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Cultivation of marine copepods for genetic and evolutionary research*

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KURZFASSUNG: **Kultur mariner Copepoden für genetische und evolutive Untersuchungen.** Einige benthische Copepoden eignen sich ausgezeichnet für das Studium genetischer Aspekte evolutiver Vorgänge im Meer. Dazu gehören speziell die Arten der Gattung *Tisbe*, weil sie (1) im Labor mit Hilfe einfacher Methoden kultiviert werden können, (2) einen kurzen Entwicklungszyklus haben, (3) eine hohe Vermehrungsrate und (4) oft genetischen Polymorphismus aufweisen. Es hat sich herausgestellt, daß dieser Polymorphismus in einigen Fällen adaptativ und balanciert ist, d. h. durch natürliche Selektion, die auf die einzelnen Genotypen verschieden einwirkt, aufrechterhalten wird. Auf diese Weise sind die Populationen befähigt, verschiedene ökologische Nischen auszunützen. Versuche haben gezeigt, daß gewisse ökologische Faktoren, wie Salzgehalt und Temperatur, stark selektiv wirken. Veränderungen in der Frequenz gewisser Genotypen einer polymorphen Art unter verschiedenen Bedingungen – in der Natur und im Labor – und die unterschiedliche Größe der genetischen Last in marinen und Brackwasser-Populationen derselben Art beweisen den Einfluß abiotischer Umweltbedingungen auf den sichtbaren Polymorphismus einerseits und auf die latente genetische Variabilität andererseits. Es darf allerdings nicht vergessen werden, daß in einer biologischen Gemeinschaft ebenfalls Faktoren, wie Anzahl und Art der miteinander lebenden Organismen, eine wichtige Rolle spielen. Die ersten Ergebnisse einer Reihe von Versuchen haben erkennen lassen, daß in gewissen Fällen die biotischen Faktoren eine mindestens ebenso große selektive Wirkung haben können wie die abiotischen. Weitere Experimente befassen sich mit der Frage der reproduktiven Isolation geographischer Populationen derselben Art und der dabei beteiligten Mechanismen.

INTRODUCTION

Some marine benthic copepods provide good material for studying genetic aspects of evolutionary processes in the sea. A few species of the genus *Tisbe* (Harpacticoida) are particularly suitable for this purpose, insofar as they (1) can be raised with the aid of comparatively simple methods; (2) possess short life cycles; (3) produce numerous offspring; (4) have a wide geographical distribution; and (5) often exhibit genetic polymorphism.

After illustrating the methods followed in our laboratory for the cultivation and

* Dedicated, with esteem and affection, to Professor T. Dobzhansky on the occasion of his 70th birthday.

handling of these copepods for crossbreeding experiments, I will provide a few examples of the kind of research which – in the framework of evolutionary genetics – can be carried out utilizing these organisms. The observations and experiments reported in this paper have been drawn mainly by some more recent lines of investigation of which a short account will be given.

COLLECTING AND REARING METHODS

The copepods are usually collected at various depths among green algae such as *Ulva* or *Enteromorpha*, or among Zosteraceae. Some species can be collected with a plankton net; for other species it is more convenient to bring the algae into the laboratory where they are left in the dark in a large glass vessel for a period of 6–8 hours. After some time the copepods escape from the asphyxial conditions in the container by swimming towards the surface. A sudden side illumination of the vessel attracts a crowd of copepods, most of which posses a positive phototropism, and, therefore, it becomes easy to collect various individuals by means of a pipette. Females of the species required, carrying egg-sacs, are then individually transferred into small glass vessels containing the culture medium.

Copepods of the genus *Tisbe* have been raised in the past by LWOFF (1927), JOHNSON & OLSON (1948), and BOCQUET (1951). LWOFF utilized test tubes or Erlenmeyers half-filled with sea water. The food consisted of fish muscles, wheat grains, rabbit red cells, or dead bacteria. JOHNSON & OLSON used instead, fresh or dried brown algae, dried fragments of molluscs, faecies of *Nereis*. Our method, which is a modification of that employed by BOCQUET, is the following: 20 cm^3 of filtered sea water are put in a cup-shaped glass vessel, 6 cm in diameter and 4 cm high. Then we add one or two fragments of *Ulva* (previously washed in fresh water for 10–15 min in order to eliminate other organisms) and a suspension of *Dunaliella, Pheodactylum* or *Nitzschia* (2 or 3 cm^3 depending on the age of the culture). *Ulva* serves as a support for the nauplii, as a source of oxygen, and, perhaps, also as food. When the copepod females lay their egg-sac and the nauplii start hatching, we add one or two very small fragments of wheat grains which have been boiled for 20 min in fresh water. The addition of wheat grains is important, perhaps as a source of carotenoids. As shown by LWOFF and by BOCQUET, the pigments of Tisbidae are carotenoid in nature and (especially for the polymorphic species *Tisbe reticulata*) the lack of wheat fragments in the cultures leads, after two or three generations, to an almost complete loss of the colour patterns. For mass cultures we use larger vessels of the same shape (10 cm diameter, 6 cm high) with 100 cm^3 of sea water and a proportionally greater supply of *Dunaliella* and other ingredients. The supply of unicellular algae, as well as of wheat, is renewed every 5 days.

In the conditions described above, the females lay their egg-sac two or three days after its emission. At a temperature of 18^0 C the nauplii reach the adult stage in 8–15 days, according to the species or the genus. Segmentation of the eggs occurs in the sac. Larval development includes 6 naupliar stages and 6 copepodite stages. The 6th copepodite stage, corresponding to the adult, is therefore reached after 11 molts.

The best culturing conditions for the contents of each egg-sac, are obtained by transferring the mother to a new vessel immediately after the deposition of a sac. This procedure reduces larval mortality (these copepods are very sensitive to crowding) and, therefore, permits collection and analysis of several hundreds of offspring from a single female.

The utilization of Tisbidae as material for genetic investigations meets with the following difficulties: (1) Small size of the copepods; (2) possibility that the females are fertilized before reaching the adult stage; (3) extreme sensitivity to protracted inbreeding, which, in most cases, leads to a strong shifting of sex-ratio in favour of males.

Difficulty number 2 requires a laborious device: in order to cross a male and a female having the desired characteristics, it is necessary to place two copepodites together to ensure that the female has not yet been fertilized. This procedure involves a large percentage of errors in the choice of the right sexes. Difficulty number 3 can be overcome only through periodical interruption of inbreeding, which makes it practically impossible to obtain true-breeding strains for many characters. The increase in the proportion of males following inbreeding depends on the peculiar determination of sex in these copepods (BATTAGLIA 1964).

PHYSICAL ENVIRONMENT AND NATURAL SELECTION

It has been found that the genetic polymorphism exhibited by some species of *Tisbe* is adaptive and balanced, that is maintained by natural selection acting differentially on the various genotypes. This enables the populations to exploit several ecological niches.

One of these polymorphic species is *Tisbe reticulata,* whose various forms differ in distribution and color of the pigment in the hypodermal cells of the cephalothorax and of the free thoracic segments. At Roscoff (France), where the species was first found, seven major phenotypes and a few minor variants were described. In the population of the lagoon of Venice (Italy) the species is represented by three forms only, and most minor variants are completely absent. In the lagoon population the genetic basis of this polymorphism is also somewhat different (BATTAGLIA 1958). At Roscoff – and it seems also at Plymouth (England) – polymorphism is under the control of a few genes, some of which are independent, whereas at Venice, polymorphism is more simply controlled by a series of alleles of the same locus. Moreover, in contrast with the Atlantic population, most genotypes present in the lagoon can also be identified in males.

We found experimental evidence that certain ecological parameters, such as salinity and temperature, act as powerful selective agents on this polymorphism which, therefore, has an adaptive meaning. Temperature, particularly, has a strong influence in this respect. In fact, in *Tisbe reticulata* it has been shown that differences in the water temperature are responsible for differences in the equilibrium frequencies of the genes which control polymorphism. This applies both to natural and to experimental populations (BATTAGLIA & LAZZARETTO 1967).

In another non-polymorphic species, which so far has been classified as *Tisbe furcata* but whose taxonomical position has to be thoroughly revised in the light of certain recent findings, we have dealt with the problem of the "concealed genetic variability". This is a kind of variability which, though not visibly manifested (as in the case of polymorphism) might, nevertheless, have an adaptive meaning in the sense that it could enable the species to survive in a multiplicity of environments.

The species in question lives in typical marine habitats as well as in marginal environments such as brackish waters. In a first series of experiments, carried out with a population from Banyuls-sur-Mer (France) and a population from Sigean (a brackish water basin not far from Banyuls) we confined our attention to the analysis of the

Table 1

Tisbe furcata. Effect of inbreeding on some population parameters in two populations of different ecological origin

Population Origin	Generation	Inbreeding coefficient (F)	Total nauplii	Survival	Net reproductive rate (R_0)
Banyuls (marine)	Control	.000	19,354	.932	340
	F_1	.250	9,170	.460	54
	F_2	.375	5,384	.432	26
Sigean (brackish water)	Control	.000	19,039	.947	297
	F_1	.250	10,571	.619	87
	F_2	.375	9,279	.520	45

effects of inbreeding on certain important population parameters. The first results are summarized in Table 1. They show that inbreeding has a significantly more severe effect on the marine population than on the brackish water population, and indirectly indicate that the former has a larger number of harmful recessive genes. Although it would be premature to speculate on the meaning of these findings, it is legitimate to assume that the differences in "genetic load" between the two populations compared are due to the ecological differences between the two environments from which the populations come.

The method reported above has proved to be quite suitable for also detecting, in monomorphic species, the effects of environmental factors on the genetic structure of populations.

BIOTIC SELECTIVE FACTORS

The experiments reported so far show how effective the physical factors of natural selection are in species such as *Tisbe reticulata* and *T. furcata.* In another polymorphic species, *Tisbe clodiensis* (BATTAGLIA & FAVA 1968), the equilibrium values of the genes controlling polymorphism seem to depend to a considerable extent on the initial composition of the population; whereas, the effect of factors such as salinity or temperature is less appreciable.

Tisbe clodiensis exhibits a polychromatism which is under the control of two alleles of the same gene. In order to compare the adaptive values of the three resulting genotypes, certain parameters – believed to be significant for the evaluation of fitness – have been measured (LAZZARETTO-COLOMBERA & POLO 1969). The parameters estimated were: (1) survival from naupliar stage to beginning of the reproductive stage; (2) net reproductive rate; (3) minimum and mean generation time; (4) intrinsic rate of natural increase (r_m). The results show that the adaptive value of the heterozygotes is superior to those of both homozygotes. Consequently, polymorphism is balanced in *Tisbe clodiensis* as well as in *Tisbe reticulata,* and heterosis plays an important role in its maintenance. Moreover, the fact that equilibria are reached suggests that also in *T. clodiensis* polymorphism has an adaptive meaning; but, in this species, the more effective selective factors seem to be biotic rather than physical. The possibility that such a hypothesis has a certain validity rests on the consideration that, in a biological association, an important parameter of the environment consists in factors such as the number and kind of the other community components. Therefore, an experiment has been devised with the purpose of testing if, and to what extent, the genetic structure of populations of a marine species can be influenced by the biotic factors of the environment.

The experiment has been carried out by raising in the same culture vessels two species of *Tisbe,* namely *T. clodiensis* and *T. reticulata,* which, both being polymorphic, can visibly show the genetic effects of co-existence. It was found that, whereas in *Tisbe reticulata* the gene frequencies at equilibrium seem to be unaffected by the presence of *T. clodiensis,* the equilibrium values in the latter are significantly different, according to the presence or absence of *T. reticulata* in the same culture vessel (Tab. 2) (BATTAGLIA & FINCO 1969). To date, no information is available on

Table 2

Frequency of the allele *p* (lack of coloured pigment) in experimental populations of *Tisbe clodiensis,* with and without *Tisbe reticulata* in the same cultures. Initial frequency = .50

Sampling	Monomorphic population	*T. clodiensis* + *T. reticulata*
1st after 40 days	.88	.59
2nd after 60 days	.86	.69
3rd after 80 days	.84	.49
4th after 100 days	.81	.60
5th after 120 days	.75	.65
6th after 140 days	.76	.69
7th after 160 days	.81	.65
8th after 180 days	.78	.61

the mechanism by which a species competing with another can modify the genetic composition of the latter at equilibrium. Perhaps interactions of a chemical nature are involved, but further research is required to throw some light on this interesting aspect of the problem.

These experiments can be considered a first approach to a problem whose implications relative to the establishment and evolution of marine communities are obvious.

REPRODUCTIVE ISOLATION AND SPECIATION IN TISBIDAE

Another line of research which we have been able to follow by utilizing laboratory cultures, concerns the study of reproductive isolation and speciation in Tisbidae. The opportunity of carrying on research of this kind was suggested mainly by the severe difficulties exhibited by the taxonomy of this group of copepods. This is, in part, due to the existence of several morphologically very similar species of *Tisbe* which, in some cases, represent real sibling species. As to the reproductive isolation, we have been able to show in Tisbidae a wide range of different conditions. The more relevant cases may be summarized as follows:

(1) *Tisbe furcata.* In this species there is a complete interfecundity between the various Mediterranean populations. Moreover, certain Atlantic and Mediterranean populations are not only interfertile but often the F_1 hybrids are heterotic (Battaglia 1967).

(2) *Tisbe reticulata.* Geographically remote populations of this species (Roscoff, France, and Chioggia, Lagoon of Venice, Italy) are interfertile, but there is evidence of an incipient reproductive isolation (lowered fecundity and viability of F_1 hybrids; strong deviations of sex ratio in favour of males) (Battaglia 1957).

(3) *Tisbe clodiensis.* Several Mediterranean populations of this species are available in our laboratory, and the following crosses have been carried out: Malta × Leghorn, Malta × Chioggia, Banyuls × Chioggia, Leghorn × Chioggia, Anzio × Chioggia, Anzio × Leghorn, Leghorn × Banyuls. Of each type of cross the reciprocal has also been made. By comparing the results of the various crosses, it has been possible to detect a certain graduality in their success (Battaglia & Volkmann-Rocco 1969). In fact, the conditions range from perfectly interfertile to practically intersterile populations; in this respect distance and discontinuous distribution seem to play a determining role. The more significant result is in the outcome of the crosses, which may be fertile if the cross is carried out in one direction, completely or partly sterile if it is carried out in the opposite direction.

Another case of such a "relative intraspecific incompatibility" is found in the copepod *Tigriopus fulvus* from the Mediterranean Sea. In this species, Božić (1960) has demonstrated a relationship between the cross incompatibility of various geographic populations and sex; moreover, the presence of a remarkable morphological heterogeneity with a continuous distribution has led this author to consider the different populations as various forms of the same species arranged along a cline.

Although reproductive isolation in *Tisbe* species is usually accompanied by morphological or physiological differentiation, there are cases of phenotypically recognizable genetic divergencies without reproductive isolation, or populations between which, in spite of the lack of appreciable phenotypical differences, the reproductive barrier is complete. This is, for instance, the case in the sibling species *T. reluctans* and *T. persimilis* (Volkmann-Rocco & Fava 1969), which are morphologically almost identical but completely intersterile.

In *Tisbe clodiensis,* in contrast to *Tigriopus fulvus,* there is no appreciable differentiation between the various Mediterranean populations, and the underlying mechanism responsible for the reproductive incompatibility is still obscure. One might suggest as a possible explanation of the non-reciprocal crossability, a cytoplasmic difference between the various strains, as in the case investigated by LAVEN (1959) in *Culex pipiens*. We hope that the matter will be elucidated by the results of research now in progress.

In his research on *Tigriopus*, BOŽIĆ (1960) has also studied the Northern species *T. brevicornis* whose geographic populations, compared to those of the Mediterranean species, are much less heterogeneous and perfectly interfertile. The case of *Tigriopus* poses the problem of the factors responsible for the greater isolation existing between the Mediterranean populations. On the other hand, the copepods belonging to this genus, because of their extremely peculiar habitat (tide pools), cannot provide a model of speciation which can be applied to other less specialized marine organisms. The Tisbidae, and especially *Tisbe clodiensis* and related forms, are certainly a more promising material in this respect.

In conclusion, I hope that the problems reviewed have illustrated that the cultivation of these copepods in the laboratory, because of their great suitability for the application of genetic tests, may disclose new perspectives in the study of adaptation and evolution in the sea.

SUMMARY

1. Methods are reported for the cultivation of harpacticoid copepods in the laboratory and their utilization for genetic research.
2. Experimental evidence has been obtained that certain ecological parameters, such as salinity and temperature, act as powerful selective agents. This has been demonstrated by considering the effects of the physical environment on the visible polymorphism as well as on the concealed genetic variability.
3. The first results of a series of experiments show that biotic factors can play a selective role which is at least as much effective as that played by physical factors.
4. Another line of investigation followed consists in the study of reproductive isolation between geographic populations of the same species.

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