Transactions of the American Fisheries Society 136:460–468, 2007

DOI: 10.1577/T06-092.1

[Article]

The Hybrid Sole *Inopsetta ischyra* (Teleostei: Pleuronectiformes: Pleuronectidae): Hybrid or Biological Species?

DANIEL L. GARRETT*[1] AND THEODORE W. PIETSCH

School of Aquatic and Fishery Sciences, University of Washington Fish Collection, Box 355100, 1140 Boat Street, Seattle, Washington 98195, USA

FRED M. UTTER AND LORENZ HAUSER

School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington 98105-5020, USA

Abstract.—Since it was first described as a biological species by Jordan and Gilbert in 1880, *Inopsetta ischyra* has been a disputed taxonomic unit, generally regarded in recent decades as an intergeneric hybrid between English sole *Parophrys vetulus* and starry flounder *Platichthys stellatus*, both of which are common in coastal waters of the eastern North Pacific. Here, we investigate this suspected hybridization with molecular genetic and morphological data. Genotyping at four diagnostic allozyme loci clearly demonstrates that *I. ischyra* is an intergeneric hybrid between English sole and starry flounder; one individual was homozygous at one locus and may have been a backcross with starry flounder. Diagnostic restriction fragment length polymorphisms of a 464-base-pair region of the cytochrome *b* gene revealed symmetric, two-way directionality of hybridization in which 50% of maternities are assigned to each of the parental species. Principal components analysis of meristic characters demonstrated that *I. ischyra* is an intermediate between the parental species. Based on the low frequency of hybrids and the rarity of backcrosses, these natural hybridization events do not appear to be ecologically or evolutionarily significant.

Interspecific hybridization, a fairly common occurrence among freshwater species (see Scribner et al. 2000 and Verspoor and Hammar 1991 for extensive reviews), has been documented less frequently for marine species. Seeb (1998) cites marine hybridizations between reef species (*Chaetodon* spp., *Acanthochromis* spp.) while describing introgressions among *Sebastes* spp. However, marine hybridizations have been most frequently reported in flatfishes (family Pleuronectidae). These include starry flounder *Platichthys stellatus* × stone flounder *Kareius bicoloratus* in the western Pacific (Hubbs and Kuronuma 1942), European flounder *P. flesus* × plaice *Pleuronectes platessa* and mud dab *Limanda limanda* in the Baltic Sea (Norman 1934), and butter sole *Isopsetta isolepis* × English sole *Parophrys vetulus* in the Puget Sound area of the eastern North Pacific (Garrett 2005).

A putative pleuronectid hybrid has had an enigmatic existence since its original description as *Parophrys ischyrus* (Jordan and Gilbert 1880; now commonly called hybrid sole or forkline sole) and subsequent reallocation to the genus *Inopsetta* by Jordan and Goss (in Jordan 1885; Figure 1B) on the basis of four specimens, all collected in Puget Sound in the eastern North Pacific Ocean off the western coast of the state of Washington. *Inopsetta ischyra* was further described by Villadolid (1927), who reported three additional specimens. The possible hybrid status was first discussed by Norman (1934), who suggested that *I. ischyra* is a cross between starry flounder and rock sole *Lepidopsetta bilineata*. Schultz and Smith (1936) corroborated Norman's (1934) suspicion after examining 12 specimens from Puget Sound; however, they suggested that starry flounder and English sole were the parental species based on intermediacy of meristics and morphometrics. Aron (1958) studied the gonads and gonadal products of *I. ischyra* and compared them with those of starry flounder and English sole. Despite the abnormal and highly variable spermatocyte morphology of *I. ischyra*, which suggested hybridization, a ripe female *I. ischyra* was successfully backcrossed with a male English sole, raising additional questions as to the reproductive viability of *I. ischyra*. Comparing meristics and caudal fin osteology between wild-caught hybrids, laboratory-reared hybrids, and three suspected parental species (starry flounder, English sole, and rock sole), Giguere (1986) found that *I. ischyra* did not display ideal meristic intermediacy between starry flounder and English sole and reported that starry flounder was the only parental species strongly supported by meristic data.

The geographic range of *I. ischyra* is poorly known.

* Corresponding author: dlgfyb@mizzou.edu

[1] Present address: Department of Fisheries and Wildlife Sciences, School of Natural Resources, University of Missouri, 302 Anheuser-Busch Natural Resources Building, Columbia, Missouri 65211-7240, USA.

Received April 18, 2006; accepted November 17, 2006
Published online March 15, 2007

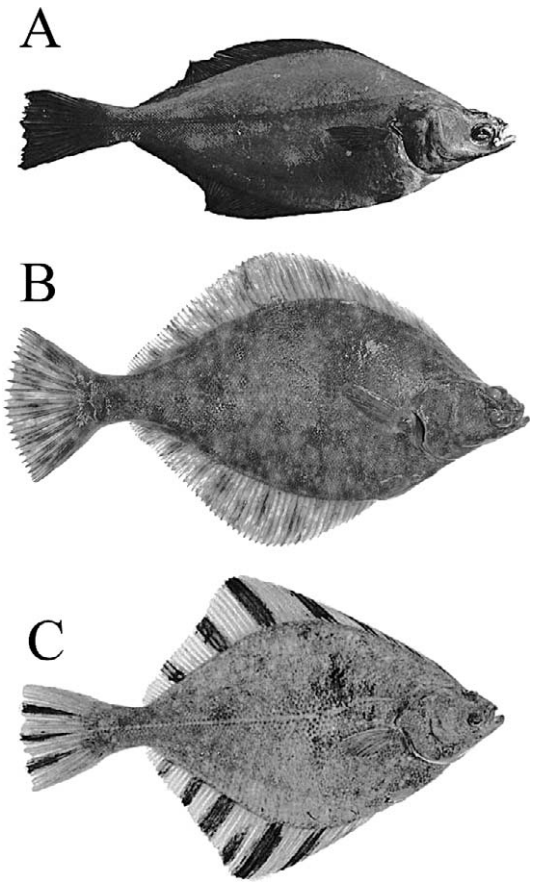

FIGURE 1.—**(A)** Adult English sole, **(B)** adult *Inopsetta ischyra* (starry flounder × English sole; specimen UW 48379, 245 mm standard length, from the Strait of Georgia near Blaine, Washington), and **(C)** adult starry flounder. Photographs (A) and (C) are taken from http://www.fishbase.org.

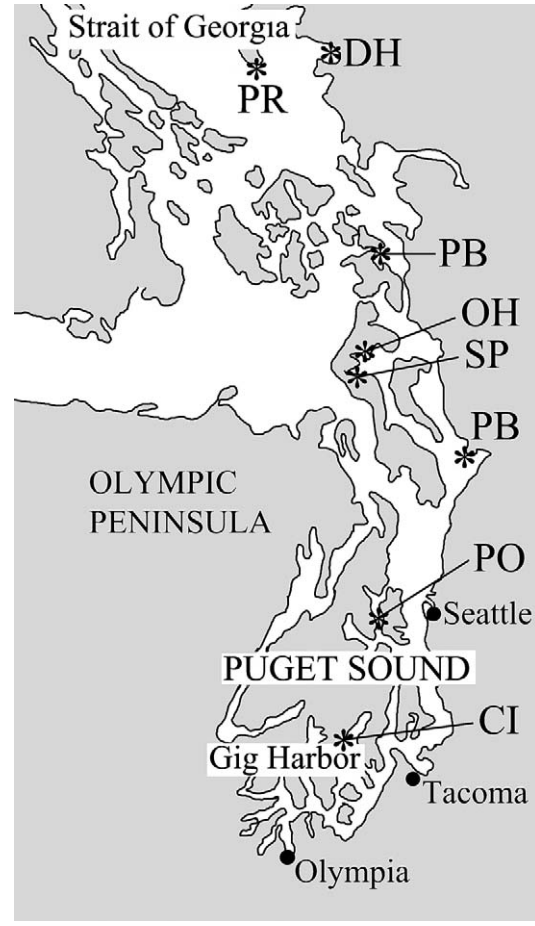

FIGURE 2.—Collection localities (asterisks) for specimens of *Inopsetta ischyra* used in this study. All specimens are archived in the University of Washington fish collection. Location abbreviations are as follows (north to south): DH, Drayton Harbor, Blaine; PR, Point Roberts; PB, Padilla Bay, near Anacortes; OH, Oak Harbor; SP, Saratoga Passage, near Coupeville; PB, Possession Bay; PO, Port Orchard; and CI, Carr Inlet.

Jordan et al. (1930) proposed a range northward to Alaska, most likely based on specimens listed by T. H. Bean in Nelson's (1887) account of an expedition to Norton Sound (Mecklenburg et al. 2002). Unfortunately, no voucher specimens exist to validate these most northerly records. Clemens and Wilby (1961) reported *I. ischyra* from southern British Columbia, but no voucher specimen was retained. Reports by Herald (1941) and Reed (1964) indicate that *I. ischyra* is found in Oregon and as far south as San Francisco Bay based on specimens at the California Academy of Sciences. These identifications were validated during the course of this study. In the last 100 years, however, Puget Sound and adjacent waters are the only regions where *I. ischyra* has been consistently collected (Figure 2).

Since the late 1960s, the assay of specific genes or DNA sequences through molecular genetic markers has provided a powerful complementary tool to traditional taxonomic methods for clarifying relationships of closely related species (e.g., Utter et al. 1973; Arnold 1997). Markers where alleles shared between two species are rare or absent (diagnostic loci) have proven particularly powerful for identifying hybrids among them (Campton 1987; Young et al. 2001).

This study combines molecular markers and traditional methods to resolve the enigma of *I. ischyra*. Nuclear markers (allozyme loci) were used to test the hypothesis that *I. ischyra* is an intergeneric hybrid between starry flounder and English sole. The maternally inherited mitochondrial DNA (mtDNA) cyto-

TABLE 1.—Sample sizes for analytical methods applied to samples of pleuronectid taxa collected from the Puget Sound area.

Species	Allozyme	Restriction fragment length polymorphism	Meristic	Source[a]
Inopsetta ischyra	8	12	15	FV *Tulip*; A. Shedlock, UW collection
English sole	20	20	20	Washington Department of Fish and Wildlife
Starry flounder	20	20	20	FV *Golden North*
Sand sole *Psettichthys melanostictus*	13	13	20	Washington Department of Fish and Wildlife
Northern rock sole *Lepidopsetta polyxystra*	0	0	20	UW collection
Rock sole	20	20	20	Washington Department of Fish and Wildlife
Butter sole	0	0	20	UW collection

[a] UW = University of Washington.

chrome *b* (cyt *b*) gene was used to determine the maternity of hybrid individuals. Meristic characters address (1) whether hybrid individuals show intermediate phenotypic traits between the putative parental species and (2) whether discrepancies exist between morphological and genetic methods of hybrid detection.

Methods

Specimen and tissue collection.—Sampling information for *I. ischyra* and six putative parental species collected in the Puget Sound area and the analyses conducted are shown in Table 1. For allozyme analyses, eye, liver, and muscle tissues were taken from each individual and stored in a −80°C freezer within 8 h of capture. A fin clip was taken from each specimen and placed in 95% ethanol for mtDNA analyses. All specimens and tissues were cataloged and archived in the University of Washington fish collection (UW). An additional five tissue samples from *I. ischyra* were collected in March 1994 by A. Shedlock (UW), four of which were only viable for mtDNA analyses. Additional specimens were provided by UW for meristic analyses.

Allozyme electrophoresis.—The activity of seven enzymes was adequately resolved to permit full screening for potential diagnostic loci (Table 2). Poor resolution during initial screening precluded further testing of seven additional enzymes, including aspartate aminotransferase (enzyme number 2.6.1.1; IUBMB 1992), fumarate hydratase (4.2.1.2), glutamate dehydrogenase (1.4.1.3), glucose-6-phosphate dehydrogenase (1.1.1.49), lactate dehydrogenase (1.1.1.27), phosphoglucomutase (2.7.5.1), and sorbitol dehydrogenase (1.1.1.14). At diagnostic loci, genotypes of 20 individuals each of starry flounder, English sole, and rock sole, 13 individuals of sand sole, and 8 individuals of *I. ischyra* were analyzed. Electrophoresis and staining protocols largely followed Aebersold et al. (1987) with some modifications. We followed Shaklee et al. (1989) regarding nomenclature for allozyme loci, except that alleles were designated by letters rather than by relative mobilities to clarify interspecific comparisons. Conformance of genotypic proportions in putative hybrids was compared with expectations from an ideal population in Hardy–Weinberg equilibrium using a Markov chain exact test in the software GENEPOP (Raymond and Rousset 1995).

Mitochondrial DNA analysis.—A 464-base-pair (bp) region of cyt *b* was amplified from 20 starry flounder and English soles and 12 *I. ischyra* using the primers

H15149AD:
5′-GCICCTCARAATGAYATTTGTCCTCA-3′
L14735:
5′-AAAAACCACCGTTGTTATTCAACTA-3′

TABLE 2.—Enzymes (enzyme numbers in parentheses; IUBMB 1992), tissue sources, and electrophoretic conditions used for allozyme analyses of pleuronactid taxa collected from Puget Sound.

Enzyme	Abbreviation	Tissue source	Electrophoretic conditions[c]
Creatine kinase (2.7.3.2)	CK[a]	Liver, muscle	
Fructose-1,6-diphosphate (3.1.3.11)	FDP[b]	Liver	B
Glucose-6-phosphate isomerase (5.3.1.9)	GPI[b]	Muscle	A
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	G3PDH[b]	Liver	B
Isocitrate dehydrogenase (1.1.1.41)	IDH[a]	Liver, muscle	
Malate dehydrogenase (1.1.1.38)	MDH[a]	Liver, muscle	
Mannose-6-phosphate isomerase (5.3.1.8)	MPI[b]	Muscle	A

[a] Nondiagnostic locus.
[b] Diagnostic locus.
[c] A = amine, citric acid buffer, pH 7.0 (Clayton and Tretiak 1972); B = tris, citric acid gel buffer or lithium hydroxide, boric acid electrode buffer (Ridgway et al. 1970).

TABLE 3.—Diagnostic loci and allelic combinations used to assign parentage to *Inopsetta ischyra*. For each locus, the allele represented by the allozyme migrating the farthest from the origin is named *a*, that migrating the next farthest *b*, and so forth. For *Inopsetta ischyra*, putative backcross is separated from first generation hybrids. Hardy–Weinberg equilibrium tests apply only to *I. ischyra* ($N = 8$).

						I. ischyra		
Tissue	Locus	Rock sole ($N = 20$)	Sand sole ($N = 13$)	English sole ($N = 20$)	Starry flounder ($N = 20$)	First-generation hybrids ($N = 7$)	Putative backcross ($N = 1$)	Hardy–Weinberg *P*-value[a]
Liver	*FDP-I**	*bb*	*bb*	*aa, cc, ac*	*bb*	*ab, bc*	*bc*	0.02
Muscle	*GPI**	*aa*	*bb, bc*	*dd, ee, ff, gg, de, df, eg*	*aa*	*ad, ae, af, ag*	*ag*	0.0198
Liver	*G3PDH-I**	*aa*	*cc, dd, ee, bd, be, ae*	*aa*	*bb*	*ab*	*ab*	0.0197
Muscle	*MPI**	*ee*	*dd*	*bb, bc*	*aa*	*ab*	*aa*	0.662

[a] Hardy–Weinberg test across all loci = 0.0001.

from Sotelo et al. (2001). The polymerase chain reaction (PCR) conditions given by Sotelo et al. (2001) were optimized to the following: a preheating step of 5 min at 94°C, then 25 cycles of 40 s at 94°C, 80 s at 50°C, 80 s at 72°C, and a final extension step of 7 min at 72°C.

Using both forward and reverse primers, 356 bp of the 464-bp cyt *b* fragment from representatives of all taxa and an additional interspecific hybrid (butter sole × English sole) from the Puget Sound area were sequenced. Sequencing reactions were carried out using a DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Piscataway, New Jersey) and a protocol consisting of 40 cycles of 20 s at 95°C, 15 s at 56°C, and 2 min at 60°C. Unincorporated nucleotides were removed from sequencing products by ethanol precipitation (Sambrook and Russell 2001) before electrophoresis on a MegaBACE 1000 (Amersham Biosciences). Sequences were aligned using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan) and BioEdit (Hall 1999) along with an outgroup witch flounder *Glyptocephalus cynoglossus* (family Cynoglossidae; GenBank accession number AF165073). Using Mega 3.1 software, maximum likelihood, maximum parsimony, and neighbor-joining (Saitou and Nei 1987) trees were generated using the Kimura two-parameter distance to analyze relationships among species groups and evaluate any discrepancies that exist among the data set and these methodologies.

Because of its efficiency, restriction fragment length polymorphism (RFLP) was used to determine the maternity of all hybrid individuals. BioEdit (Hall 1999) was used to identify restriction sites that discriminated between starry flounder and English sole. The cutting enzymes (*Bcc* I and *Msp* I) for these sites were then used to restrict cyt *b* fragments of all individuals and assign maternities by comparing fragment lengths between the hybrids and the parental species. Expected fragment lengths were calculated by aligning the 356-bp fragment sequenced with a GenBank sequence (e.g., outgroup sequence) that encapsulated the 464 bp of cyt *b* and corresponding primers (Sotelo et al. 2001). Fragment lengths calculated by BioEdit (Hall 1999) were then corrected to correspond with the entire PCR product.

Restriction digests followed protocols specified by the manufacturer (New England BioLabs). The DNA fragments were separated by polyacrylamide gel (6%) electrophoresis, stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes), and visualized with an FMBIO fluorescence scanner (MiraiBio).

Meristic comparisons.—The following meristic characters differed most between the parental species and were used for comparisons: rakers on the first gill arch, rakers on the second gill arch, dorsal fin rays, anal fin rays, interorbital scales, supraorbital pores, and the extension of the accessory dorsal branch (ADB) of the lateral line (to the nearest dorsal fin ray). Meristic data from specimens of *Lepidopsetta* spp. were provided by J. W. Orr of the Alaska Fisheries Science Center. *Inopsetta ischyra* was initially compared with 20 specimens each of six species (starry flounder, English sole, sand sole, butter sole, northern rock sole, and rock sole) and one hybrid (English sole × butter sole). There was no correlation between specimen size and meristic characters, so length was not considered in meristic analysis. Principal component analysis (PCA; SPSS statistical software, version 11.5) was conducted on the correlation matrix of meristic characters. Parentage validation via PCA was determined by visually assessing the intermediacy of *I. ischyra* data points relative to the data point clusters of starry flounder and English sole. To further evaluate the meristic intermediacy of *I. ischyra* relative to the parental species, a second PCA was run that excluded other taxa.

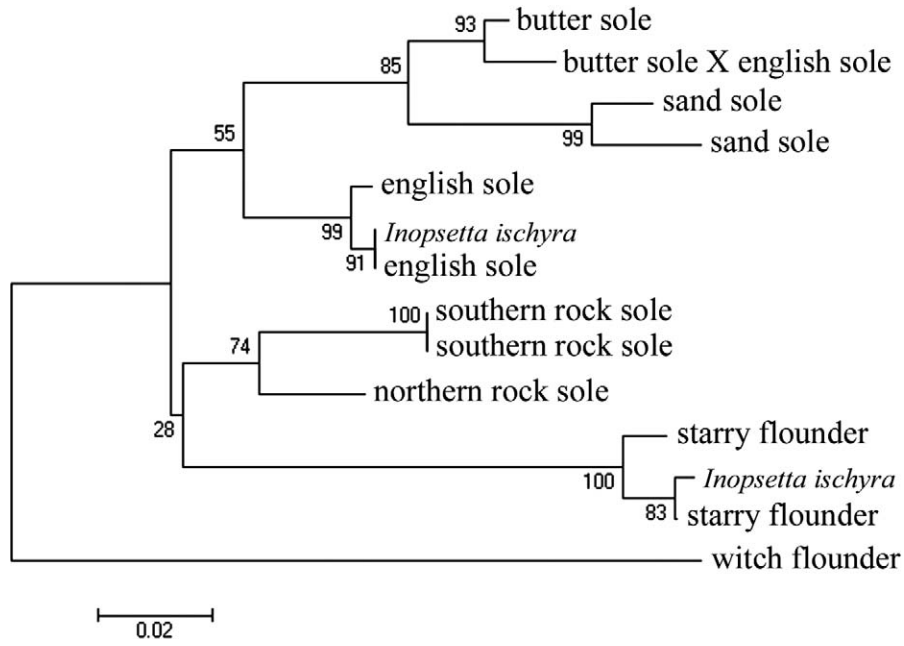

FIGURE 3.—Neighbor-joining tree including bootstrapping (1,000 replications) of cytochrome *b* sequences demonstrating the relationships among six pleuronectid species and the maternities of two pleuronectid hybrids found in Puget Sound and adjacent waters. All termini represent single individuals; southern rock sole = rock sole.

Results

Allozyme Analysis

Of the 14 enzymes screened for polymorphisms, 4 were encoded by putative loci that were useful for interspecific discrimination (Table 3). Combinations of genotypes at these loci unambiguously identified rock sole, sand sole, English sole, and starry flounder. Each locus distinguished starry flounder and English sole, the presumed parental species of *I. ischyra* (Schultz and Smith 1936). English sole expressed two alleles of *FDP-1** (**a*, **c*), four alleles of *GPI** (**d*, **e*, **f*, **g*), and two alleles of *MPI** (**b*, **c*) that did not occur in the other species. Four alleles of *G3PDH-1** (**b*, **c*, **d*, **e*) distinguished sand sole from rock sole and English sole, both of which expressed only the **a* allele.

TABLE 4.—Molecular sizes (base pairs) of restriction fragments resolved by digestion of starry flounder and English sole mtDNA cytochrome *b* fragments, Puget Sound.

Restriction enzyme	Parental species	Fragment sizes[a]
Msp I	Starry flounder	177, 287
	English sole	70, 177, 217
Bcc I	Starry flounder	464
	English sole	85, 279

[a] Undigested fragment is 464 base pairs.

Of the eight individuals of *I. ischyra* sampled (Table 3), seven were found to be heterozygous at all four loci, each expressing single diagnostic alleles of starry flounder and English sole. Thus, these individuals were identified as F_1 hybrids between these two species. A single individual was homozygous for the *MPI*a* allele (Table 3) and was interpreted as a backcross between *I. ischyra* and starry flounder.

Mitochondrial DNA Analysis

Of the original 464-bp cyt *b* fragment from six species and two hybrids, 356 bp were successfully sequenced. As expected, polymorphisms were primarily observed as transitions (86%) at third-codon positions (95%). Maximum likelihood, maximum parsimony, and neighbor-joining (Saitou and Nei 1987) methods produced identical trees (Figure 3) with high bootstrap support for conspecific (79–100%) and congeneric (i.e., *Lepidopsetta* spp., 52–86%) clades. One *I. ischyra* clustered with starry flounder and one with English sole, while the butter sole × English sole hybrid clustered with butter sole.

Twenty of the 54 variable sites showed fixed nucleotide differences between starry flounder and English sole, and three of these diagnostic sites were within restriction sites for *Bcc* I and *Msp* I. The

TABLE 5.—Counts and measurements for starry flounder, *Inopsetta ischyra*, and English sole used for principal components analysis, Puget Sound.

Character	Starry flounder ($N = 20$)		*Inopsetta ischyra* ($N = 15$)		English sole ($N = 20$)	
	Range	Mean	Range	Mean	Range	Mean
Upper gill rakers (first arch)	3–4	4.0	4–6	5.0	4–6	5.2
Lower gill rakers (first arch)	7–9	8.0	9–10	9.8	11–13	12.1
Upper gill rakers (second arch)	1–1	1.0	1–2	1.3	1–2	1.9
Lower gill rakers (second arch)	8–10	9.1	10–12	11.0	14–16	14.6
Dorsal fin rays	55–61	58.5	69–77	71.7	71–86	79.4
Anal fin rays	40–44	41.8	52–58	54.1	54–67	59.7
Interorbital scales	1–3	2.3	3–4	3.5	1–3	2.1
Supraorbital pores	0–0		1–4	1.8	5–11	8.4
Accessary dorsal branch extension to nearest dorsal-fin ray	0–0		5–12	9.0	18–27	22.9

expected restriction fragment lengths for starry flounder and English sole are given in Table 4.

Of the 12 individuals of *I. ischyra*, 6 individuals shared identical RFLP haplotypes with starry flounder and 6 with English sole, indicating symmetric two-way directionality of hybridization between starry flounder and English sole.

Meristic Comparisons

The ranges and means of meristic characters for *I. ischyra* and the parental species are given in Table 5. Results of a PCA on nine meristic characters from *I. ischyra*, six selected species, and one additional hybrid (English sole × butter sole) demonstrated intermediacy of *I. ischyra* between English sole and starry flounder (Figure 4A). Principal component (PC) 1 strongly loaded on upper gill rakers of the first arch and lower gill rakers of the second arch, whereas PC2 strongly loaded on supraorbital pores (Table 6).

Principal component analysis of meristic characters including only *I. ischyra* and the two parental species yielded similar results (Figure 4B). Accounting for most of the variation, PC1 demonstrated intermediacy of hybrids between the parental species, strongly loading on dorsal fin ray counts and the extension of the ADB to the nearest dorsal fin ray (Table 6). In both plots, the backcross showed no meristic deviation from F_1 hybrids.

Discussion

Genotyping at four diagnostic allozyme loci clearly demonstrates that *I. ischyra* is an intergeneric hybrid between English sole and starry flounder, which is in concurrence with previously published morphological data (Schultz and Smith 1936) and negates its earlier identity as a biological species (Jordan and Gilbert 1880). Despite limited sampling of pleuronectid species in the Puget Sound area, the diagnostic allelic differences between English sole and starry flounder, coupled with the observed genotypes of *I. ischyra*, are sufficient evidence for parentage assignment. Further, we conducted a Hardy–Weinberg equilibrium test using GENEPOP (Raymond and Rousset 1995) to test for a heterozygote excess among individuals of *I. ischyra* at all four loci. With the exception of the *MPI** locus where the putative backcross was observed (*P*-

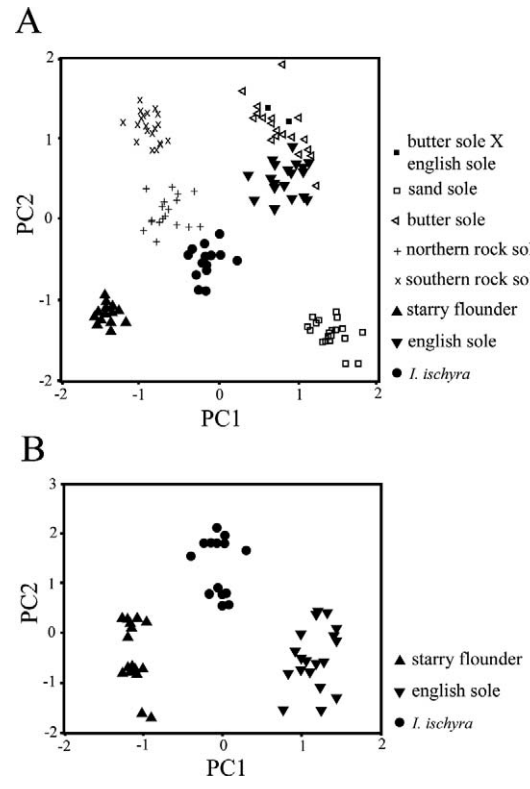

FIGURE 4.—Principal component (PC) scores of meristics for *Inopsetta ischyra* vis-à-vis **(A)** six biological species and one other hybrid found in Puget Sound and adjacent waters (PC1 and PC2 account for 60% and 40% of the variability, respectively) and **(B)** parental species (PC1 accounts for 74.6% of the variability).

TABLE 6.—Character loadings for principal component (PC) analyses of meristic characters of (A) six species and two hybrids found in Puget Sound and adjacent waters and (B) *Inopsetta ischyra* and parental species.

	A		B	
Character	PC1	PC2	PC1	PC2
Upper gill rakers (first arch)	0.81	−0.41	0.77	0.28
Lower gill rakers (first arch)	0.75	−0.58	0.95	−0.03
Upper gill rakers (second arch)	0.76	−0.48	0.82	−0.12
Lower gill rakers (second arch)	0.79	−0.39	0.95	−0.11
Dorsal-fin rays	0.76	0.59	0.96	0.18
Anal-fin rays	0.67	0.69	0.95	0.22
Interorbital scales	0.64	−0.33	−0.13	0.96
Supraorbital pores	0.35	0.81	0.91	−0.19
Accessory dorsal branch extension to nearest dorsal-fin ray	0.78	0.57	0.98	−0.06

value = 0.662), all loci demonstrated a significant excess of heterozygotes (P-value < 0.05), providing further support for the hybrid origin of *I. ischyra* (Table 3). The data also concur with a preliminary allozyme analysis (F. M. Utter, unpublished data from 1987; sample sizes in parentheses) of *I. ischyra* (2) and five pleuronectid species from the Puget Sound area: rock sole (4), sand sole (2), butter sole (1), starry flounder (4), and English sole (6), where genotypes at four different diagnostic loci (*FH-1**, *sAAT-1**, *G3PDH-1**, *IDHP-1**) similarly indicated that two *I. ischyra* were F_1 hybrids between starry flounder and English sole. Cumulatively, eight loci have been identified that demonstrate all specimens of *I. ischyra* collected to be hybrids ($N = 10$).

The putative backcross with starry flounder suggests partial fertility of *I. ischyra* beyond the first generation, though alternative explanations are possible. A possible scoring error of the *MPI** homozygote is unlikely because the genotype was validated on three separate occasions using different homogenates of the tissue. Although the possibility of a recessive null allele cannot be excluded (Richardson et al. 1986), the absence of null genotypes among 20 starry flounders coupled with the potential lethality of a missing glycolytic enzyme further reduce the likelihood of this explanation. We must finally consider the possibility that *MPI*a* (Table 3) is found in English sole at a low frequency and went undetected in the parental species but was found in a hybrid. After binomial sampling distribution, the combined probability of this observation in both English soles and the hybrids is highest at an *MPI*a* allele frequency of 0.025 ($P_{\text{vetulus}} = 0.36$, $P_{\text{hybrid}} = 0.168$, $P_{\text{combination}} = 0.061$). Thus, the explanation of a rare allele cannot be excluded at the 0.05 significance level.

The putative backcross could not be differentiated from other F_1 hybrids based on PCA of meristic characters. The range of meristics for backcrosses can vary widely between the F_1 hybrids and the parental species depending upon the number of generations of backcrossing and the genes underlying meristic phenotypes (e.g., Neff and Smith 1979); thus, this result was not interpreted as evidence for misidentification. The absence of records of naturally occurring backcrosses among flatfish hybrids may reflect that (1) past studies on naturally occurring flatfish hybrids (e.g., Norman 1934; Hubbs and Kuronuma 1942) were conducted before the availability of molecular techniques; and (2) the extent of hybridization or introgression often cannot be determined from morphological data, given that F_1 hybrids may not be individually distinguishable from backcrosses (Campton 1987). Further molecular genetic studies with larger sample sizes are required to assess the existence and extent of backcrossing in starry flounder × English sole hybrids.

The observed two-way directionality of hybridization (maternity of 6 out of 12 hybrids was assigned to each of the parental species) is much more common than unidirectional hybridization in fishes (e.g., Munehara et al. 2000; Brown et al. 2004), although sex biases usually exist (e.g., Avise and Saunders 1984; Ryan and Wagner 1987; Avise et al. 1997; Grant and Grant 1997; Ostberg et al. 2004; Bettles et al. 2005) that are caused by unequal sex ratios in the parental species and, more commonly, by sexual selection (Bettles et al. 2005). Because flatfishes are broadcast spawners (Garrison and Miller 1982; Seeb 1998), factors such as sexual selection are unlikely to play a role in hybridization and may partially explain the reciprocal directionality. The directionality of hybridization can also vary between populations of parental species (e.g., Dowling et al. 1989; Bettles et al. 2005), giving importance to screening multiple populations to detect directionality when possible. In this study, all of the samples collected for genetic analyses were obtained in one poorly defined geographic area (Strait of Georgia near Blaine, Washington), preventing assessment of population-specific effects.

Other morphological characters in addition to meristic comparisons also support the assignment of starry flounder and English sole as parental species. The elongate head and cycloid scales of *I. ischyra* are clearly derived from English sole, the only pleuronectid in Puget Sound with these characters (Hart 1973). Further, the dorsal and anal fins of *I. ischyra* have faint dark bands of pigment similar to those of starry flounder.

Only seven known individuals were collected over a 2-year span of bottom trawling. Although the task of

collecting hybrids was confined to one commercial fishing vessel, the apparent difficulty by which specimens were retained suggests that *I. ischyra* is fairly uncommon with respect to other commercially fished species that are more prevalent in these waters (e.g., starry flounder, English sole, sand sole, and rock sole; Hart 1973). Like the only other hybrid flatfish known from Puget Sound and adjacent waters (butter sole × English sole), the occurrence of *I. ischyra* is most likely a product of the geographic and temporal overlap in broadcast spawning of the parental species. Between February and April, both starry flounder and English sole spawn over sand, silt, and mud bottoms in relatively shallow areas (<70 m; Garrison and Miller 1982). However, starry flounder tend to spawn in shallower estuarine environments (<45 m) and both species form spawning aggregations with conspecifics (Garrison and Miller 1982). Thus, hybridization between English sole and starry flounder may be restricted to very small areas of contact during the spawning season that limits hybrid numbers, despite the genetic compatibility of the parental species.

Although the behavioral and reproductive ecology of the flatfish assemblages in Puget Sound remain poorly understood, these natural hybridization events do not appear to be ecologically and evolutionarily significant based on the low number of hybrids and backcrosses observed. Future research on hybridization in flatfishes may seek to integrate behavioral and reproductive ecology to further understand relationships between intergeneric isolating mechanisms and hybridization.

Acknowledgments

We are especially indebted to Captain S. Waters, Captain K. Johnson, and the Washington Department of Fish and Wildlife, who collected specimens that were so crucial for the success of this project. We thank J. W. Orr (Alaska Fisheries Science Center), who provided meristic data for specimens of *Lepidopsetta* spp., assisted with statistical analyses, and reviewed the manuscript. J. R. Dunn (UW) also reviewed the manuscript. L. Conquest (UW) assisted with statistical analyses, K. P. Maslenikov (UW) assisted with curation of tissues and specimens, and C. P. Kenaley (UW) assisted with illustrations.

References

Aebersold, P. B., G. A. Winans, D. J. Teel, G. Milner, and F. M. Utter. 1987. Manual for starch gel electrophoresis: a manual for the detection of genetic variation. NOAA Technical Report NMFS 61.

Arnold, M. L. 1997. Natural hybridization and evolution. Oxford University Press, New York.

Aron, W. 1958. Cytological and histological studies on the hybrid of *Platichthys stellatus* × *Parophrys vetulus*, with notes on its backcross to *P. vetulus*. Copeia 1958:105–111.

Avise, J. C., P. C. Pierce, M. J. Van Den Avyle, M. H. Smith, W. S. Nelson, and M. A. Asmussen. 1997. Cytonuclear introgressive swamping and species turnover of bass after an introduction. Journal of Heredity 88:14–20.

Avise, J. C., and N. C. Saunders. 1984. Hybridization and introgression among species of sunfish (*Lepomis*): analysis by mitochondrial DNA and allozyme markers. Genetics 108:237–255.

Bettles, C. M., M. F. Docker, B. Dufour, and D. D. Heath. 2005. Hybridization dynamics between sympatric species of trout: loss of reproductive isolation. Journal of Evolutionary Biology 18:1220–1233.

Brown, K. H., T. M. Gardner-Brown, and G. H. Thorgaard. 2004. Equivalent survival and different development rates in reciprocal apache trout × rainbow trout hybrids. Copeia 2004:378–382.

Campton, D. E. 1987. Natural hybridization and introgression in fishes. Pages 161–192 *in* N. Ryman and F. M. Utter, editors. Population genetics and fishery management. University of Washington Press, Seattle.

Clayton, J. W., and D. N. Tretiak. 1972. Amine–citrate buffers and pH control in starch gel electrophoresis. Journal of the Fisheries Research Board of Canada 29:1169–1172.

Clemens, W. A., and G. V. Wilby. 1961. Fishes of the Pacific Coast of Canada, 2nd edition. Fisheries Research Board of Canada Bulletin 68.

Dowling, T. E., G. R. Smith, and W. M. Brown. 1989. Reproductive isolation and introgression between *Notropis cornutus* and *Notropis chrysocephalus* (family Cyprinidae): comparison of morphology, allozymes, and mitochondrial DNA. Evolution 43:620–634.

Garrett, D. L. 2005. A new intergeneric hybrid flatfish (Pleuronectiformes: Pleuronectidae) from Puget Sound and adjacent waters. Copeia 2005:673–677.

Garrison, K. J., and B. S. Miller. 1982. Review of the early life history of Puget Sound fishes. University of Washington, Fisheries Research Institute,Publication 8216, Seattle.

Giguere, D. M. 1986. Reexamination of parentage in a hybrid flounder, *Inopsetta ischyra* (Jordan and Gilbert). Master's thesis. University of Washington, Seattle.

Grant, P. R., and B. R. Grant. 1997. Hybridization, sexual imprinting, and mate choice. American Naturalist 149:1–28.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98.

Hart, J. L. 1973. Pacific fishes of Canada. Fisheries Research Board of Canada Bulletin 180.

Herald, E. S. 1941. First record of the hybrid flounder, *Inopsetta ischyra*, from California. Bulletin of California Fish and Game 27(2):44–46.

Hubbs, C. L., and K. Kuronuma. 1942. Hybridization in nature between genera of flounders in Japan. Papers of the Michigan Academy of Science, Arts, and Letters 27:267–306.

IUBMB (International Union of Biochemistry and Molecular Biology). 1992. Enzyme nomenclature 1992. Academic Press, San Diego, California.

Jordan, D. S. 1885. A catalogue of the fishes known to inhabit

the waters of North America, north of the Tropic of Cancer, with notes on species discovered in 1883 and 1884. Report of the U.S. Fish Commission789–973.

Jordan, D. S., B. W. Evermann, and H. W. Clark. 1930. Checklist of the fishes and fishlike invertebrates of North and Middle America north of the northern boundary of Venezuela and Columbia. Report of the U.S. Fish Commission for 1928, Appendix 10.

Jordan, D. S., and C. H. Gilbert. 1880. Description of two new species of flounders (*Parophrys ischyrus* and *Hippoglossoides elassodon*) from Puget's Sound. Proceedings of the U.S. Natural History Museum 3:276–280.

Mecklenburg, C. W., T. A. Mecklenburg, and L. K. Thorsteinson. 2002. Fishes of Alaska. American Fisheries Society, Bethesda, Maryland.

Munehara, H., Z. Kanamoto, and T. Miura. 2000. Spawning behavior and interspecific breeding in three Japanese greenlings (Hexagrammidae). Ichthyological Research 47:287–292.

Neff, N. A., and G. R. Smith. 1979. Multivariate analysis of hybrid fishes. Systematic Zoology 28:176–196.

Nelson, E. W. 1887. Field notes on Alaskan fishes, with additional notes by Tarleton H. Bean. U.S. Government Printing Office, Archived serial publication issued in connection with the Signal Service, U.S. Army, No. 3., Washington, D.C.

Norman, J. R. 1934. A systematic monograph of the flatfishes (Heterostomata), Psettodidae, Bothidae, and Pleuronectidae, volume 1. Trustees of the British Museum of Natural History, London.

Ostberg, C. O., L. Slatton, and R. J. Rodriguez. 2004. Spatial partitioning and asymmetric hybridization among sympatric coastal steelhead (*Oncorhynchus mykiss irideus*), coastal cutthroat trout (*O. clarki clarki*) and interspecific crosses. Molecular Ecology 13:2773–2788.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249.

Reed, P. H. 1964. Recent occurrences of intergeneric hybrid flounders, *Inopsetta ischyra* (Jordan and Gilbert), from California and Oregon. Bulletin of the California Fish and Game 50:118–121.

Richardson, B. J., P. R. Baverstock, and M. Adams. 1986. Allozyme electrophoresis. Academic Press, Sydney, Australia.

Ridgway, G. J., S. W. Sherburne, and R. D. Lewis. 1970. Polymorphism in the esterases of Atlantic herring. Transactions of the American Fisheries Society 99:147–151.

Ryan, M. J., and W. E. Wagner. 1987. Asymmetries in mating preferences between species: female swordtails prefer heterospecific males. Science 236:595–597.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning, 3rd edition, volume 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schultz, L. P., and R. T. Smith. 1936. Is *Inopsetta ischyra* (Jordan and Gilbert) from Puget Sound, Washington, a hybrid flatfish? Copeia 1936:199–203.

Scribner, K. T., K. Page, and M. Bartron. 2000. Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. Reviews in Fish Biology and Fisheries 10:293–233.

Seeb, L. W. 1998. Gene flow and introgression within and among three species of rockfishes, *Sebastes auriculatus*, *S. caurinus*, and *S. maliger*. Journal of Heredity 89:393–403.

Shaklee, J., F. Allendorf, D. Morizot, and G. Whitt. 1989. Genetic nomenclature for protein-coding loci in fish: proposed guidelines. Transactions of the American Fisheries Society 118:218–227.

Sotelo, C. G., P. Calo-Mata, M. Jose Chapela, R. I. Perez-Martin, H. Rehbein, G. L. Hold, V. J. Russell, S. Pryde, J. Quinteiro, M. Izquierdo, M. Rey-Mendez, C. Rosa, and A. T. Sanotos. 2001. Identification of flatfish (Pleuronectiformes) species using DNA-based techniques. Journal of Agriculture, Food, and Chemistry 49:4562–4569.

Utter, F., F. Allendorf, and H. Hodgins. 1973. Pacific salmon and related trout based on protein variations. Systematic Zoology 22:257–270.

Verspoor, E., and J. Hammar. 1991. Introgressive hybridization in fishes: the biochemical evidence. Journal of Fish Biology 39(Supplement A):309–334.

Villadolid, D. V. 1927. The rediscovery of *Inopsetta ischyra*, a rare species of flounder. Annals of the Carnegie Museum 17:395–397.

Young, W. P., C. O. Ostberg, P. Keim, and G. H. Thorgaard. 2001. Genetic characterization of hybridization and introgression between anadromous rainbow trout (*Oncorhynchus mykiss irideus*) and coastal cutthroat trout (*O. clarki clarki*). Molecular Ecology 10:921–930.