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Thoughout most of the Tertiary the Atlantic and Pacific oceans were continuous and connected over much of the area that is now Central America and the northwestern corner of South America. Presumably populations of many marine species were also continuous in this range. The rise of the Isthmus of Panama 2–5 million years ago (Woodring, 1966) split this vast biota into two disjunct populations, the western Atlantic and the eastern Pacific. The land bridge has remained as a geographic barrier between the oceans and as such has provided a unique opportunity to study the evolution of many marine organisms. The Panama Canal has had little effect on the distribution of most groups because of the fresh water of Gatun Lake. Few marine species can survive a canal transit. (Concerning fishes see Hildebrand, 1939; R. W. Rubinoff and I. Rubinoff, 1968 and 1969).

The species that were separated have continued to evolve independently and diverge from each other with time. Some groups are still morphologically identical on both coasts. Others have changed radically, probably in response to different selective pressures.

These pairs of populations, with an obvious common origin, have been called "geminate." It seems likely that some of the "geminate" populations have evolved potential reproductive isolating mechanisms. But since the presence of reproductive isolation is not necessarily correlated with morphological divergence, it would be difficult to predict which pairs are still conspecific. The Isthmus of of Panama is, both historically and logistically (the oceans are only 50 miles apart), ideally suited to an experimental laboratory study of isolating mechanisms.

Most studies of isolating mechanisms have involved field work on species that are already sympatric. Some experimental studies on allopatric populations have been conducted with a limited number of terrestrial and fresh water animals (Littlejohn, 1969), but marine species have been largely ignored.

THE PROBLEM

A number of questions can be asked. Are closely related species on both coasts of the Isthmus still capable of exchanging genes, or have they evolved isolating mechanisms in 2–5 million years? And if they have, what types? Are the potential isolating mechanisms that may exist between these allopatric species—and have not been selected for—different from those that exist between the sympatric species? And finally, what is the correlation between morphological divergence and reproductive isolation?

The objective of the following study was to determine the extent to which Panamanian species of the fish genus *Bathygobius* had evolved isolating mechanisms, using artificial hybridization experiments and laboratory breeding tests.

Four types of *Bathygobius,* a small inshore goby, have been found in Panama (Ginsburg, 1947).

B. soporator, Cuvier and Valenciennes—Atlantic
B. curacao, (Metzelaar)—Atlantic
B. ramosus, Ginsburg—Pacific
B. andrei, Sauvage—Pacific

The allopatric populations have been called species simply because they are

TABLE 1. *Values of coefficient of difference (C.D.) between species of Panamanian Bathygobius.*

	soporator ramosus Allopatric	*soporator andrei* Allopatric	*curacao andrei* Allopatric	*curacao ramosus* Allopatric	*andrei ramosus* Sympatric	*soporator curacao* Sympatric
Tail Length	0.259	0.135	0.330	0.462	0.130	0.183
Body Depth	0.178	0.033	0.641	0.122	0.340	0.362
Caudal Depth	0.387	0.000	0.769	0.345	0.526	0.585
Head Length	0.000	0.734	2.249	1.366	0.851	1.152
Snout Length	0.136	0.076	1.391	0.992	0.231	1.136
Eye Diameter	0.078	0.000	0.305	0.313	0.078	0.308
Interorbital	0.149	0.298	0.714	0.652	0.242	0.294
Snout-Dorsal	0.288	0.670	2.303	1.358	1.131	1.193
Snout-Anal	0.208	0.032	1.160	0.872	0.194	1.094
Snout-Ventral	0.124	0.193	1.012	0.493	0.323	0.674
Ventral Length	0.852	0.024	0.887	2.321	1.191	0.054
Pectoral Length	0.631	0.507	0.672	0.812	0.065	0.169
Eye-1st Dorsal Scale	1.667	2.165	0.376	0.480	0.859	1.929
Scales	0.243	0.480	2.749	3.280	0.321	2.692
Gill Rakers	0.438	0.506	0.600	0.526	0.070	0.060
Pectoral Rays	0.877	2.549	4.000	2.362	1.428	1.709
SUM OF 16 *C.D.*'s	6.515	8.402	20.158	16.756	7.980	13.594

morphologically distinct and we will refer to them by these names for convenience. This genus was chosen for several practical reasons. It is abundant, easy to collect and can be maintained and bred in the laboratory with comparative ease. In addition, the courtship, spawning behavior, and embryology of *B. soporator* had been described (Tavolga, 1950, 1954, 1956).

MORPHOLOGICAL ANALYSIS

Before the experimental studies were begun, a comparative morphological analysis of the four species was undertaken. We hoped to find a measure of relative morphological divergence between the species which would in turn be correlated (or not) to the extent of reproductive isolation. There appeared to be no particularly suitable multiple character index which fulfilled this need (several computer programs were investigated without success). For this reason, we used a method based on the coefficient of difference (*C.D.*) described in Mayr et al., (1953). The *C.D.* between two populations for one character $=(M_b-M_a)/(S.D._a+S.D._b)$. We calculated the *C.D.* for 16 characters and added them. The resulting sum of the coefficients of difference (*S.C.D.*) was used to evaluate the intrageneric relationships (Rubinoff, 1963).

It is recognized that this method has certain limitations, the most serious of which is that it cannot be applied to intergrading characters which are not discrete or easily quantifiable (e.g., color and branching patterns of fin rays). *C.D.*'s and *S.C.D.*'s for the four species of *Bathygobius* are presented in Table 1.

Three of the species (*B. soporator, B. ramosus, B. andrei*) are very similar and appear to have diverged almost equally from each other. Certainly the magnitude of the differences is too small to presume a closer phyletic relationship between any two species. The fourth species (*B. curacao*) is quite distinctive and is no doubt a more distant relative, perhaps an older species and probably not part of the "geminate complex." In some ways this was just as well; subsequent collecting revealed so few *curacao* that it was necessary to drop them from the experimental program, particularly since it is a much smaller species and only the very largest adults could have been used in interspecific

TABLE 2. *Artificial hybridization experiments.*

Cross			*% Eggs Completing Embryogenesis	
B. soporator ♀	×	*B. ramosus* ♂	85.2%	(3)+
B. soporator ♀	×	*B. andrei* ♂	91.4	(3)
B. soporator ♀	×	*B. soporator* ♂	83.5	(3)
B. ramosus ♀	×	*B. soporator* ♂	83.6	(3)
B. ramosus ♀	×	*B. andrei* ♂	82.9	(3)
B. ramosus ♀	×	*B. ramosus* ♂	84.3	(3)
B. andrei ♀	×	*B. soporator* ♂	39.8	(1)++
B. andrei ♀	×	*B. ramosus* ♂	39.9	(2)
B. andrei ♀	×	*B. andrei* ♂	58.5	(2)

* The % eggs completing embryogenesis = no. of eggs with fully expanded chorion membranes/ no. of embryos viable after 96 hours. Individual experiments initiated with ca. 500–700 eggs.

+ No. of experiments indicated in parentheses.

++ It was difficult to obtain ripe *andrei* ♀♀ and, therefore, eggs used were not always in optimum condition for fertilization.

breeding tests. In spite of the overall morphological similarity of the three species certain external key characters could be used to distinguish between them, and even field identifications were rarely a problem.

EXPERIMENTAL ANALYSIS

Methods

Quinaldine (Eastman Kodak Organics), a fish anesthetic, was used to collect *Bathygobius* from tidepools on the Atlantic and Pacific coasts of the Canal Zone and adjacent areas of the Republic of Panama. Stock and experimental animals were held in 45 and 90 liter acrylic aquaria and fiberglass troughs 76 cm × 76 cm × 20 cm deep, supplied with continuously running sea-water. Water temperature was maintained at 23–30C, salinity 24‰ to 36‰. Indoor lighting was regulated to a 12 hr light and 12 hr dark period. The fish were fed chopped shrimp, clams and squid two times daily.

I. *Artificial Hybridization Experiments.*— The method used was generally that described by Tavolga (1950) for *B. soporator*. Eggs from gravid females were stripped directly onto 8 cm × 3 cm glass slides (ca. 100 eggs in a single row) and immediately immersed into large 18 cm finger bowls containing sperm solutions from heterospecific males or conspecific males as controls. (If the slides were first placed in sea-water and then flooded with sperm solution the chorion membranes of a large percentage of eggs did not fully expand—particularly when eggs of *B. andrei* or *B. ramosus* were used.) After 10 minutes each slide was placed into a 10 cm finger bowl containing fresh sea-water. Water was changed twice daily and 20,000 units of penicillin G was added (24 hrs after fertilization to avoid possible adverse effects) to control bacterial and secondary fungal infections. The embryos were kept at 25C ± 1C and hatched in 4 days. The results of these experiments are summarized in Table 2.

There appeared to be no significant differences between development in control and hybrid groups during embryogenesis and we concluded that gametic or zygotic mortality as a post-mating isolating mechanism had not evolved. A possible case of reduced viability in hybrid embryos from crosses using *B. andrei* females may be obscured by a very poor control series for the group. Unfortunately, at that time, we were not able to rear the larvae to determine if they would continue to develop normally to sexual maturity.

TABLE 3. *Single pair "No Choice" mating experiments.*

	Cross			Total No. of days	No. of Spawnings
Allopatric	*B. soporator* ♀	×	*B. ramosus* ♂	141(4)	0
	B. ramosus ♀	×	*B. soporator* ♂	153(5)	0
	B. soporator ♀	×	*B. andrei* ♂	206(2)	0
	B. andrei ♀	×	*B. soporator* ♂	211(3)	0
Sympatric	*B. ramosus* ♀	×	*B. andrei* ♂	67(3)	0
	B. andrei ♀	×	*B. ramosus* ♂	261(3)	0
Controls	*B. soporator* ♀	×	*B. soporator* ♂	296(4)	4
	B. ramosus ♀	×	*B. ramosus* ♂	371(8)	11
	B. andrei ♀	×	*B. andrei* ♂	357(8)	6

* The number of days shown for each cross represents the total of a number of single pair experiments (no. of experiments indicated in parentheses).

II. *Single Pair "No Choice" Mating Experiments.*—Single pair "no choice" situations (one ♀ species A and one ♂ species B), were used to test for behavioral isolation.

Adult males were isolated in 45-liter aquaria several days before a female was introduced. The pairs were then left together for indefinite periods of time. Each aquarium contained a triangular shaped clay tile which served as a nest site. Reciprocal heterospecific combinations and conspecific pairs as controls were established. Table 3 summarizes the results of these breeding tests.

Spawnings were recorded in all of the control groups. No spawnings were ever recorded for a heterospecific pair although the experimental conditions were identical. Observed differences in the courtship and spawning behavior of the three species seemed to substantiate these results. For example, a conspicuous feature of the behavior of *B. soporator* is the pre-courtship color pattern of the male. A dramatic darkening of the chin is contrasted by a lightening of over-all body color. It was interesting to find that the pre-courtship color pattern of a *B. ramosus* male was exactly the opposite, a very white chin and a dark (almost black) body color. *B. andrei* males seem to fall between the two and have a mottled chin and a less intense body color change.

We also conducted a series of experiments which manipulated the mating situation in an attempt to induce the species to interbreed. These included: restricting pairs to the nest site (Tavolga, 1954, reported that this method reduced courtship behavior, but did not inhibit spawning in *B. soporator*); infusing solutions of conspecific gonadal material into tanks with heterospecific pairs; and interrupting conspecific spawnings to give each member of the pair a new heterospecific partner. All of these methods failed to produce any heterospecific spawnings.

The results of these experiments led us to believe that behavioral discrimination between the three species was highly developed and that reproductive isolation was complete at the premating level between both the sympatric and the allopatric species.

However, there seemed to be proportionately more spawnings in our stock tanks (which contained 10–15 individuals) than in the aquaria containing a single conspecific pair. This indicated that an alteration in the design of our breeding experiments might produce different results.

TABLE 4. *Group "No Choice" mating experiments.*

	Cross			Duration Days	No. of Spawnings
Allopatric	*B. soporator* ♀♀	×	*B. ramosus* ♂♂	*139	0
				119	0
	B. ramosus ♀♀	×	*B. soporator* ♂♂	153	9
				119	0
	B. soporator ♀♀	×	*B. andrei* ♂♂	138	6
				119	6
	B. andrei ♀♀	×	*B. soporator* ♂♂	138	9
				119	14
Sympatric	*B. ramosus* ♀♀	×	*B. andrei* ♂♂	119	0
	B. andrei ♀♀	×	*B. ramosus* ♂♂	170	0

* All experiments contained 6 ♀♀ × 6 ♂♂ except * which contained 3 ♀♀ × 3 ♂♂.

III. *Group "No Choice" Mating Experiments.*—Group "no choice" situations were set up as follows: Six females of species A and six males of species B were introduced into large troughs which contained seven tiles for nest sites. Conspecific controls were not used simply because we did not have enough troughs.

Table 4 summarizes the results of the group "no choice" experiments. Under these conditions we observed very different results than in the single pair experiments. There were frequent spawnings between allopatric species, particularly between *B. andrei* and *B. soporator*. The sympatric species, *B. ramosus* and *B. andrei,* never spawned. Hypotheses to explain the success of group matings can be suggested. Perhaps more than one male is involved in courting a female, thus maintaining a higher (or longer) level of excitation; or intraspecific aggression between males might reduce their discriminatory abilities. A very preliminary investigation into this problem was attempted by setting up the crosses with unbalanced sex ratios summarized in Table 5. The results of these experiments imply that neither sex is totally responsible for species discrimination but, in view of the negative data obtained, it would seem that a detailed behavioral analysis would probably be the most profitable approach to determine the effects of gregarious situations on breeding in *Bathygobius*. Certainly under natural field conditions (i.e., small tide pools) *Bathygobius* would usually be found in "group" situations similar to those we have simulated in the laboratory.

The results of these experiments do indicate that sympatry has reinforced behavioral discrimination between Panamanian *Bathygobius*. However, many species that are known to be reproductively isolated in nature will often breed in "no choice" laboratory experiments (Phillips,

TABLE 5. *Effects of unbalanced sex ratios on interspecific spawnings.*

	Cross				No. of Days	No. of Spawnings
1	*B. andrei* ♀	×	6	*B. soporator* ♂♂	95	0
1	*B. soporator* ♀	×	6	*B. andrei* ♂♂	95	0
6	*B. soporator* ♀♀	×	1	*B. andrei* ♂	95	0
6	*B. andrei* ♀♀	×	1	*B. soporator* ♂	77	0
6	*B. soporator* ♀♀	×	6	*B. soporator* ♂♂	87	7
6	*B. andrei* ♀♀	×	6	*B. andrei* ♂♂	77	4

TABLE 6. *Group "Choice" mating experiments.*

	Females (6)		Males (3)		Males (3)	Spawnings Conspecific	Spawnings Heterospecific
Allopatric	*B. soporator*	×	*B. soporator*	×	*B. ramosus*	4	2
	B. ramosus	×	*B. ramosus*	×	*B. soporator*	9	1
	B. soporator	×	*B. soporator*	×	*B. andrei*	0	2
	B. andrei	×	*B. andrei*	×	*B. soporator*	11	0
Sympatric	*B. ramosus*	×	*B. ramosus*	×	*B. andrei*	8	0
	B. andrei	×	*B. andrei*	×	*B. ramosus*	8	0
		(Duration of experiment—177 days)					

No. of heterospecific allopatric spawnings	5
No. of conspecific spawnings	24
No. of heterospecific sympatric spawnings	0
No. of conspecific spawnings	16

1915; Patterson and Stone, 1952). Therefore, a more meaningful test for reproductive isolation would be one in which a choice of mates was offered. Presumably if behavioral isolation is complete, there would be no heterospecific spawnings if a conspecific mate is available (Blair and Howard, 1944; Gordon, 1947).

IV. *Group "Choice" Mating Experiments.*—Six females, three conspecific males, and three heterospecific males were introduced into troughs with conditions similar to those used for the group "no choice" experiments. A choice of males was offered, rather than females, to facilitate identification of which species participated in the spawning. The female is present in the nest only during the short time she is ovipositing. In contrast, the male guards the nest for 4 days and is easily captured, identified and returned to the nest during that time. (It could be argued that a ripe female might not have a choice of a mate at all times if the three conspecific males were brooding simultaneously. However, in our experiments we never encountered more than two males guarding nests in a given trough and, in addition, a male will often fertilize and incubate a second and even third batch of eggs if a number of ripe females are available.) Results of the group "choice" experiments are presented in Table 6.

In the troughs containing allopatric species "mistakes" were made and heterospecific spawnings occurred. Again, in the troughs containing sympatric species no heterospecific spawnings were recorded. There were 24 conspecific spawnings and 5 heterospecific spawnings in the four troughs containing allopatric species, indicating an error in mate selection of almost 18% for this experiment. Therefore, it would seem that behavioral isolation has evolved in sympatric species of *Bathygobius,* but has only partially evolved between the allopatric species.

In the group "no choice" experiments (Table 4) there were many more spawnings in the *B. soporator* × *B. andrei* crosses (35 spawnings) than in the *B. soporator* × *B. ramosus* crosses (9 spawnings). This raised suspicions that perhaps evolutionary divergence between *B. soporator* and *B. andrei* was not as great as between *B. soporator* and *B. ramosus.* However, in the last group of experiments—group "choice" (Table 5)—there was an almost equal ratio of heterospecific to conspecific spawnings between *B. soporator* and *B. ramosus* (3:16) and between *B. soporator* and *B. andrei* (2:16). Obviously, there is much variability in the breeding

potential of individual *Bathygobius* under these circumstances. And, since it is not practical (due to space and generation time) to replicate the experiments many times, the conclusions drawn must of necessity be qualitative rather than quantitative.

V. *Hybrid Viability*.—Frequently some form of isolating mechanisms may operate at a post-mating level before behavioral isolation is achieved, particularly between previously allopatric populations in which selection against gametic wastage has not been an influencing factor (Mayr, 1963).

The initial artificial hybridization experiments led us to conclude that all embryos were completely viable up to the hatching stage. But, the possible existence of hybrid inviability or sterility still loomed as large unanswered questions. It thus became imperative to reattempt to rear some of the pelagic larvae which resulted from our group mating experiments.

The method used was basically one that was developed for rearing another goby, *Lophogobius* (Delmonte, I. Rubinoff, R. Rubinoff, 1968). Plastic swimming pools (182 cm in diameter, 20 cm deep) were placed outdoors under an overhead cover, and were filled with sea-water a few days before the larvae were introduced. On the fourth day after oviposition the eggs, still attached to the tile nest, were removed from the trough and placed into a bucket of sea-water until they hatched. Examination of these eggs revealed almost 100% of both hybrid and conspecific spawnings developing normally. Also, there seemed to be no difference in the numbers of embryos that hatched in both groups.

After hatching, the larvae were placed into the pools. Penicillin G (500,000 units) was added to the pools every other day and a small amount of water (ca. 1/5 of total volume) was changed daily. Liquifry (a commercial fish food) was introduced three times daily. Those pools that had a rich original innoculation of plankton, particularly copepods, were the ones in which the larvae were able to survive. Although this method was not always successful, we were able to raise enough individuals to answer some basic questions.

Hybrid larvae of a cross between *B. soporator* and *B. andrei* were reared past metamorphosis (at 3 weeks) to sexual maturity at 5 months. The F_1 appear to be completely viable under laboratory conditions, although too few were actually raised to compare hybrid success with that of the controls. Some of these individuals have now spawned, which indicates that hybrid F_1 sterility would not act as an isolating mechanism between the two species.

We have also attempted to backcross hybrids to both parental species. To date, only backcrosses of the hybrids to *B. soporator* have been successful but again, the number of experiments is too small to draw any definite conclusions concerning the mating of *B. andrei* and the hybrids. We have reared the progeny of one of the backcrosses. A large number have reached sexual maturity and bred. Preliminary examination of the hybrids and backcrosses for a few key characters has revealed morphological intermediates.

An interesting aspect for future study would be to rear the hybrids of an artificial cross between the sympatric species to determine if any post-mating barriers are present between species that have evolved complete behavioral isolation.

Discussion

The results of these investigations indicate that the sympatric species of *Bathygobius* are effectively isolated—as one would expect. And it is most likely that behavioral discrimination at the premating level is responsible for reproductive isolation at the present time. Even under the stress of "no choice" forced laboratory breeding tests there were no heterospecific spawnings between *B. ramosus* and *B. andrei*. Since the two Pacific species are often collected from the same tide pool

TABLE 7. *Summary of reproductive isolation between species of* Bathygobius.

Isolating Mechanism	Between Sympatric Species	Between Allopatric Species
Premating		
Temporal or Ecological	No	No
Mechanical	No	No
Behavioral	Yes	Partial
Post-Mating		
Gametic or zygotic/mortality	No	No
Hybrid inviability/mortality	?	*No
Hybrid sterility	?	*No
Backcross inviability/mortality	?	*No

* Only *B. soporator* × *B. andrei* tested.

and are known to spawn throughout the year, it is unlikely that any form of temporal or ecological isolation exists. No post-mating barriers are evident through embryogenesis.

However, the allopatric species have not evolved complete reproductive isolation in the absence of the reinforcing influence of selection. Errors in mate selection, such as those made in our group "choice" experiments, would probably occur if the species were to become sympatric under natural conditions. Nor is it likely that any other form of ecological or temporal isolation would prevent hybridization (*B. soporator* is found in similar habitats and also breeds throughout the year). Successful rearing experiments have demonstrated that the hybrids of at least one F_1 combination (*B. soporator* × *B. andrei*) resulting from interspecific spawnings would be fertile, and that they would be able to backcross to the parental species. Therefore, it seems reasonable to assume that introgression (at least at first) would occur. Although the hybrids (both F_1 and backcross) reared in laboratory circumstances appear completely viable, we do not know their fitness or success under natural competitive conditions, and we are not able to predict whether selection will continue to reinforce behavioral discrimination or whether gene flow will be great enough to produce hybrid swarms.

Bossert has developed a model that will determine the expected gene flow between two populations after they have become sympatric—if the initial mating error (premating isolation) and heterozygotic fitness (post-mating isolation) are known. We have applied our data to this graph (Fig. 1) and can draw some very broad conclusions.

The mating error between *B. andrei* and *B. soporator* is sufficiently high to assume that unless the hybrids were almost totally inviable in the field (<10%) the probable separation of the species is unlikely. And, if the hybrids are as fit in nature as they are in the laboratory (>80%), fusion of the species is likely.

We do know that for Panamanian species of the genus *Bathygobius* 2–5 million years of geographic isolation and sufficient morphological divergence to be considered good species (taxonomically) is not enough evidence to assume a correlation with reproductive isolation.

SUMMARY

The objective of this investigation was to determine the extent to which three species of Panamanian *Bathygobius* (two Pacific—*B. andrei, B. ramosus* and one Atlantic—*B. soporator*) had evolved reproductive isolation. Breeding tests and artificial hybridization experiments were designed to establish the presence of isolating mechanisms at the pre- and post-mating levels.

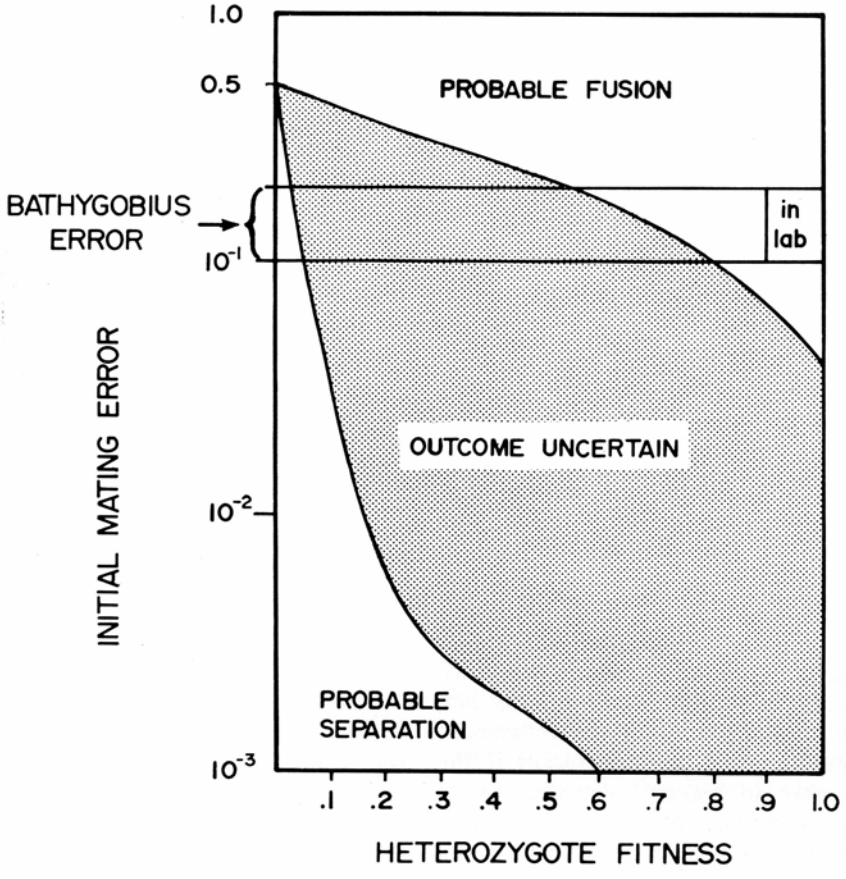

FIG. 1. Possible outcome if *B. andrei* and *B. soporator* become sympatric. Modified from Bossert (in Wilson, 1965).

Single pair "no choice" mating experiments (one ♀ species A + one ♂ species B) produced no heterospecific spawnings between allopatric or sympatric species, suggesting a high level of behavioral discrimination between the three species.

However, group "no choice" mating experiments (six ♀♀ species A + six ♂♂ species B) resulted in frequent spawnings between allopatric species. There were no spawnings between the sympatric species although conditions were identical.

In group "choice" experiments (six ♀♀ species A + three ♂♂ species A and three ♂♂ species B) heterospecific spawnings still occurred between allopatric species even though a conspecific mate was available. Errors in mate selection were made in 18% of the total spawnings.

The artificial hybridization experiments indicated that through embryogenesis there were no post-mating barriers to hybridization between the three species.

Hybrid larvae of a cross between *B. soporator* × *B. andrei* have been reared to sexual maturity. They are fertile and have produced an F_2. In addition, hybrid F_1 larvae have been successfully backcrossed to one of the parental species (*B. soporator*) and their progeny have also matured and bred.

Therefore, allopatric species of *Bathygobius* that have been isolated from each other for 2–5 million years are still capable

of genetic interchange. Behavioral discrimination, which acts as an effective isolating mechanism between the sympatric species has only partially developed between the geographically isolated populations. No post-mating barriers to hybridization are apparent under laboratory conditions. Morphological divergence is not, within this geminate complex, correlated to reproductive isolation.

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