

Use of salinity tolerance data for investigation of phylogeny of *Paramecium* (Ciliophora, Peniculia)

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Summary

The purpose of our research was to test the hypothesis concerning the possibility to use tolerance polygons method in the taxonomy of ciliates. 13 species of *Paramecium* were used in the experiments. Resemblance of tolerance polygons of investigated species was estimated. Template of resemblance was clustered through the weighed pair-group method. There are 3 clusters in the graph of tolerant polygons and potential tolerance resemblance. The largest, which includes 7 species (*P. caudatum*, *P. jenningsi*, *P. multimicronucleatum*, *P. primaurelia*, *P. biaurelia*, *P. sexaurelia*, *P. polycaryum*) coincides with «aurelia» group. The small cluster that consists of 2 species - *P. putrinum* and *P. bursaria* corresponds to Jankowski's subgenus Helianter. Euryhaline species corresponding to «woodruffi» group - *P. calkinsi*, *P. duboscqui*, *P. nephridiatum* and *P. woodruffi* form the third cluster.

Key words: *Paramecium*, potential tolerance, tolerance polygon, salinity

Introduction

Use of ecological characteristics of species in systematics has some difficulties. First, comparison of ecological features of species it is possible only in case it is made according to one plan for all samples. Therefore, there is a problem of a choice uniform for all species «systems of co-ordinates». Secondly, the chosen system of co-ordinates should be a rather stable ecological characteristic. Besides there is always a danger that the grouping made according to ecological features will result the outlining of ecological groups not having systematic importance. We offer to use 2 interconnected approaches, which seem to allow a successful comparison of the ecological characteristics of species.

The term of potential tolerant range is connected with the essence of acclimation idea, which assumes that tolerance range depends on previous salinity conditions (existence of experimental matter conditions). The tolerant range and the values of limits can

be reversible in the acclimation process. As the result of consistent acclimation to increasing factor the organism will be capable of surviving in a wide range than initially. Continuing acclimation in the direction of reduction or increasing one can obtain the value of salinity when further acclimation is impossible. The potential tolerance does not depend on conditions of habitation or preliminary acclimation and is a stable ecophysiological characteristic of species.

The using of tolerance polygons for the characteristic of species relation to ecological factors was offered for the first time in 1942 (Fry et al., 1942). To build them up, it is necessary to demonstrate graphic dependence of the upper and lower tolerance limits from conditions of acclimation. Polygon of tolerance, as a rule, has the form of trapezoid or parallelogram. Similar graphics form was obtained both in researches of temperature tolerance (Fry et al., 1946; Hart, 1952; McLeese, 1956) and salinity tolerance (Khlebovich and Kondratenkov, 1973; Smurov, 2000) of hydrobionts.

Table 1. *Paramecium* species which were used in experiments.

Species	Stock	Place of the stock origin.	Date
<i>P. caudatum</i>	KAR98-1	Russia, White Sea	1998
<i>P. sexaurelia</i>	Psex-3	Germany, Stuttgart	1995
<i>P. calkinsi</i>	OCE4-1	Russia, White Sea	1994
<i>P. nephridiatum</i>	I1-5-4	Jerusalem Zoo, Israel	1997
<i>P. bursaria</i>	PK-60	Russia, Stary Peterhof	1994
<i>P. putrinum</i>	PG-5	Russia, Leningrad region, Gatchina	1995
<i>P. multimicronucleatum</i>	JYA3-3	Japan, Jamaguchi	1999
<i>P. jenningsi</i>	IA	India, Bangalor	1958
<i>P. primaurelia</i>	HV15-2	Vietnam, Hanoi	1994
<i>P. biaurelia</i>	T10-5	Russia, Ircutsk region	1989
<i>P. polycaryum</i>	Sh2-4	China, Shanghai	1999
	Pp5-5	Russia, Toliatti	1988
	K-2	Ukraina, Crime	1996
<i>P. woodruffi</i>	BB-5	Russia, Baltic Sea, Gulf of Finland	1995
<i>P. duboscqui</i>	AHW3-3	USA, Woods Holl	1997

Plot for the particular factor has “stability”, preserving the form even in the case of interaction of several factors (McLeese, 1956). Plot of tolerance has two important features. First, the change of tolerance limits relatively to the change of salinity during acclimation will be approximately linear or hyperbolic. Therefore, it is easy to calculate the square of tolerance polygon. Second, the isoosmotic line ($x=y$) will be diagonal of this polygon of tolerance. The second feature is a property of the graph itself: the lower and upper limits of the tolerance range correspond to minimal and maximal salinities to which acclimation is possible (Filippov, 1998). In fact, plot of tolerance does not only show the zone of potential tolerance of species (projection of diagonal of polygon on horizontal axis), but also showed the dependence of tolerance on acclimation conditions. Salinity tolerance polygon is determined by genotype of species in particular degree. Therefore it seems possible to use salinity tolerance polygon in taxonomic analysis. It is easier to estimate resemblance of species tolerance polygons using all known quotients of similarity.

The purpose of our research was to test the hypothesis concerning the possibility to use tolerance polygons method in the taxonomy of ciliates. 13 species of *Paramecium* were used in the experiments. The choice of objects was dictated by the availability of a unique collection the living cultures containing almost all valid morphological species of paramecia, and by the existence of systematic paramecia studies which were made on the same species with the use of other procedures. Last circumstance enables possibility to

compare our results with the data on morphological and molecular-biological analyses (Fokin and Chivilev, 1999, 2000; Struder-Kypke et al., 2000).

Material and Methods

Tolerance of paramecia species acclimated to the media of various salinities on the basis of marine salt was investigated. 13 morphological species were used in the experiments (see Table 1).

Cells were cultivated on lettuce medium inoculated with *Enterobacter aerogenes* (Sonneborn, 1970). The necessary salinity was obtained using artificial marine water (Chubravyi, 1983), diluted with lettuce medium. Before the experiment, the ciliates were maintained in freshwater or marine lettuce medium for not less than two months. The ciliate cultures were fed twice a week.

Salinity tolerance polygon is a graph of dependence of upper and lower tolerance limit on salinity acclimation. To construct tolerance polygon it was necessary to obtain the values of tolerance limits for several values of salinity acclimation.

Determination of tolerant limits is based on a technique (Smurov and Fokin, 1998) which takes into account certain features of protists biology. For most unicellular organisms one could say, «dividing therefore existing», analogous to «thinking therefore existing». Therefore we propose to regard the value of lethal salinity not for individual cells but for cells population as tolerant limit. In this case we take into account the fact that individual cells subjected to salinity impact can

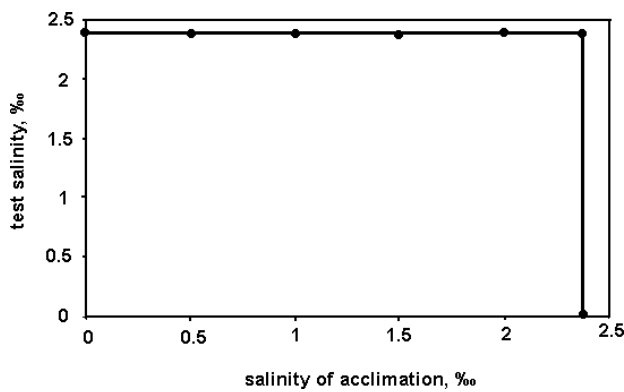


Fig. 1. Salinity tolerance polygon of *P. putrinum*.

survive and exist for a long time but cannot produce viable posterity. This method was used to investigate all species.

The approach to the data obtained for construction of tolerance polygon consists in acclimation to several salinities inside the tolerance range. After finishing acclimation (about 1.5 months during each experiment) to all values of salinity acclimation the tolerance limits were determined. Cultures of paramecia acclimated to salinity range outside the initial tolerant limits were obtained with the help of alteration of tolerance limits as a result of acclimation. New cultures were again tested. The procedure was iterated up to achievement of potential tolerance limits. As a result, a detailed graph of dependence of tolerance limits values (upper and lower) on salinity acclimation (salinity tolerant polygon) were constructed for each species.

Resemblance of tolerance polygons was estimated using the formula: $2C / (A+B)$, where C - value of common square of tolerance polygons, A - square of the first polygon, B - square of the second polygon. Resemblance of potential tolerance ranges was estimated analogously. Template of resemblance was clustered through the weighed pair-group method.

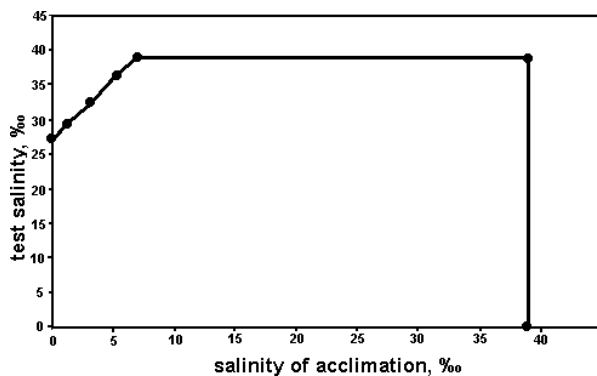


Fig. 2. Salinity tolerance polygon of *P. bursaria*.

Results

Salinity tolerance polygons of all investigated species had a usual form corresponding to salinity tolerant polygons of multicellular hydrobionts. As the most important characteristics of polygon we considered: value of the top tolerant limit at acclimation to freshwater lettuce medium (initial ordinate); the existence of the lower tolerant limit different from 0 (zero according to freshwater mineralisation); scope of potential salinity tolerance zone; square of tolerance polygon; scope of zone, in which the value of the upper tolerant limit at changing the salinity of acclimation increases.

P. putrinum and *P. bursaria* demonstrate less square of the tolerance salinity polygons. These species cannot be adapted to salinity above 2-3 ‰ (Fig. 1, 2). *P. putrinum* cannot be acclimated at all. *P. bursaria* can be acclimated in the range from fresh water to 0.5 ‰.

P. caudatum, *P. multimicronucleatum* and *P. jenningsi* have greatest square of the tolerance salinity polygons. The initial ordinate of these species has the range 3-4.5 ‰. It was acclimated to salinity 6.5-8.5 ‰ (Fig. 3,4,5).

The species of aurelia complex has the same initial ordinates as the 3 species mentioned above, but the square of the tolerance salinity polygons was greater. The species of aurelia complex can be acclimated to salinity 12-15 ‰ (Fig. 6,7,8). The tolerance salinity polygon of the *P. polycaryum* has almost the same features as the polygons of the aurelia complex (Fig. 9).

The euryhaline paramecia species has the greatest square of the tolerance polygon (Fig. 10, 11, 12, 13). The initial ordinate greatly exceeded of critical salinity value. The scope of zone, in which the value of the upper tolerant limit at changing the salinity of acclimation increases, is almost the same for other paramecia species.

There are 3 clusters in the graph of tolerant polygon resemblance (Fig. 14). The largest includes 7 species (*P. caudatum*, *P. jenningsi*, *P. multimicronucleatum*, *P. pri-*

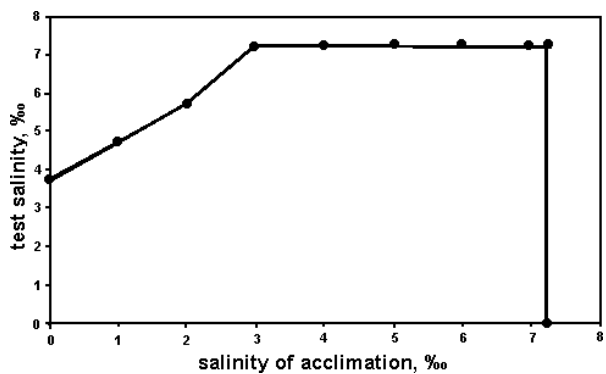


Fig. 3. Salinity tolerance polygon of *P. caudatum*.

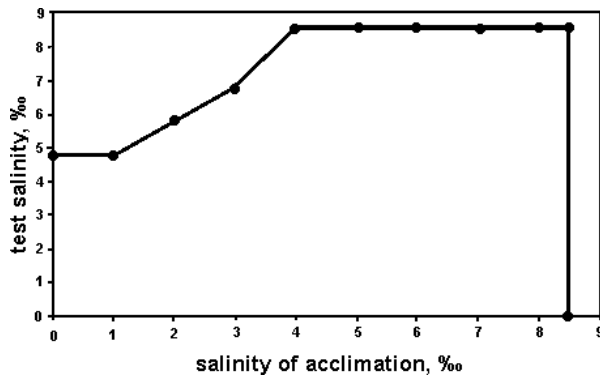


Fig. 4. Salinity tolerance polygon of *P. multimicronucleatum*.

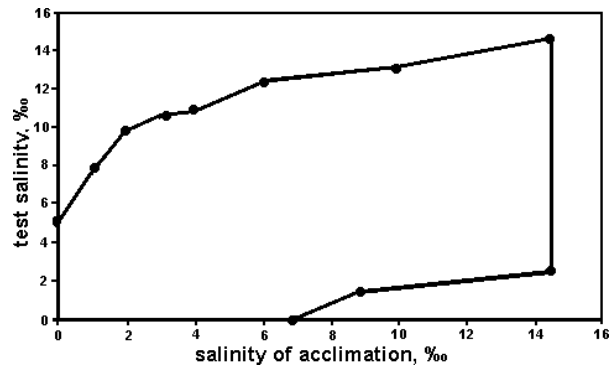


Fig. 6. Salinity tolerance polygon of *P. primaurelia*.

maurelia, *P. biaurelia*, *P. sexaurelia*, *P. polycaryum*). The small cluster includes of 2 species - *P. putrinum* and *P. bursaria*. Euryhaline species - *P. calkinsi*, *P. duboscqui*, *P. nephridiatum* and *P. woodruffi* have derivated the third cluster. The graph of resemblance of potential tolerance ranges has same 3 clusters (Fig. 15), but details are distinguished.

The large clade of potential salinity diagram consists from 2 subclade. In the first subclade includes species of aurelia complex and *P. polycaryum*. The second subclade includes *P. caudatum*, *P. jenningsi* and *P. multimicronucleatum*. In the euryhaline species group *P. duboscqui* united with *P. nephridiatum*, *P. calkinsi* united with *P. woodruffi*.

The diagram of resemblance of potential salinity polygons differed from the diagram of resemblance of potential tolerance ranges predominantly by the branching of the large clade.

Discussion

Various authors distinguish two species groups inside *Paramecium*, which have morphological features clearly differing: «aurelia» and «bursaria» (Woodruff,

1921; Wichterman, 1986). Jankowski (1969, 1972) recognised three such groups: «putrinum», «woodruffi» and «aurelia». This author regarded them as taxonomic subgenera Helianter, Cypreostoma and Paramecium. Studies carried out during last years using both molecular-biological methods (Struder-Kypke et al., 2000) and morphometric analysis (Fokin and Chivilev, 1999, 2000), have confirmed the existence of at least 2 clusters of species, which corresponds to «woodruffi» and «aurelia» groups. According to molecular-biological analysis (Struder-Kypke et al, 2000) aurelia species group is a monophyletic cluster. The species of this group have high morphological similarity. *P. jenningsi* is grouped at the base of the branch leading to the aurelia complex. *P. nephridiatum*, *P. woodruffi* and *P. calkinsi* seem to form a monophyletic cluster like the species of the aurelia group. *P. polycaryum* cannot be assigned to the aurelia group, but is now affiliated with «woodruffi» group. The final clearness in taxonomic position of some species inside the genera does not exist. There are 3 species - *P. duboscqui*, *P. putrinum* and *P. bursaria*, which it is impossible to refer to any species groups.

Morphometric analysis (Fokin and Chivilev, 1999, 2000) has shown that *P. duboscqui* is more primitive

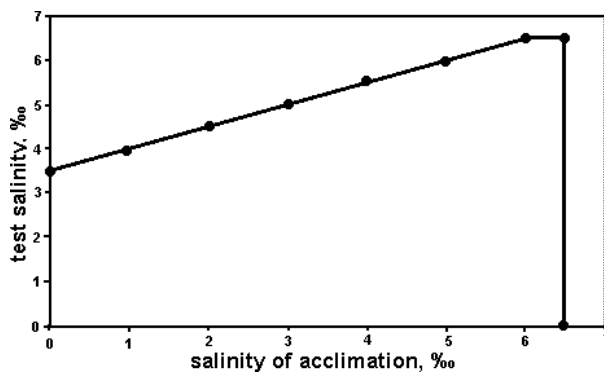


Fig. 5. Salinity tolerance polygon of *P. jenningsi*.

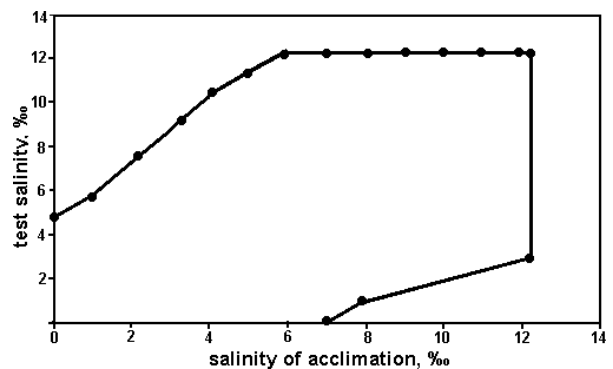


Fig. 7. Salinity tolerance polygon of *P. biaurelia*.

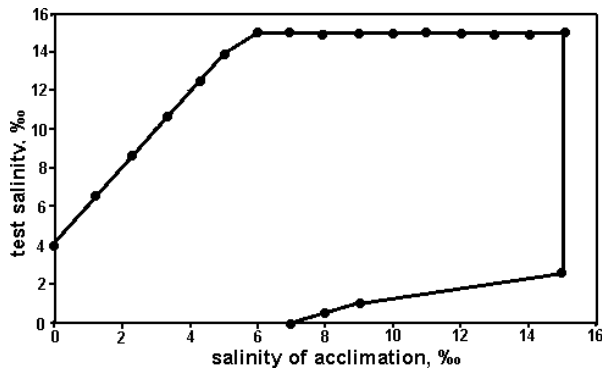


Fig. 8. Salinity tolerance polygon of *P. sexaurelia*.

than species of «woodruffi» group - *P. nephridiatum*, *P. woodruffi* and *P. calkinsi*. *P. polycaryum* can be united with «woodruffi» group to a greater extent than *P. dubosqui*. *P. putrinum* and *P. bursaria* according to morphometric analysis do not fall into any cluster.

According to our results the largest cluster, which includes 7 species, corresponds to with «aurelia» group. The small cluster, which consists of 2 species - *P. putrinum* and *P. bursaria*, corresponds to Jankowski's (1972) subgenus *Helianter*. According to morphological and molecular-biological data, these species cluster do not exist. However analysis of sequence of gene SSrRNA has shown that they represent the most ancient parametia (Struder-Kypke et al., 2000). Euryhaline species corresponding to «woodruffi» group form the third cluster. On the to whole, it is possible to conclude that the results obtained to a greater extent correspond Jankowski's system.

On the basis of the results obtained during analysis of SSrRNA sequences of a gene, morphometric analysis, and our data it is possible to see that the aurelia species combined into subclade coupled with such species as *P. caudatum*, *P. jenningsi* and *P. multimicronucleatum*. It is believed that aurelia species were descended from the forms similar to the three species

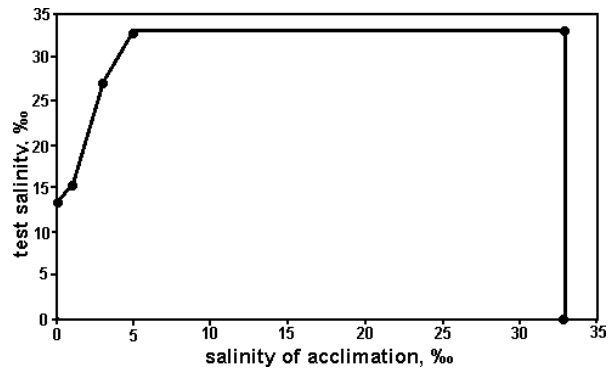


Fig. 10. Salinity tolerance polygon of *P. calkinsi*.

above mentioned, which can be considered more primitive than aurelia species. *P. caudatum*, *P. jenningsi* and *P. multimicronucleatum* are typical freshwater species that exist within the limits up to approximately 8 ‰. Aurelia species are more euryhaline in comparison with these species and are capable of existing at salinity up to 12-15 ‰.

One of the most ancient species in the genus is *P. bursaria* and *P. putrinum*. They demonstrate significant specialisation according to many attributes, including relation to salinity factor. They can exist at salinities no more than 2-3 ‰. It is logical to believe that a similar specialisation is possible at a parentage from the ancestors capable of existing in limits from freshwater up to 8 ‰. Therefore this cluster of species was generated in freshwater localities.

The discrepancy of morphometric results and analysis of SSrRNA sequences of the gene on the one hand, and analysis of adaptive opportunities of the lowest parametia to the salinity factor on the other, can be explained by non-synchronous evolution of different cell characters. So, for example, according to SSrRNA structure of a gene genetical distance between *P. bursaria* and *P. aurelia*, which have similar morphology, appears as large as between the representatives of suborders Tetrahymenina and Ophryoglenina, which

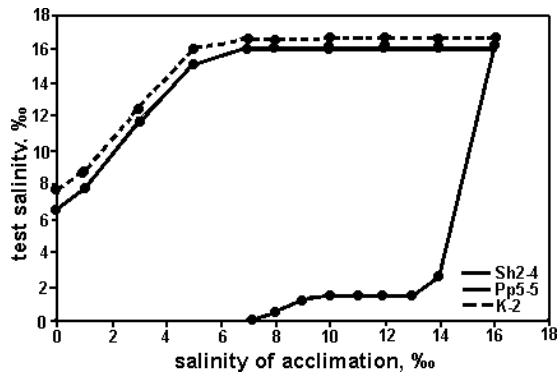


Fig. 9. Salinity tolerance polygon of *P. polycaryum*.

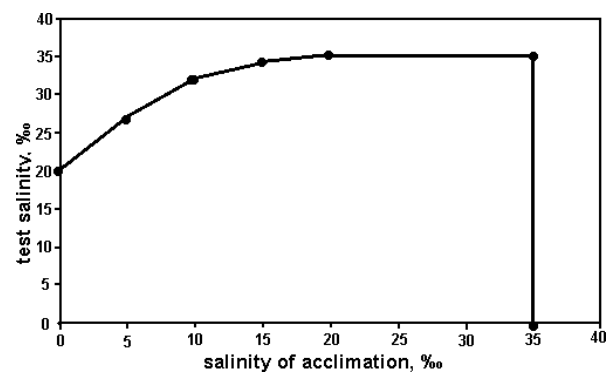


Fig. 11. Salinity tolerance polygon of *P. woodruffi*.

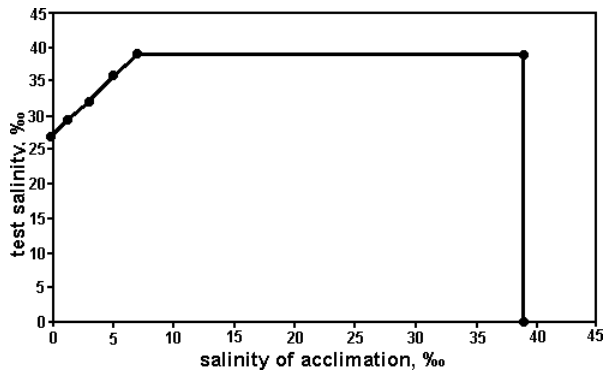


Fig. 12. Salinity tolerance polygon of *P. duboscqui*.

morphologically differ very strongly. The dendrogram constructed for the representatives of “woodruffi” bunch on the basis of DNP pattern shows significant difference *P. nephridiatum* from other euryhaline paramecia species. This fact does not find confirmation by other methods of analysis of resemblance of these species. It is indicated that the divergence of this character in paramecia species took place differently from other characters.

The problem of *Paramecium* origin is complicated by the presence of euryhaline species, which can exist both in freshwater and in seawater. We can conclude that most of paramecia species evolved in freshwater.

Thus, our data are similar to the data obtained by other methods and confirm the possibility of salinity tolerance polygons used in systematic investigations of water organisms, at least for low rank taxa. Now the problem is whether the data obtained by our procedure will be adequate for systematic of higher rank taxa?

The modern investigators have shown the existence of groups, well distinguished by potential salinity tolerance, within types, classes and sometimes even families (Aladin, 1996). It is interesting that the tolerance gradation of these groups is

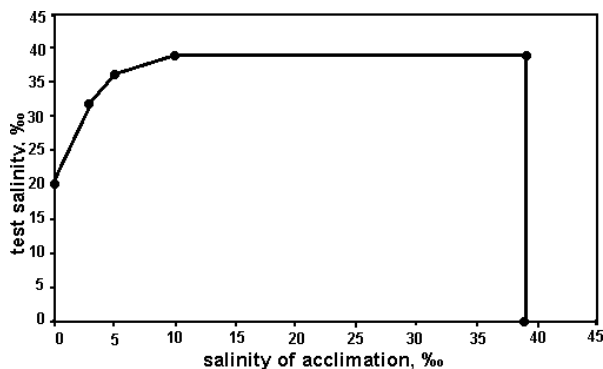


Fig. 13. Salinity tolerance polygon of *P. nephridiatum*.

practically the same in different phyla of multi-cellular organisms. Therefore «the rise of rank» when using our procedure on ciliates will most likely result in outlining of groups which have no systematic status but demonstrate the main stages of salinity adaptive evolution of organisms.

The results obtained allow observing some ecological groupings of species, which can exist in freshwater. In case these groups are the same as for the multi-cellular hydrobionts, than combined analysis of phylogenetic relations within *Paramecium* and ecological species groups in relation to salinity within the genus will demonstrate the ways of their origination.

There are at least 4 such ecological group. The central group includes species capable of existing in a range from freshwater up to approximately 6-8‰. The group which can be called true freshwater species (stenohaline freshwater species according to Zenkevich, 1961) originated from the group above mentioned. These species can exist at salinity not exceeding 2.5 - 3‰, and accordingly can regulate internal osmolarity in the same range. An analogous ecological group exists in molluscs.

Besides, they are capable of long, but limited (about 2-2.5 weeks) time of existence in salinities 3-3.5‰. Within the limits of potential salinity tolerance their direct transfer without preliminary acclimation is possible. It might be that there is a group among ciliates which includes, except 2 paramecia species (*P. putrinum* and *P. bursaria*), a large number of the usual forms, for example species of *Spirostomum* and *Loxodes*. These forms exist in basins with high level of common mineralisation (2-3 g/l) in which absolute contents of Na and Cl ions is insignificant. It is a feature, which is connected with the regulation of internal concentration Ca and K.

The most interesting group is formed by the numerous species of aurelia complex and *P. polycaryum*. These species can exist in considerably more salty waters up to 12 - 16‰. The left part of their tolerant polygons approximately corresponds to the tolerant polygons of the central group, the right part can be considered a new formation. It does not give any special benefits and actually only enables to survive at salinity above 8-9‰, as the rate of cell division sharply decreases. An analogous ecological group which can exist in range from freshwater up to 12 - 18‰ was found in ostracods (Aladin, 1996).

The final group includes forms, which can be called true euryhaline. They can stand direct transfer to freshwater from upper limit of potential salinity tolerance. The upper limit of potential salinity tolerance is almost equal to ocean salinity.

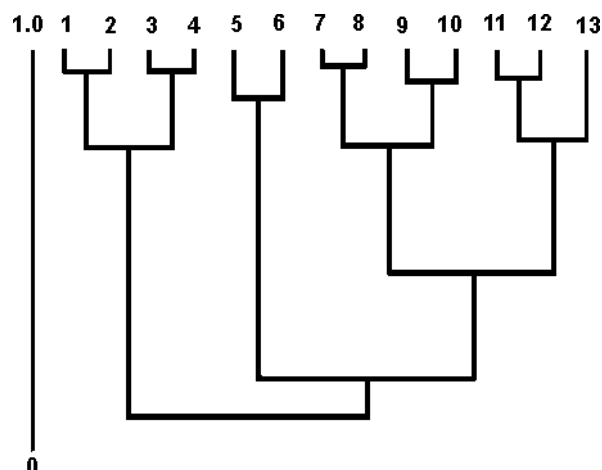


Fig. 14. The diagram of resemblance of salinity tolerance polygons of *Paramecium* species. 1 - *P. nephridiatum*, 2 - *P. duboscqui*, 3 - *P. woodruffi*, 4 - *P. calkinsi*, 5 - *P. putrinum*, 6 - *P. bursaria*, 7 - *P. primaurelia*, 8 - *P. biaurelia*, 9 - *P. sexaurelia*, 10 - *P. polycaryum*, 11 - *P. caudatum*, 12 - *P. multimicronucleatum*, 13 - *P. jenningsi*.

The concept of ecological groups is of great importance for creation of a classification of eukaryotes attitude to salinity.

Many seas and continental reservoirs have varying salinity regime. At strong salinity variations it becomes a limiting factor, which limits the fauna of a different origin. At present the study of this phenomenon goes in three directions: experimental estimation of the scope of tolerant salinity ranges, research of types of osmotic relations of the internal medium of water organisms with environment, and analysis of information about salinity limits of organisms expansion in nature (Aladin, 1988). All three aspects are investigated well enough only in a small number of the representatives of different phyla and classes of water animals. However, the amount of information obtained by the investigators inside each direction is rather great enough.

The problem of salinity limits of expansion of hydrobionts is developed in most detail. The most successful places for such research are the reservoirs where salinity varies in a wide range. The classical place for such researches is the Baltic sea, which has a smooth salinity gradient from freshwater up to almost oceanic salinity. Remane (1934, 1940) and Valikangas (1926, 1933) were the first to solve this problem for this reservoir. The logic of researches in this direction necessarily results in an attempts of brackish water classification according to their fauna, or of classification of the fauna according to the salinity of water, where it exists (Khlebovich, 1974).

The attempts at classification were also made later (Dahl, 1956; Segestråle, 1959), including the

recent past (Williams, 1972, 1991; Williams et al., 1990). It should be noted that some authors have offered more than one system, which can be connected with relative insufficiency of all systems. In fact, the authors created systems for a certain reservoir or a group of reservoirs.

It is obvious that the distinctions between systems are substantially explained by interaction of the salinity factor with others, including biotical factors. The value of interaction of the factors in this case is well known (Filippov, 1995). The necessary logical addition to these systems should be knowledge of potential tolerance ranges, which will emphasize the effect of the salinity factor «in the pure state». As already shown, the analysis of potential tolerance ranges of species results in division of ecological groups. The analysis of potential tolerance ranges of species in connection with the types of their osmotic relations with the environment will make possible to create a classification of the relation of organisms to the salinity of the environment. This classification will unite the results obtained by researches of all three directions.

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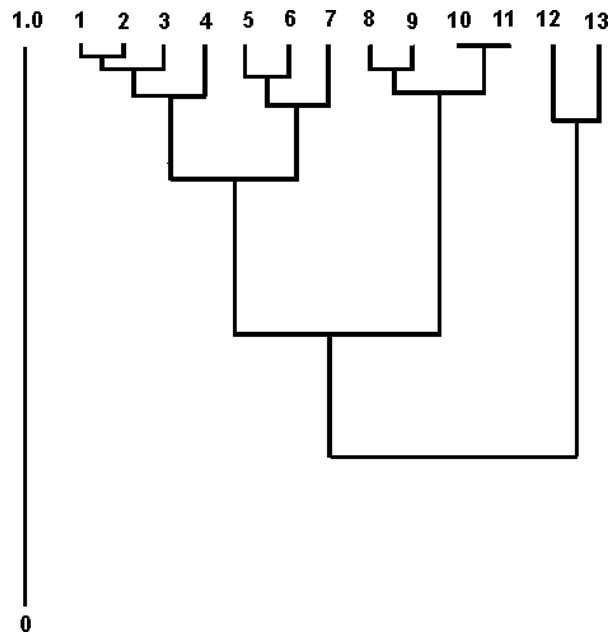


Fig. 15. The diagram of resemblance of salinity potential tolerance of *Paramecium* species. 1- *P. sexaurelia*, 2 - *P. polycaryum*, 3 - *P. primaurelia*, 4 - *P. biaurelia*, 5 - *P. caudatum*, 6 - *P. jenningsi*, 7- *P. multimicronucleatum* 8 - *P. woodruffi*, 9 - *P. calkinsi*, 10 - *P. nephridiatum*, 11 - *P. dubosqui*, 12 - *P. putrinum*, 13 - *P. bursaria*.

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