4.0b10 (Swofford, 2002). The model selected was Hasegawa, Kishino and Yano (HKY) using the hierarchical likelihood ratio tests. Maximum-likelihood analyses were performed heuristically with 1000 random additions, tree bisection and reconnection (TBR) swapping and Multitrees option. Branch robustness was tested using 1000 bootstrap replicates. All phylogenetic trees were generated using TreeView version 1.6.5 (Page, 1996).

RESULTS

VISUAL IDENTIFICATION OF GUT CONTENTS

Juvenile *S. fuscescens* ranged from 36 to 54 mm with a mean standard length (L_S) of 45 mm. Visual identification of gut contents revealed the presence of ingested seagrasses and algae (Fig. 1). Seagrass was observed in every sample. *Zostera muelleri* was the most frequently identified seagrass species, with a visually confirmed presence in 38 of the 40 samples (Fig. 2). Other seagrass species were detected in a small number of samples: *S. isoetifolium* (4), *C. serrulata* (4) and *H. ovalis* (8) (Fig. 2). Two seagrass species found in the bay, *H. spinulosa* and *H. uninervis*, were not identified in any samples. The macroalga *Caulerpa taxifolia* was identified in the gut contents of some fish used for preliminary dissections; however, none was found in the fish included in this study. The majority of the gut samples (38 of 40) contained at least some seagrass fragments that were too small or degraded to be identified

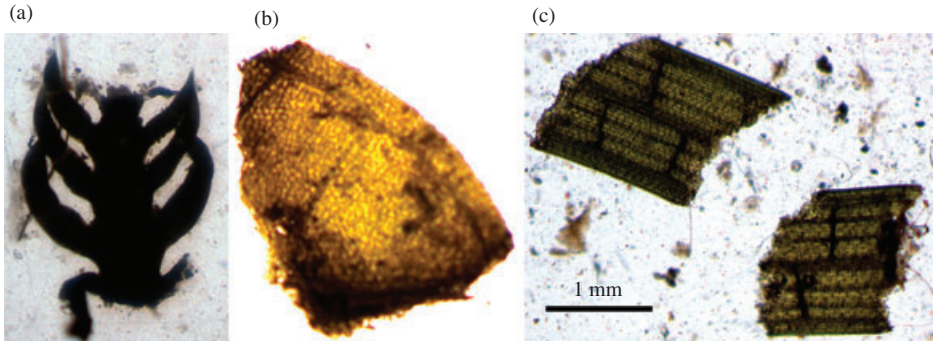

FIG. 1. Fragments of an alga and seagrasses collected from the guts of *Siganus fuscescens* at One Mile Harbour, Dunwich, Moreton Bay, showing that visual identification of ingested seagrass is possible using light microscopy (×12 magnification): (a) *Caulerpa taxifolia*, (b) *Halophila ovalis* and (c) *Zostera muelleri*.

using morphological features. Many fish appeared to have multiple species of seagrass in their gut, however, this was difficult to confirm due to the large amount of unidentifiable fragments.

MOLECULAR IDENTIFICATION OF GUT CONTENTS

PCR of the extracted plastid *trn*H-*psb*A DNA yielded positive results for 32 of the 42 *S. fuscescens* gut content samples and from all the seagrass-positive controls. The size of the amplified *trn*H-*psb*A DNA product varied with species (*Halophila* spp. *c*. 300 bp; *v*. *Z. muelleri*, *C. serrulata* and *S. isoetifolium c*. 400–450 bp). The *trn*H-*psb*A DNA SSCP profile of *Z. muelleri* was unique, but *C. serrulata* and *S. isoetifolium*, and the two *Halophila* species formed two similar profiles, respectively (Fig. 3). The presence of *Z. muelleri*, *C. serrulata* and *S. isoetifolium* and *Halophila* spp. was detected in the *S. fuscescens* gut contents using SSCP profiles consistent with specific seagrass controls (Fig. 3). The SSCP profile consistent with *Z. muelleri* was detected in the gut contents of 30 fish (*e.g.* S3, S5, S6, S15, S22, S34 and S39; Fig. 3). The SSCP profile consistent with both *Z. muelleri* and *C. serrulata* and *S. isoetifolium* was detected in one fish (S10; Fig. 3), and the *Halophila* spp. SSCP profile was detected in four fish (S2, S14, S41, SSCP profiles not shown; S42; Fig. 3).

DNA sequencing and phylogenetic analysis of *trn*H-*psb*A PCR products from representative fish gut samples confirmed the identity of several SSCP profiles (S6, S15, S27, S34 and S39: *Z. muelleri*; S41 and S42: *Halophila* spp.). A maximum-likelihood tree (Fig. 4), produced from aligned *trn*H-*psb*A DNA representative sequences, groups the seagrasses from the gut contents into robust *Z. muelleri* and *Halophila* spp. clusters (bootstrap values = 100%).

DISCUSSION

The use of the intergenic spacer *trn*H-*psb*A enabled discrimination of all three control seagrass families by SSCP profiles followed by DNA sequencing. This is the first

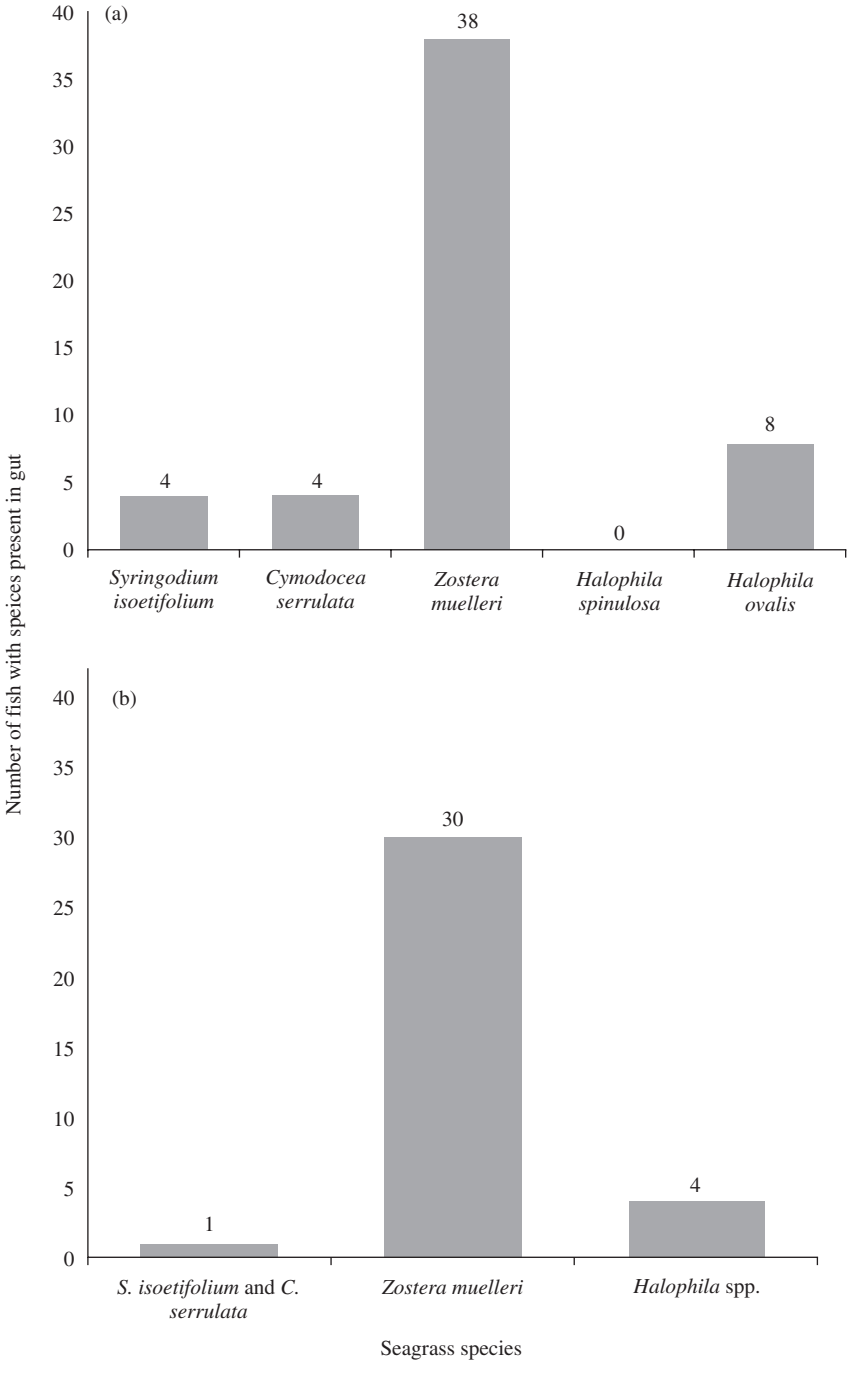

FIG. 2. Comparison of the number of each seagrass species contained in the gut of *Siganus fuscescens* collected from One Mile Harbour, Dunwich, Moreton Bay using (a) visual inspection and (b) molecular analysis. Numbers above bars indicate sample size for each species or species grouping.

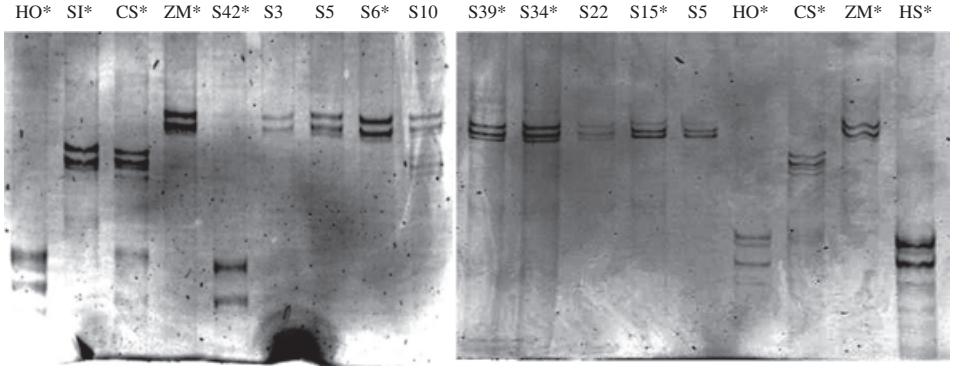

FIG. 3. Single-strand conformational polymorphism profiles of representative PCR-amplified plastid *psb*A-*trn*H DNA from free-living control seagrass and gut contents of *Siganus fuscescens*. CS, *Cymodocea serrulata*; HO, *Halophila ovalis*; HS, *Halophila spinulosa*; SI, *Syringodium isoetifolium*; ZM, *Zostera muelleri*; gut contents = S3, S5, S6, S10, S15, S22, S34, S39 and S42. ⁎, the PCR product was sequenced and added to the phylogenetic tree in Fig. 4.

study to successfully use *trn*H-*psb*A for the DNA barcoding of seagrasses, supporting this gene as an effective locus for the discrimination of angiosperms (Kress *et al*., 2005), at least to family level. Three families of seagrass, Zosteraceae, Cymodoceae and Hydrocharitaceae, were detected in the gut contents of *S. fuscescens*. *Zostera muelleri* was the most frequently found species, with 30 samples showing bands consistent with control samples. *Cymodocea serrulata* and *S. isoetifolium* and *Halophila* spp. were, however, found in only a small number of samples (one and four, respectively). These findings are consistent with the results of visual inspection, which showed that a majority of fish (38 of 40) consumed *Z. muelleri*, and only a small number (12 of 40) consumed the other species of seagrass present in Moreton Bay.

The molecular phylogeny of the seagrass species used in this study (Fig. 4) was consistent with those of Les *et al*. (1997, 2002) who used another plastid gene *rbc*L and the nuclear ITS intergenic spacer DNA. Using the intergenic spacer *trn*H-*psb*A DNA, *Z. muelleri*/*Z. marina*, *C. serrulata*/*S. isoetifolium* and *H. ovalis*/*H. spinulosa* formed robust groupings corresponding with the Zosteraceae, Cymodoceae and Hydrocharitaceae, respectively; matching genus and family placements in the *rbc*L phylogenetic tree by Les *et al*. (1997). The robust family or species level phylogeny obtained from *trn*H-*psb*A sequences underpins the value of this gene for SSCP barcoding. Importantly, the polymorphism observed in this SSCP study reflects the family level of differences from the phylogeny using amplified *trn*H-*psb*A DNA. This supports the efficacy of using *trn*H-*psb*A for the DNA barcoding of seagrasses to determine the seagrass diet of fishes. The identification of gut contents to species or family level by sequencing justifies future use of SSCP alone as an accurate, efficient and cheaper method for DNA barcoding of seagrasses consumed by herbivorous fishes. Until now, none of the seagrass species used in this study had nuclear and plastid genetic sequences available in open access databases. Future use of molecular methods to identify seagrass species and understanding the phylogeny and evolution of seagrass species will depend on the development of an extensive nuclear or plastid DNA database of seagrass taxa.

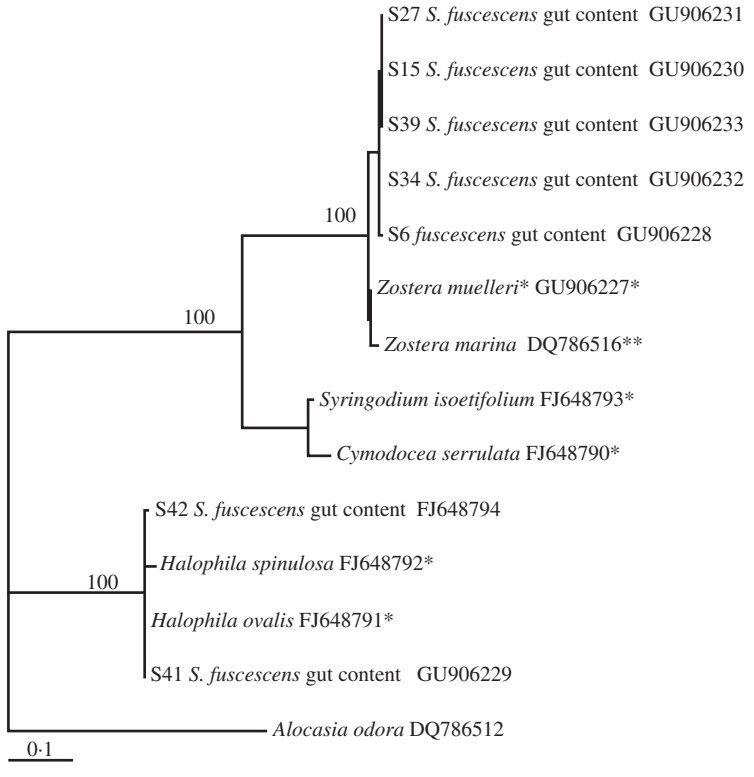

FIG. 4. Maximum-likelihood tree of seagrass plastid *psb*A-*trn*H DNA. Seagrass control species (⁎) from free-living plants, gut contents of *Siganus fuscescens* and an outgroup organism are included in the analysis. The *Zostera marina* sequence (⁎⁎) was obtained from GenBank. All GenBank accession numbers are shown in the tree. Only bootstrap values >95% are shown. Distance represents the number of substitutions per 100 bases.

Visual inspection of fish gut contents identified four species of seagrass. The success of the visual method was based largely on the morphological characteristics of each individual species, as some seagrasses can be easily identified and confirmed (*e.g. Z. muelleri*, which is the only species in Moreton Bay to have characteristic cross-veins; Waycott *et al*., 2004). The identification of ingested fragments was not possible, however, for the majority of gut contents, as many fragments were either too small or lack identifying characteristics. In this particular study, 38 of 40 fish had unidentifiable fragments in their guts. In cases where gut contents are even more macerated and plant fragments cannot be identified visually, molecular methods such as amplification of species marker genes by PCR and barcoding methods such as SSCP will be helpful as they give more definitive and fewer ambiguous results.

While challenges remain to the broad utility of SSCP for the molecular identification of herbivorous fish gut contents, the potential to improve the assay and extend its use is significant, especially in studying the trophic ecology of fishes. The current method is capable of identifying major diet components, and the use of a more variable coding region, if one is identified, or the use of multiple coding regions in combination, may increase its sensitivity and taxonomic resolution.

Following initial confirmation with reference species DNA, SSCP analysis will also be effective in dealing with mixed species diets where direct sequencing will prove difficult and arduous. Visual techniques remain most useful in the limited number of cases where gut contents are easily identified, while the current molecular method reduces the uncertainty typically associated with visual identification. Improvements to this application of SSCP may eventually replace the visual method, providing an unambiguous and comprehensive method for the detection of a broad range of food species in the alimentary tracts of fishes.

We thank J. Testa, J. Schofield and R. Day for assistance with this study, and K. Pitt for comments on the manuscript. We appreciate comments from the two anonymous reviewers, which enhanced the quality of this manuscript. The laboratory work was funded by PADI Project Aware Foundation grant to D.D.B.

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