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Genetic characterization of *Zostera asiatica* on the Pacific Coast of North America

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Abstract

We gathered sequence information from the nuclear 5.8S rDNA gene and associated internal transcribed spacers, ITS-1 and ITS-2 (5.8S rDNA/ITS), and the chloroplast maturase K (*matK*) gene, from *Zostera* samples collected from subtidal habitats in Monterey and Santa Barbara (Isla Vista) bays, California, to test the hypothesis that these plants are conspecific with *Z. asiatica* Miki of Asia. Sequences from approximately 520 base pairs of the nuclear 5.8S rDNA/ITS obtained from the subtidal Monterey and Isla Vista *Zostera* samples were identical to homologous sequences obtained from *Z. marina* collected from intertidal habitats in Japan, Alaska, Oregon and California. Similarly, sequences from the *matK* gene from the subtidal *Zostera* samples were identical to *matK* sequences obtained from *Z. marina* collected from intertidal habitats in Japan, Alaska, Oregon and California, but differed from *Z. asiatica* sequences accessioned into GenBank. This suggests the subtidal plants are conspecific with *Z. marina*, not *Z. asiatica*. However, we found that herbarium samples accessioned into the Kyoto University Herbarium, determined to be *Z. asiatica*, yielded 5.8S rDNA/ITS sequences consistent with either *Z. japonica*, in two cases, or *Z. marina*, in one case. Similar results were observed for the chloroplast *matK* gene; we found haplotypes that were inconsistent with published *matK* sequences from *Z. asiatica* collected from Japan. These results underscore the need for closer examination of the relationship between *Z. marina* along the Pacific Coast of North America, and *Z. asiatica* of Asia, for the retention and verification of specimens examined in scientific studies, and for assessment of the usefulness of morphological characters in the determination of taxonomic relationships within Zosteraceae.
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Keywords: *Zostera marina*; *Z. japonica*; *Z. asiatica*; *ITS*; *matK*

1. Introduction

Despite taxonomic controversies associated with the relationships within the seagrass family Zosteraceae (Posluszny and Tomlinson, 2001; Les et al., 2002; summarized in Kuo and den Hartog, 2001), all treatments consider that the genus *Zostera* (subgenus *Zostera* according to Les et al., 2002) comprises at least four species. These include *Z. asiatica* Miki, *Z. caespitosa* Miki, *Z. caulescens* Miki and *Z. marina* L., which all inhabit the North Pacific (Short et al., 2001). *Z. marina* occurs throughout the Northern Hemisphere, while *Z. asiatica*, *Z. caespitosa* and *Z. caulescens* are found only in the North Pacific region (Short et al., 2001). In addition, *Z. japonica* Asch and Graebn., in the genus *Nanozostera* (Posluszny and Tomlinson, 2001) or subgenera *Zosterella* (Kuo and den Hartog, 2001; Les et al., 2002), occurs in the North Pacific (Short et al., 2001; Aioi and Nakaoka, 2003; Lee and Lee, 2003; Wyllie-Echeverria and Ackerman, 2003). Three of these species, *Z. asiatica*, *Z. marina* and *Z. japonica*, inhabit the Pacific Coast of North America (Wyllie-Echeverria and Ackerman, 2003).

Z. marina populations are thought to have been present in North Pacific waters during the Pliocene (Domning, 1976), and are now found along the coast and in bays and estuaries from Alaska to Mexico. Morphological variation and differences in

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biotic processes, primarily influenced by water temperature (Setchell, 1927, 1929; Phillips and Lewis, 1983; Phillips et al., 1983) led to the identification of five varieties along the Pacific coast (Backman, 1991). Varietal status is linked to variation in somatic characteristics such as leaf width, seed morphology and frequency of flowering (Setchell, 1927, 1929; Backman, 1991; Wyllie-Echeverria et al., 2003). Tall, wide-leaved (6–20 mm) plants with heavier seeds live primarily in subtidal regions whereas short, thin-leaved (1.5–4 mm) plants with lighter seeds grow primarily in intertidal areas (Backman, 1991; Wyllie-Echeverria et al., 2003).

Z. japonica, probably introduced early in the 20th century through oceanic trade with Japan (Harrison and Bigley, 1982), is a short, thin-leaved (0.75–1.5 mm) plant morphologically distinguished from intertidal *Z. marina* by the presence of an open leaf sheath and retinacula (Den Hartog, 1970; Phillips and Menez, 1988; Kuo and den Hartog, 2001). This species commonly co-occurs with intertidal *Z. marina* in the Pacific Northwest from southern British Columbia to southern Oregon (Harrison and Bigley, 1982; Wyllie-Echeverria and Ackerman, 2003). Recently the species was found in Humboldt Bay, California (Susan Schlosser, University of California Sea Grant Extension Program, Eureka, CA, USA, personal communication) but no populations are reported elsewhere in California, or in Mexico.

Phillips and Wyllie-Echeverria (1990) provided evidence that the third *Zostera* species, *Z. asiatica*, occurs in subtidal waters along the California coast of the Pacific. Diagnostic characteristics used to describe this taxon included the plant's restriction to deep water (−5 to −17 m mean lower low water), a wide leaf (12–18 mm), the notched characteristic of the vegetative leaf tips, a smooth seed coat, and the flowering and seed phenology, all characteristics of *Z. asiatica* (Miki, 1932). Leaf tip morphology and ''appearance of the seed surface'' are also suggested by Kuo and den Hartog (2001) as appropriate characteristics to differentiate ''large *Zostera* species'' such as *Z. asiatica* and subtidal *Z. marina*.

While the Jepson Manual (Thorne, 1993) accepts the occurrence of a third *Zostera* species in California, the flora gives precedence to the name *Z. pacifica* S. Watson. Nevertheless, because *Zostera* in general, and *Z. marina* in particular, demonstrates wide plasticity in a number of morphological and life history characteristics (Backman, 1991), it is possible this taxon represents a deep-water adapted *Z. marina* instead. Because genetic markers routinely used in phylogenetic reconstructions can also be used to clarify species status, we used nuclear and chloroplast DNA markers to determine the status of this wide-leaved *Zostera* found in California waters.

The nuclear ribosomal DNA-ITS markers [i.e., internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) and associated nuclear DNA (nrDNA) sequences; Jansen et al., 1998], have been nominated as a target for DNA barcoding for plants (Stoeckle, 2003; Kress et al., 2005). Although nrDNA-ITS genes are repeated throughout the plant nuclear genome, rapid concerted evolution often promotes intra-genomic uniformity of repeat units (Baldwin et al., 1995; Dubouzet and Shinoda, 1999; but see Gaut et al., 2000; Hughes et al., 2002) and as a result of relaxed constraint on mutation, the ITS regions are characterized by high levels of polymorphisms at lower taxonomic levels (Baldwin et al., 1995). Furthermore, ITS regions are small (<700 base pairs (bp) in most angiosperms), facilitating their use as taxon-specific markers particularly when working with substandard DNA sources, such as herbarium specimens. DNA from such sources is typically degraded, precluding polymerase chain reaction (PCR) amplification of larger fragments of less polymorphic loci.

Here we use the nrDNA ITS-1 and ITS-2 regions' potential as a molecular diagnostic marker to determine the specific status of an unknown marine angiosperm. The present study, involving ITS sequencing, and independent corroborative evidence from the chloroplast DNA (cpDNA) maturase K (*matK*) gene, was undertaken to test the hypothesis that wide-bladed, subtidal *Zostera* occurring off the coast of Monterey Bay and the Santa Barbara Channel, California, is *Z. asiatica*.

2. Materials and methods

2.1. Voucher specimens examined and certification of species

All sequences were compared against sequence information accessioned in GenBank (http://www.ncbi.nlm.nih.gov/) by Les et al. (2002) and Olsen et al. (2004) for the nrDNA 5.8S rDNA/ITS-1 and ITS-2 gene region (5.8S rDNA/ITS) of *Z. marina* and *Z. japonica*. Similarly, cpDNA *matK* sequences were compared with those accessioned in GenBank by Tanaka et al. (2003) and Kato et al. (2003) for *Z. marina*, *Z. japonica*, and *Z. asiatica*. No data are available in public databases for the 5.8S rDNA/ITS family for *Z. asiatica*. We therefore include in our analyses homologous sequence information for both genes from herbarium specimens (Kyoto University Museum, KYO) determined to be *Z. asiatica* (KYO 547-2, 547-3 and 547-4). An accessioned specimen representing North Pacific *Z. marina* was used to obtain homologous information from both loci (UAM Herb 43460, collected from Wide Bay, Alaska Peninsula, AK, USA). Similarly, a specimen representing the individuals used by Phillips and Wyllie-Echeverria (1990) to describe *Z. asiatica* from Tomales Bay, California, was used to represent the original described specimen (WEH 94937-1).

2.2. Plant material

Twelve samples from extant *Z. marina* populations were obtained from the intertidal regions of Alaska [Izembek ($n = 2$) and Kinzarof ($n = 2$) lagoons on the Alaska Peninsula; Crab Bay ($n = 2$) in the Alexander Archipelago in southeastern Alaska], Yaquina Bay, Oregon ($n = 2$) and Keil Cove ($n = 4$), San Francisco Bay, California. Four samples were obtained from Hokkaido, Japan (Notsuke Bay, $n = 2$; Lake Furen, $n = 2$). Samples of *Z. japonica* were obtained from Boundary Bay, British Columbia ($n = 2$), Puget Sound, Washington ($n = 1$), and Humboldt Bay, California ($n = 2$). Specimens representing the North American Pacific coast *Z. asiatica* were collected along the California coast, off the mouth of Corral Creek near