Vol. 3: 309–315, 1980 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published December 15

Genetic Evidence for Naturally Occurring Fertile Hybrids Between Two Goby Species, *Pomatoschistus minutus* and *P. lozanoi* (Pisces, Gobiidae)

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Abstract: Sympatric populations of the closely related sand goby species *Pomatoschistus minutus* and *P. lozanoi* were examined electrophoretically. Out of a total of 33 protein loci resolved, three were completely species diagnostic and one was virtually so. In samples containing, in all, 1051 *P. minutus* and 604 *P. lozanoi*, five individuals with a hybrid genotype at all these loci were found. As well as these F_1 individuals, one fish of unusual genotype at these four loci was discovered. On the criterion of the distribution of the alleles at these four loci the probability that this fish is an F_1 hybrid is $<5 \times 10^{-6}$; that it is a backcross, only slightly higher. We conclude that this individual is most likely to be an F_2 resulting from a cross between two F_1 individuals.

INTRODUCTION

Hybridisation between animal species in the wild is generally uncommon in most groups except fish, in which it is seen relatively frequently (Hubbs, 1955; Fryer and Iles, 1972; Lagler et al., 1977), particularly in freshwater species, and those with external fertilisation.

There are, however, many instances of pairs of fish species hybridising in captivity and producing viable offspring although hybrids of the same species in nature are unknown. This difference might be explained by lack of proximity of the species, ethological reasons, or insufficient scrutiny.

Introgression, that is gene exchange between species, is rarer still as it depends on at least partial fertility of hybrid individuals. It suggests that the species concerned have accumulated relatively few critical genetic differences, and if it occurs to any great extent there will be a loss of genetic distinction between the two taxa (Skibinski and Beardmore, 1979). This gene flow may represent a source of genetic variation for adaptation (Lewontin and Birch, 1966). Hybrids of two species may themselves be better adapted in some way to a particular environment, and there may be the establishment of a hybrid zone, (Hagen, 1967), or even hybrid speciation. Hybrid sterility on the other hand, constitutes a form of disruptive selection helping to maintain firm species boundaries, and indicates fuller development of the speciation process. Clark Hubbs (1970) has pointed out the importance of experimental fish hybridization studies as a means of assessing interspecific relationships, the success of hybridisation being dependent upon the degree of phylogenetic divergence.

In this paper we present clear genetic evidence for the existence of naturally occurring F_1 hybrids between two species of sand goby, and for offspring of later hybrid generations.

The past few years have witnessed a rapidly growing use and acceptance of gel electrophoresis as a taxonomic aid (Avise, 1974, 1976; Johnson, 1975; Skibinski et al., 1978; Thorpe 1979). The great advantage of the technique over morphological studies is that differences between species can be estimated at an empirical level, that of gene loci. These differences are therefore wholly discrete, independent of one another as separate characters and real in that they represent genetic variation of DNA due to base substitutions within loci coding for specific polypeptides. Unlike many morphological features, electrophoretic differences are not usually influenced by age or sex, and are unaffected by environmental changes during the lifetime of the individual. Whilst it may be argued

0171-8630/80/0003/0309/$ 02.00

that the proteins routinely utilised in such surveys do not constitute a random selection of loci, and care must be taken in making phyletic inferences from such phenetic data, the immense power of electrophoresis at a discriminatory level cannot be disputed (Manwell and Baker, 1963; Thorpe et al., 1978). It has thus proved very useful in hybridisation studies (Cross, 1978; Cross and O'Rourke, 1978; Solomon and Child, 1978), and for measuring genetic isolation between related species, particularly where sympatry is involved (Hunt and Selander, 1973; Skibinski et al., 1978). It is with three such populations of closely related goby species that this paper is concerned.

Pomatoschistus minutus (Pallas), known commonly as sand goby, is the largest of a genus currently housing some 10 species (Miller, 1973). The pale semi-translucent basic colouration and orange/brown melanophores give ideal camouflage for life over sand and shingle, to which the fish attaches itself by means of two fused pelvic fins forming a weak suction disc. It is abundant around the coasts of Europe from Norway to the Mediterranean commonly in depths of up to 25 m. Collett (1903) described a distinct deep-sea form off the coast of Norway, which he nominated *P. minutus norvegicus*, now *P. norvegicus*. De Buen (1923) recognised that the inshore *P. minutus* is composed of two distinct forms to which he gave subspecific status: *P. minutus minutus* and *P. minutus lozanoi*. These were diagnosed as separate species, *P. minutus* and *P. lozanoi*, by Webb (1980).

Pomatoschistus lozanoi tends to be a little smaller, pinker and paler, the pigmentation being in discrete freckles, rather than more finely dispersed as in the overall greyer *P. minutus*. This is particularly noticeable around the head. During the breeding season, males have black pigmented stripes on the body and an intense blue-black spot on the first dorsal fin. The number and distribution of these features differ between the two species and present a further means of identification. Large differences in seasonal abundance occur due to different breeding times (Table 1) supporting the findings of Fonds (1973) and Healey (1971). For a fuller description of the two species see Fonds (1973). A feature often used to distinguish between goby species, which are notorious for their superficial similarity, is the pattern of dermal papillae on the head (Sanzo, 1911; De Buen, 1930; Miller, 1963; Fonds, 1971). *P. minutus* and *P. lozanoi* have distinct patterns, but these are not easy to see on juveniles and are usually damaged during the process of netting and freezing the fish. Even useful characteristics such as these also display some variation according to the age of fish. Individuals with intermediate patterns, sometimes with one species pattern on one side of the head and the other on the opposite side have been found. Using a multi-disciplinary approach, Webb (1980) has shown that these are hybrids, cross-breeding having been successfully realised in aquaria (Fonds 1973). It is clear from the distribution of the two species in the wild, and their performance in several laboratory experiments that *P. minutus* represents an estuarine species, and *P. lozanoi* a more neritic form (Fonds, 1973; Fonds and Veldhuis, 1973).

Table 1. Catches of *Pomatoschistus minutus* and *P. lozanoi* and their hybrids from three locations by push-netting

Date	Location	*P. minutus*	*P. lozanoi*	F_1s	F_2s
1977 Nov. 11	Pendine	41	2	–	–
Dec. 12	Pendine	24	7	–	–
1978 Jan. 11	Pendine	38	27	2	–
Feb. 09	Pendine	4	5	–	–
Mar 08	Pendine	10	13	–	–
Apr. 05	Pendine	–	20	–	–
Jun. 23	Oxwich	–	5	–	–
Sep. 30	Pendine	26	3	1	–
Oct. 30	Saundersfoot	100	5	–	–
1979 Jan. 30	Saundersfoot	63	44	–	–
Feb. 28	Saundersfoot	127	47	2	–
Mar. 28	Saundersfoot	75	21	–	–
Apr. 24	Oxwich	39	148	–	–
Apr. 27	Saundersfoot	1	85	–	–
Jun. 12	Saundersfoot	1	113	–	1
Jul. 11	Saundersfoot	5	50	–	–
Aug. 09	Saundersfoot	232	7	–	–
Sep. 06	Saundersfoot	85	1	–	–
Oct. 07	Saundersfoot	89	–	–	–
1980 Feb. 18	Saundersfoot	91	1	–	–

MATERIALS AND METHODS

Three sites on the South Wales coast supporting sympatric populations of *Pomatoschistus minutus* and *P. lozanoi* were sampled: Oxwich, Pendine and Saundersfoot. Fish were caught at low spring tides using a push-net (mesh size 8 mm) in depths of up to 1 m. These were then transferred to jars of fresh, aerated filtered sea-water, or placed directly in liquid nitrogen for transport. On return, fish to be electrophoresed were placed in a deep freeze at −70 °C until needed. For the enzyme loci under consideration here, crude homogenates of muscle were prepared, and horizontal starch gel electrophoresis carried out in a manner essentially similar to that of Ward & Beardmore (1977). The enzymes in question are alanine aminotransferase (ALAAT), creatine kinase (CK), peptidase (PEP), phosphoglucomutase (PGM) and phosphoglucose isomerase (PGI), and were stained using slightly modified techniques of Harris and Hopkinson (1976). Materials and methods used will be described in greater detail elsewhere (Wallis and Beardmore, in preparation).

RESULTS

A total of 16 enzyme systems and a general protein stain were found to yield scorable results giving 33 putative loci. Although we lack the breeding data to verify the heritable nature of these systems, their tissue distributions and the appearance of heterozygotes are consistent with other work on teleosts in which these data are available. The genetic identity (I) of the two species is estimated as 0.845, a genetic distance (D) of 0.168 ± 0.075 (Nei, 1972), fairly typical of closely related species (Avise and Smith, 1974; Ayala, 1975; Patton et al., 1972; Gorman et al., 1976; Ward and Galleguillos, 1978). These and other measures will be presented and discussed more fully in other communications. Here we are interested in diagnostic loci, that is to say those loci that have become more or less completely differentiated for different alleles in the two species (Table 2). *ALAAT A* and *CK A* are fixed for alternative alleles in each species. (Figs. 1–2). *PEP A* also shows no overlap between the two species, but is polymorphic in both. (Fig. 3). (We use the term polymorphic to describe loci at which the frequency of the most common allele <0.99). *PGI B* is essentially monomorphic for different alleles in *Pomatoschistus minutus* and *P. lozanoi*, but some rare variants in *P. minutus* cause a small degree of overlap. Hybrids between the two species are therefore seen as being rare heterozygotes at these loci (Fig. 4). *PGM B* and *PGI A* are also of some use as discriminatory loci as the former is polymorphic only in *P. minutus* and the latter only in *P. lozanoi*. *ALAAT A, PEP A* and *PGI* heterozygotes show the three bands characteristic of dimeric enzymes, and *CK A* heterozygotes show only two bands of equal staining intensity despite the fact that this enzyme is also dimeric (Ferris and Whitt, 1978). The two PGI systems hybridise giving a third central interlocus hybrid zone (Avise and Kitto, 1972). 6-Phosphogluconate dehydrogenase (PGD) stains faintly with PGI and CK, adenylate kinase (AK) also appearing on the latter, but do not affect scoring

Table 2. *Pomatoschistus lozanoi* and *P. minutus*. Allele frequencies at the six most useful loci for species distinction. The numbers assigned to alleles are those used in zymograms. RAM = Approximate relative anodal mobility of allozymes; N = Number of individuals

Enzyme locus	Allele	RAM	*P. lozanoi*	*P. minutus*
CK A	2	110	1.000	–
	1	100	–	1.000
			N = 369	N = 694
ALAAT A	2	150	1.00	–
	1	100	–	1.00
			N = 41	N = 30
PEP A	6	120	0.4748	–
	5	110	–	0.0393
	4	105	0.5252	–
	3	100	–	0.9570
	2	95	–	0.0028
	1	90	–	0.0009
			N = 278	N = 535
PGI A		200	0.0036	–
	2	170	0.5782	0.0042
	1	100	0.4182	0.9922
		10	–	0.0036
			N = 422	N = 840
PGI B		115	–	0.0006
		110	–	0.0208
		105	–	0.0006
	5	100	–	0.9738
	4	98	0.0011	–
	3	85	0.9966	0.0024
	2	72	–	0.0018
	1	70	0.0023	–
			N = 437	N = 840
PGM B		120	–	0.0066
		110	0.0029	0.4746
		102	0.9929	0.1958
		100	–	0.2268
		95	0.0042	0.0100
		90	–	0.0851
		70	–	0.0011
			N = 350	N = 452

In samples containing in all 1051 *Pomatoschistus minutus* and 604 *P. lozanoi*, 5 individuals with a totally hybrid genotype at the *ALAAT A, CK A, PGI B* and *PEP A* loci, and 1 individual with hybrid *ALAAT A* and *CK A, lozanoi PGI B* and *minutus PEP A* were found. This fish also displayed heterozygosity for *PGI A* that is largely restricted to *P. lozanoi*.

These individuals had pigmentation dispersal patterns intermediate to the parental forms and appeared to have normal ripening gonads when caught with other maturing fish.

DISCUSSION

As we are considering 4 virtually entirely differentiated loci, the chance of these individuals being coinciding rare variants is of course so infinitesimally small as to be negligible. The forms with hybrid patterns at all four diagnostic loci are presumably first generation hybrids resulting from *Pomatoschistus minutus* × *P. lozanoi* crosses. Whilst a temporal isolating mechanism exists as regards the breeding season of the two species, there must still be a fair degree of overlap during which time representatives of both species are displaying reproductive behaviour, and interspecific matings may occur. *P. minutus* leave the immediate shore line in April, the bulk of their offspring arriving

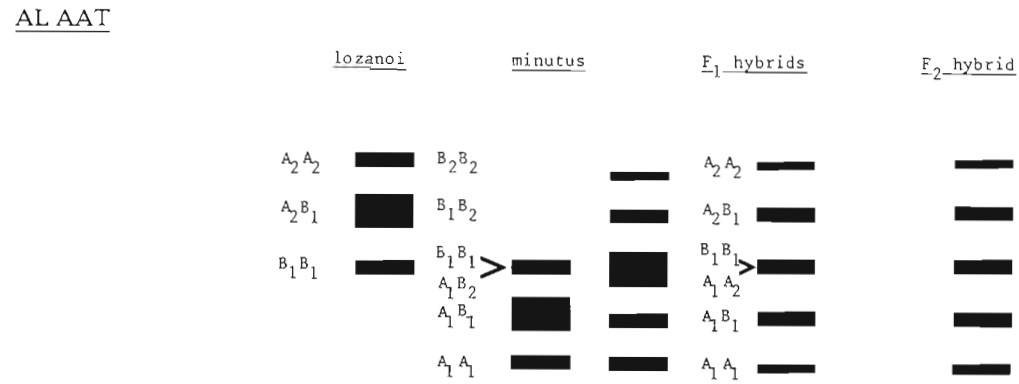

Fig. 1. *Pomatoschistus minutus* and *P. lozanoi*. Diagrammatic zymograms and genetic interpretations of isoenzyme banding patterns in the two species and their hybrids. Letters represent loci, with numerical subscripts for alleles; these are numbered from the origin upwards in the direction of anodal migration. ALAAT patterns are interpreted as two hybridising systems, A and B. The zones of equal anodal migration in both species (B) have been considered homologous, the other zone (A) being faster in *P. lozanoi* and slower in *P. minutus*. The appearance of the hybrids support this, but an alternative hypothesis is that there are two diagnostic loci rather than one

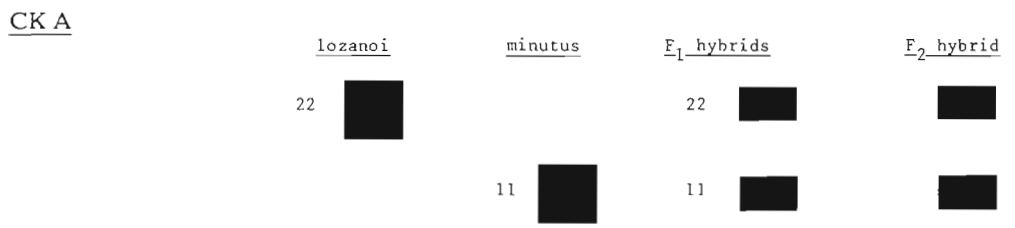

Fig. 2. *Pomatoschistus lozanoi* and *P. minutus*. Diagrammatic zymograms and genetic interpretations of isoenzyme banding patterns in the two species and their hybrids. For details consult legend to Figure 1

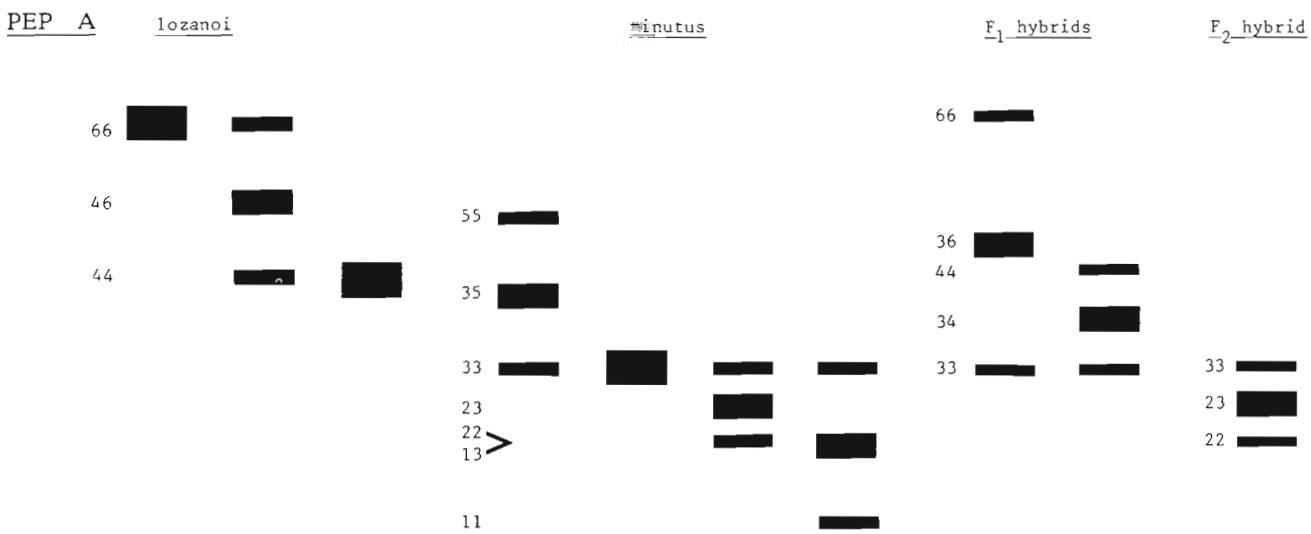

Fig. 3. *Pomatoschistus lozanoi* and *P. minutus*. Diagrammatic zymograms and genetic interpretation of isoenzyme banding patterns in the two species and their hybrids. For details consult legend to Figure 1

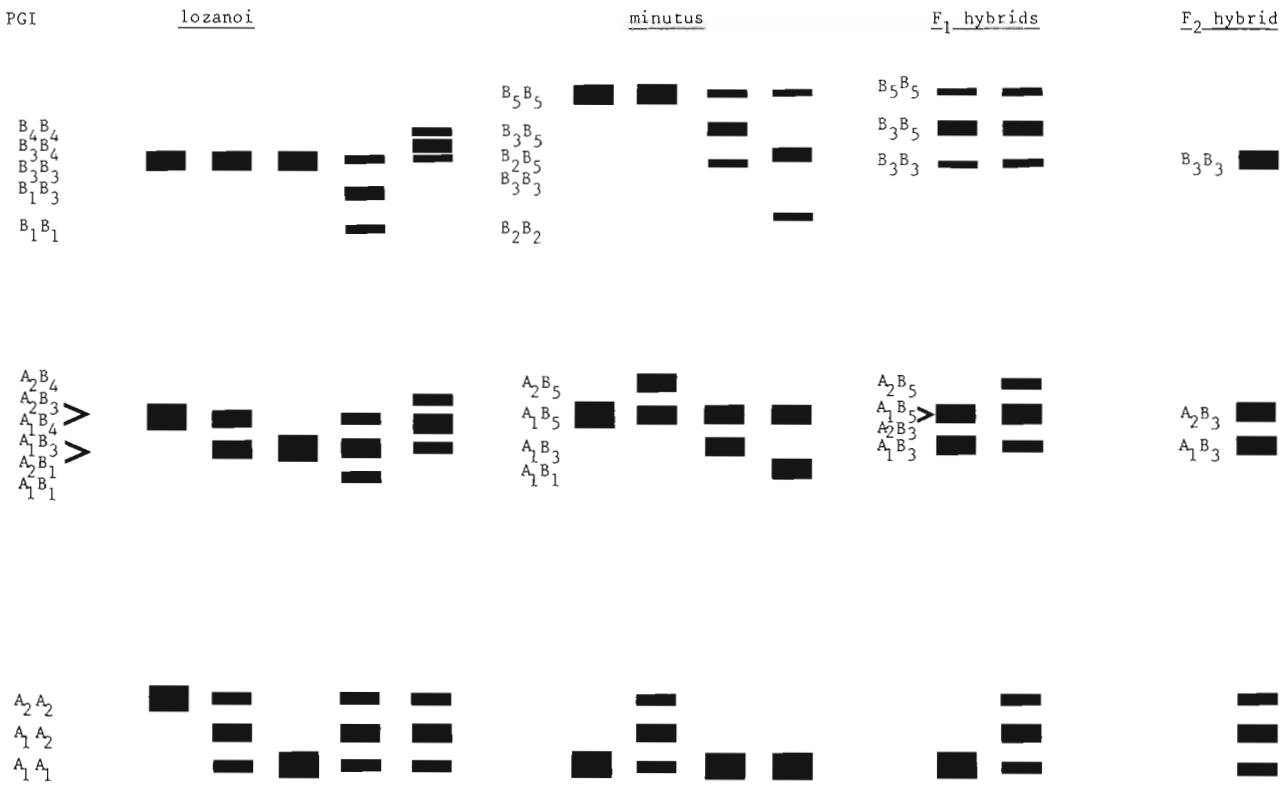

Fig. 4. *Pomatoschistus lozanoi* and *P. minutus*. Diagrammatic zymograms and genetic interpretations of isoenzyme banding patterns in the two species and their hybrids. For details consult legend to Figure 1. PGI patterns are interpreted as two hybridising systems, A. and B. Some of the rare variants that are confined to one species only have been omitted for the sake of simplicitiy

again in July and August as *P. lozanoi* start to leave. Of course, there is nothing to suggest much geographic separation of the species at this time. They are both very abundant and there is considerable overlap in ecological niches; it is probably that first one species migrates to a more localised area within the niche as a whole, then the other. The efficacy of this pre-mating isolating mechanism may well be altered by physical anomalies such as extreme temperature, unusually strong currents or heavy seas. For instance, *P. minutus* spawns at temperatures as low as 5 °C, albeit sluggishly, whereas *P. lozanoi* shows no nesting behaviour or spawning below 10 °C (Fonds, 1973).

Goby species have highly specific courtship display patterns by which means the male entices the female to lay her eggs in the nest he has dug out, usually under an empty shell (Nyman, 1953; Kinzer, 1960). There is evidence to suggest that *Pomatoschistus lozanoi* on average utilises smaller bivalve species than *P. minutus* for this purpose (Fonds, 1973). It seems most likely that occasionally fish mistake a partner for a member of the same species, and hybridisation results. It could also be possible that nests are confused, or even that there are opportunist males that in some way intercept an original pairing and deposit their own sperm over the eggs.

Webb (1980) has studied the chromosome complements of *Pomatoschistus minutus*, *P. lozanoi* and *P. norvegicus*, and has shown that despite the high degree of relatedness of the three taxa, considerable differences exist. However, these appear to be largely due to differences in the arrangement of the genetic material; the DNA content and the number of chromosome arms is fairly consistent across the group. *P. lozanoi* is intermediate in chromosome number, and has an unpaired metacentric and a trivalent association at first metaphase. From these observations, Webb concludes that the karyotypic differences may primarily be a result of a number of Robertsonian transformations, indeed *P. lozanoi* appears to possess such a polymorphism. Thus any interspecific hybrids will be heterozygous for several Robertsonian changes and one might reasonably expect a concomitant and not inconsiderable reduction in fertility of these individuals through malorientation and unequal disjunction at meiosis. This situation should represent a considerable restraint to gene flow between species.

The individual of unusual genotype could not result from a backcross of a hybrid to either parent, given that our genetic interpretation of the isozymes visualised is correct and they are inherited in the expected Mendelian fashion. It would seem that the most likely expla-

nation for the existence of a genotype possessing a *P. minutus PEP A* and homozygous *P. lozanoi PGI B* is as a consequence of an $F_1 \times F_1$ cross producing a true F_2.

As, in this paper, we rely completely on this individual as evidence for F_1 hybrid fertility, it is desirable to estimate the chance of this fish not being what it appears. One can assess the probability of it being an F_1 resulting from parents of rare genotypes. The genotypes at the *CK A* and *ALAAT A* loci are the expected heterozygotes, and the *PGI A* heterozygote genotype is consistent with the possibility of it being an F_1. However, to be a *PGI B lozanoi* type homozygote would require the *P. minutus* parent to have been a rare heterozygote ($P = {}^4/840$). Likewise, to be a *PEP A minutus* type heterozygote would require the *P. lozanoi* parent to have possessed an allele that has not been found in 278 fish of that species ($P<{}^1/278$). It would further be necessary that both of the rare alleles were passed on by the two parents ($P = {}^1/4$). Thus based on the *PEP A* and *PGI* loci there is probability of $< 4.28 \times 10^{-6}$ of this fish being an F_1. The chance of it being a backcross rather than an F_1 is slightly higher, but of course this would not refute the hypothesis of hybrid fertility.

With hybrids at such a low frequency ($\simeq 0.3$ %), the production of an F_2 individual appears an unlikely event, but this ignores the possibility of a degree of recognition of one hybrid by another. It is reasonable to suppose that hybrids behave in a way intermediate to, or at least different from both parents, but more importantly, it is likely that F_1 hybrids will behave similarly to each other. This may be true as regards timing of their departure from the shore, and the same may be true of their appearance and breeding display patterns. Dr. D. Colombera of the Institute of Animal Biology, Padova (Italy), kindly informs us that some species of the genus *Gobius* have been shown to possess steroid derivative water-soluble pheromones provoking specific responses from individuals of the opposite sex. It is conceivable that a hybrid between two species will have an intermediate form of pheromone recognisable as such by other hybrids. Whilst a thorough knowledge of ethological mechanisms in the two species would be useful, is does not seem unreasonable to suppose that the chance of a mating between two F_1 hybrid individuals is considerably greater than the frequency of such individuals would, on its own, predict.

Such situations are of relevance to evolutionary studies as they can help to understand the speciation process, and the degrees of divergence involved. Although we have no evidence for introgression in this particular case, as no backcrosses have been found, this would represent gene flow between species, and very little gene flow is necessary to overcome the weak stochastic process of genetic drift.

Acknowledgements. We are very grateful to Dr Charles Webb for letting us use unpublished information, and for the time he and Dr. Peter Miller have given up in helpful discussion. Thanks are also due to Drs. Mark Fonds, John Thorpe and Bob Ward for their interest shown and useful suggestions made, and to Brendan McAndrew for his help as a fishing colleague. The Natural Environmental Research Council provided the financial support for this work, and we express our gratitude to them.

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This paper was presented by Professor J. A. Beardmore; it was accepted for printing on July 5, 1980