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THEORETICAL PAPERS AND REVIEWS

Analysis of Nucleotide Diversity at the Cytochrome *b* and Cytochrome Oxidase 1 Genes at the Population, Species, and Genus Levels

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Abstract—Algorithms of nucleotide diversity measures and other measures of genetic divergence at the molecular level are analyzed. Based on a database of *p*-distances, we have compared genetic divergence of populations (1) and taxa of different rank, such as sibling species (2), species within a genus (3), and species from different genera within a family (4). Based on the theory and algorithms of distance calculation from the primary DNA sequences, as well as the actual distances estimated from literature, it is recommended to use in analysis of experimental data a specific model selected from the eight available ones. The empirical data for more than 24 000 vertebrate and invertebrate species demonstrate that the data series are realistic and interpretable when *p*-distance or its various estimates are used. This testifies to the applicability of *p*-distance for most interspecies and intraspecies comparisons of genetic divergence up to the family level by two genes compared. Data on *p*-distances revealed various and increasing levels of genetic divergence of the sequences of genes *Cyt-b* and *Co-1* in four groups compared. Mean unweighted scores of distances for the four groups were as follows: *Cyt-b* (1) 1.55 ± 0.56, (2) 5.52 ± 1.34, (3) 10.69 ± 1.34, (4) 18.51 ± 2.09 and *Co-1* (1) 0.55 ± 0.19, (2) 4.91 ± 0.83, (3) 9.66 ± 0.72, (4) 14.69 ± 1.02. Differences in divergence between the genes themselves at the four levels were also found, although the total mean distances for the two genes did not show statistically significant differences. This conforms to the ample evidence showing different and nonuniform evolution rates of these and other genes and their various regions. The results of the analysis of the nucleotide and allozyme divergence within species and higher taxa of animals, first, are in a good agreement with these results, including data on protein gene markers, and, second, this evidence suggests that in animals, phyletic evolution is likely to prevail at the molecular level, and speciation mainly corresponds to the type D1 (geographic model). The prevalence of the D1 speciation mode does not mean that the other modes are absent. There are at least seven various modes of speciation. Recognition of speciation modes is a task that seems to require construction of a quantitative genetic model (theory) of speciation. Although, in view of a vast diversity of the possible causes of reproductive isolating barriers (RIBs) and speciation initiation, as well as the "empirical nature" of the formalized approach, proposed in the present work, some newly arising questions may be left without an answer. Their solution probably lied in increasing the number of descriptors and members of equations, proposed in this study, on the basis of DNA markers and other genomic characteristics.

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INTRODUCTION

The modern experimental and theoretical population genetics has acquired a strong impetus after the advent of molecular methods of analysis of genetic variation. On the other hand, investigation of the divergence of organisms in time, i.e., their evolution, on the basis of molecular gene markers must take into account fundamental genetic properties of the organisms and their groups, forming in nature such reproduction units, as populations and biological species. Thus, it seems logical to combine the issues of population genetics and molecular evolution. The temporal population genetic dynamics cannot be separated from the spatial population dynamics and understanding the bases of intraspecies genetic differentiation. Carried away by colossal possibilities of phylogenetic reconstructions inferred from the primary DNA sequences, some authors reject analysis of spatial divergence, opposing the phylogenetic concept of the species to the biological one [1, 2]. However, many geneticists are far from such extreme views, because they understand the common nature of a number of intraspecies and interspecies divergence mechanisms [3–8]. These, as well as many other, issues are considered in the present review, which is intended for geneticists of different specialties. Since the authors of the review are marine biologists, many examples are from the literature in this field, involving fish and marine invertebrates.

In the review, we have summarized and analyzed mainly the evidence on the proportion of different nucleotide substitutions in populations within species, as well as in taxa of various ranks, although there were earlier reviews on this topic [9–14]. Nevertheless, in current reviewing, we were motivated by two reasons: first, the information amount in this field rapidly increases and, second, the estimates for different genes had not been compared. In the last decade, mitochondrial genes for cytochrome *b* (*Cyt-b*) and cytochrome oxidase 1 (*Co-1*) have been most frequently used for taxonomic and phylogenetic analysis at the species–family level. These genes proved to be advantageous over other genes for estimating divergence in taxa up to the family level in many animal groups [13, 15–17]. A survey of the evidence on intraspecies divergence of mitochondrial genes in 256 vertebrate, mostly sexually reproducing species indicated that 56% of them form distinct intraspecies maternal lines, which typically are confined geographically [18]. Thus, the polytypic character or subdivision into groups of most species is documented by sources that are independent from other biological data and in good agreement with the latter.

In the present study, we do not examine heterozygosity and standardized variances at molecular genetic or protein markers, which have been repeatedly reviewed ([3–5, 9, 19–24] and other publications), though we briefly discuss these and related data. Also, we practically omit problems, related to construction and analysis of phylogenetic trees and a related issue of phylogenetics. This is a separate and extensive topic, the discussion of which the interested readers can find in literature [9, 14, 25–32].

The main objective of this study is considering the levels of molecular genetic divergence in animal populations and taxa of various ranks. For convenience, we refer to these categories as comparison groups. In connection with the main objective, the aims of the review are as follows: (1) comparing statistical algorithms for analysis of molecular variation and evolution; (2) comparing estimates of nucleotide divergence of proportion of different nucleotide substitutions per site (*p*-distance), as well as genetic distances (similarities) at allozyme (protein) markers and present a brief analysis of these data; and (3) shortly summarizing the views on the species in genetic terms and showing whether and how molecular genetic divergence is related to speciation.

Characteristics of the Data Sets Used

Primary nucleotide gene sequences. The conclusions of this review are mainly based on information from a database on *p*-distances of two genes, *Cyt-b* and *Co-1,* presented in the table (see Appendix). A considerable part of this table was obtained directly from *Cyt-b* [13] and *Co-1* [15, 16] gene sequences. Most sequences were retrieved by the authors of the cited works from GenBank, Release 103.0, 131. For genes *Cyt-b,* 2821 gene sequences were examined and for *Co-1,* 655 and 13 320 sequences; the sequences were analyzed in the sources [13, 15, 16]. Sequence length was taken as 200 bp for *Cyt-b* [13] and varied in different group comparisons from 619 to 669 bp for gene *Co-1* [15]. In each group compared, the *p*-distance was estimated (see Section 1.1). Our analysis consisted in computing and comparing the mean values, from which we formed our database, including in it many data from other sources (see table in the Appendix for references). The information was retrieved from the literature sources by means of the following three methods. (1) If the distance matrices were available, the arithmetic means were calculated directly, using each of the pairs one time relative to the other units of comparison: e.g., 1–2 and 1–3, but not 2–3 of the three possible pairs. This principle, which permits avoiding restriction of random choice, imposed by the matrix, was also employed earlier [13]. Hebert et al. [16] compared all possible pairs of $n(n-1)/2$, while in [15], the comparison principles utilized by the authors were different for different taxa compared. (2) When the distance matrixes were not available, we extrapolated the distances from the values presented on plots and dendrograms (this can be readily accomplished, using the scales of graphs and dendrograms). (3) In many works, the *p*-distances between the comparison groups required were directly presented. Note that virtually all values from [13] were computed from plots. This procedure inevitably entails some approximation. However, in view of very high intragroup (intrataxon) distance variance, these errors were insignificant for comparative group analysis. In addition to distances, we analyzed some other data on DNA markers.

Protein markers. These markers were also involved in analysis, though to a lesser extent. The priority in their choice was given to publications that presented sufficiently representative set of loci (as a rule, more than 18). For instance, for intraspecies data, the mean number of loci in samples was 24.7, and for interspecies data, 26.8. The number of animals in samples in intraspecies (population) studies was generally more than 50. In comparisons of taxa, the sample size typically exceeded 20. The main information contained in these tables was empirical series of similarity index estimates, beginning from populations within a species and ending with genera within a family. The numbers of analyzed taxa were 40 for subspecies, 23 for semispecies and sibling species, 265 for species, and 56 for genera (xls files are available on request).

The literature data were screened using Tompson Institute Scientific Information, Science Citation Index, SCI data base. Articles of 1995 through 2005 were examined. Our work also included analysis and obtaining analytical expressions for the statistics used. Since this part of the work is related to examination of factual data on molecular variation, but not essentially, it is briefly outlined in Section 1.1. Statistical analysis was performed using the STATISTICA software package [33]. From this package, we employed the basic mod-

ule for calculating mean and variance parameters, as well as those for parametric analysis of variance (ANOVA) and Kruskall–Wallis nonparametric analysis of variance.

1. INTRASPECIESAND INTERSPECIES DNA VARIATION

The biological species (BSC) concept implies that a species is an isolated reproductive community. Molecular data, especially pertaining to mitochondrial DNA (mtDNA) show that, on the one hand, natural hybridization between species often leads to introgression of genes from one gene pool to the other one. On the other hand, comparison of variability of DNA markers and divergence of taxa of various levels show that genetic differentiation increases with the rank of the taxon, according to the data on nucleotide sequences of individual genes [13, 15]. Hence, we believe it is expedient to compare the data on nucleotide divergence for several genes and from several data sources and, in addition, to substantiate the variation parameters used and present their analytical expressions. The latter is important for understanding the essence of estimating divergence at the DNA level.

1.1. Polymorphism of DNA Sequences. Nucleotide Diversity

From the viewpoint of genetics, understanding DNA sequence polymorphism as a result of nucleotide substitution is of primary interest. What items and how should be measured? If the nucleotide sequence for a particular set of loci or alleles in a population sample is known, DNA polymorphism can be assessed in a several ways. The basic measures of DNA polymorphism are nucleotide diversity and the proportion of nucleotide differences per site, *p*-distance as *P* or its estimate $\hat{p}$ [7, 29]: $\hat{p} = n_d/n$, where n_d is the number of nucleotides differing between the DNA sequences *X* and *Y*, and *n* is the total number of analyzed nucleotides. To understand the essence of the process of substitution, an appropriate mathematical model should be used. We have analyzed the substantiation of the measures proper, their analytic expressions (models), and the variance estimates in detail on the basis of four sources [7, 11, 29, 32]. Two popular models are Jukes–Cantor's and two-parameter Kimura's, which assume equal substitution rates for all nucleotides and different proportions for transitional (α) and transversional (β) models. In all, at least eight such models are known: (1) Jukes–Cantor; (2) two-parameter Kimura, K2P; (3) Equal-input; (4) Tamura; (5) Hashigawa–Kishino–Yano, HKY; (6) Tamura–Nei, TrN; (7) General time reversible, GTR; and (8) Unrestricted.

In the Kimura (K2P) model, the equilibrium frequencies of all four nucleotides are 0.25. However, the proposed algorithms could be applied, irrespective of the initial frequencies [29a, 29, p. 38]. In this respect, this model is similar to that by Jukes–Cantor, and, as the latter, can be applied to a wider range of empirical data than the remaining six models. Note that in the Kimura model, $R = \alpha/2\beta$, but many authors and software program packages employ the ratio $k = \alpha/\beta$. This should be kept in mind to avoid unfortunate errors in comparisons. Furthermore, an examination of the model algorithms showed that a thoughtful choice of the model for data analysis is advisable. Sometimes it may be worthwhile to spend some additional time and select a more complex model of estimation of the nucleotide substitution number (nos. 3–8) instead of following the routine software option, leading to K2P, in order to get more correct results. However, we would like to note that in the more complicated models, a great number of parameters results in relatively higher standard errors in their estimation [29]. Note that in the analyzed array of studies, the authors most often (29% according to the table data, see Appendix) use the Kimura model, but sometimes employ simply an uncorrected $\hat{p}$ value. There are examples of using the HKY model [34–36], Tamura–Nei model [35, 37], and General time reversible model [35, 38]. A numerical simulation for an infinite number of nucleotides showed that if the substitution number is low (<20%), all models give close values ([29, Fig. 3.1]). However, as the substitution number and homoplasy increase, the $\hat{p}$ value is the first to be biased. Correction by means of the gamma-distribution is an important tool to correct measures in relation to non-uniform substitution rate in different sequence regions [11, 26, 29, 32]. The MODELTEST program (3.06 and later versions) [39] is widely used for selecting a model suitable for concrete empirical data. Valuable information on the properties of the models and their applicability to various data types is presented in [29–32]. Different options for computing *p*-distances are implemented in software packages PAUP* [40], MEGA2 (MEGA3 [41, 42]), and others. A helpful interface and various statistic possibilities, including those for analysis of DNA sequences and markers, are presented in the ARLEQUINE package [43]. A very good guide for phylogenetic analysis is given by Hall [30]. It is mainly intended for PAUP*, but also present in a popular form general principles of phylogenetic analysis of DNA variation.

1.2. Divergence at DNA Markers within Species and at Different Levels of the Taxonomical Hierarchy: Analysis of Empirical Data

Intraspecies differentiation at DNA markers. With exclusion of deletions and insertions, the most suitable measure of polymorphism at the nucleotide sequence level is nucleotide diversity, π [7]. However, examining large samples for population analysis often proves implausible because of nucleotide sequence analysis being labor-consuming. Typically, RFLPs are analyzed

for this purpose, and more recently, microsatellites, which permit to estimate allele frequencies and other differentiation measures, F_{st}, etc. However, these data are beyond the scope of the present review.

In spite of high labor costs, intraspecies nucleotide diversity has been studied in many species. Nei [7] presented a summary of these results for various mtDNA regions and nuclear genes (β-globin, alcohol dehydrogenase, histone H4, hemagglutinin, insulin, and two immunoglobulins) of human, monkeys, and other organisms (in total, nine species). Most of these estimates were obtained using restriction analysis. The nucleotide diversity varies from 0.002 to 0.019 in eukaryotes and is rather similar for mitochondrial and nuclear genes: mean $\hat{\pi} = 0.007$, i.e., 0.7% for both groups ([7, Table 10.6]; means in our recalculation). In two fish species, nucleotide diversity in a *Cyt-b* gene fragment $\pi = 0.59\%$ and $\pi = 0.08\%$ [44]. The intraspecies nucleotide diversity in the control mtDNA region in fish *Pterois miles* reaches $\pi = 1.9\%$ [45]. Latitudinal differences in nucleotide diversity were found in two copepod species. The subarctic species *Calanus finmarchicus* ($\pi = 0.37\%$, $SD = 0.26$) proved to have lower diversity that the species from temperate waters, *Nanocalanus minor* ($\pi = 0.50\%$, $SD = 0.32$) [46]. The *p*-distance (K2P) for a 600-bp *Co-1* gene sequence was estimated for 107 intraspecies groups of various species from five butterfly families (Lepidoptera: Arctidae, Geometridae, Noctuidae, Notodontidae, and Sphingidae) and shown to exhibit low variation [15]. For the mean values, the variation ranged from 0.17 to 0.36%. Recalculation for these groups produced the grand mean K2P = 0.25 ± 0.04% (here and in further text, ± is followed by standard error of mean). On average, the intrapopulation *p*-distances for genes *Cyt-b* and *Co-1*: $M = 1.55 \pm 0.56\%$ and $M = 0.55 \pm 0.19\%$, respectively (table, Appendix). A close value was obtained for a 2214-bp mtDNA fragment, treated with restriction endonuclease *Hin*dIII in five individuals of *Oncorhynchus mykiss* from different populations [47]: mean for $\hat{p} = 0.254 \pm 0.025\%$. These variation values, related to single nucleotides, show a vast potential of intraspecies variability, if they are recalculated for a relatively short gene, say, 1000 bp in size: $0.25 \times 1000 = 250$, or 250 variable sites per gene. In some cases, however, nucleotide diversity is fairly low. For instance, mtDNA of Indians from Venezuela is completely monomorphic [48]. Apparently, this population has recently passed through a bottleneck. Very low nucleotide diversity was recorded for *Co-1* in knidarias [16].

Even relatively small series of *p*-distances for two genes at the population level (see table, Appendix) show that intraspecies divergence is under a strong influence of common population-genetic factors, predominantly isolation (migration), population size, and, apparently, natural selection (some evidence on selection is considered below). The space limits and the aim of this article do not allow us to dwell on these issues. We only would like to note that mammal populations (the genus *Apodemus*) from main Japanese islands are less differentiated at *Cyt-b* (K2P = 0.96%) than populations from small islands (K2P = 1.54%), while geographically distant populations of another group of mammals (the genus *Martes*) have far longer distances (*TrN* = 3.2%), than the geographically close populations (*TrN* = 0.4%) (table, Appendix). Clearly, the reproduction system may also play here an important role. In the review, we focus on sexually cross-reproducing animals. However, among obligate hermaphrodites or parthenogenetic forms, nucleotide divergence between lines of different geographic distances within nominal species can reach high values. For instance, in freshwater crustaceans *Potamoneutes* $P = 1.5$–2.0% [49], and in *Artemia* $P = 3.8\%$ [50]. In such organisms, genetic differentiation may develop because of their isolation and relatively small effective population size *Ne* with the total population size of hundreds of millions [46].

Generally, mtDNA shows maternal inheritance and exists in form of haplotypes. Hence, *Ne* values for mtDNA are expected to be equal to one-fourth of the value obtained from nuclear gene variation, which must reduce the level of drift–mutation equilibrium, and, consequently, $\hat{\pi}$. However, the substitution rate is higher for mtDNA than for the nuclear genome. Recent estimates, reported for 14 to150 taxa of various insect groups, showed that even by the conservative estimates based on the Jukes–Cantor model, the ratio of *Co-1/EF-1*α (i.e., mitochondrial and nuclear genes) substitution rates varied from 1.9 ± 0.3 to 5.4 ± 1.7 [51]. The effects of these two compensating factors, probably, lead to a situation, when mean π values are nearly equal in mitochondrial and nuclear genomes.

Silent polymorphism. It is of interest to examine polymorphism that does not manifest phenotypically even at the simplest level of amino acid sequences in proteins. Ample literature exists on the subject, to which we address the reader for extensive information [7, 11, 14, 26, 52–54]. Here, we confine ourselves to discussing several simple issues. The neutrality theory predicts that this so-called silent polymorphism occurs more frequently than polymorphism implemented at the level of amino acid sequences, because silent mutations would undergo less strong selection, than non-silent ones. Conversely, if polymorphism is generally maintained by selection (in case of a negligibly small drift effect), silent polymorphism would be less frequent than non-silent polymorphism. One of the ways to test these assumptions is examining polymorphism at the first, second and third codon positions in structural genes, as well analyzing pseudogenes. Li et al. [55, 56] were among the first authors addressing these issues. In their studies of the myoglobin gene in comparison with four pseudogenes in human, mouse, rabbit, and goat, these authors have shown that (1) the nucleotide substitution rate in functional genes is the highest at the third codon position and (2) the nucle-

otide substitution rate in pseudogenes is twice as high as the corresponding parameter even at the third codon position. The R index ($R = \sigma^2/\mu$, [11, p. 232]), estimated for 20 loci, supports these old conclusions for more extensive data for non-synonymous (N) and synonymous (S) substitutions, the means being $R_N = 8.26$ and $R_S = 14.41$ ([11, Table 8.8]). The differences in the nucleotide substitution rates (r) of nuclear genes in human (47 genes) and *Drosophila* (32 genes) are even more contrasting: $r_N = 0.74$ (0.67; in brackets is standard deviation); $r_S = 3.51$ (1.01) and $r_N = 1.91$ (1.42), $r_S = 15.6$ (5.5), respectively ([11, Tables 7.1, 7.6]). Earlier, using another gene set, the substitution rates for N- and S-codons in evolution were also shown to differ: 8.26 and 14.41, respectively [57]. On average, the nucleotide substitution rate in pseudogenes is 4.7×10^{-9} per nucleotide per year and is thought to be close to the neutral process [7]. Analysis of another multigene family, amylases, revealed clear differences in p-distances for synonymous (1) and nonsynonymous (2) nucleotide substitutions in the primary nucleotide sequences in three *Drosophila* species: (1) $P = 0.398 \pm 0.043$ and (2) $P = 0.068 \pm 0.008$ [58]. In all, summarized analysis of extensive data showed that for a randomly selected coding sequence, the ratio of synonymous and nonsynonymous substitutions is approximately 25 : 75%, while this proportion is inverse (69 : 31%) for the third position ([11, Table 1.4]). Note that the N/S ratio is significantly higher in human and close anthropoid ape species than in other monkey groups, owing to greater N [59]. The increased proportion of nonsynonymous substitutions in hominids is attributed to the rapid adaptive evolution in this group. The above evidence suggests that (1) genes and their regions with and without functional significance accumulate mutations and diverge at different rates and (2) the presence of purifying selection on coding sequences of structural genes is a well-established fact.

Genealogical relationships of genes within and among populations. The phylogenetic relationships for one gene or DNA fragment may be inferred from the DNA polymorphism in nucleotide sequences or restriction sites. If a phylogenetic tree is constructed on the basis of genes sampled from several populations connected by migration, theoretically we deal with mixed genealogies [60]. MtDNA in human races, which today are actively intermixing, provide an example of such genealogical mixture. The character of clustering of members of various races in a phylogenetic tree unequivocally demonstrate its "mixed" branches [61]. Similar results were obtained in other studies of humans [62, 63]; this ambiguous clustering was interpreted as showing migration among races [63]. The most ancient mtDNA divergence, dating back 300 000 years, occurred in the members of Mongoloid and Negroid races, with exclusion of one individual. Apparently, the mtDNA divergence preceded the divergence of the races themselves, as follows from the estimates of their divergence based on other genes [7]. Note, however, that genealogical mtDNA mixing for various populations is expected, even in the absence of migration, if the ancestral population was polymorphic and the time since divergence was relatively short [7]. Lineage sorting of individuals by populations, which are isolated after that, yield a phenomenon of older age of gene genealogies, than population lineages. Later, this will result in difference between gene and species trees.

The evidence given in the previous section demonstrates a relatively low percentage of nucleotide divergence within species. Yet, the available data suggest that the divergence of populations within a species in some cases produces stable, geographically distinct spatial groups, phyletically marked by mitochondrial genes. This was found for bottle-nosed dolphin *Tursiops truncates* [64], Canadian goose *Branta canadiensis* [65], in fish *Fundulus heteroclitus* and *Stizostedion vitreum* [66, 67], and in a number of other organisms [68]. Thus, migration gene flow can be restricted, while intraspecies phyletic groups are as real as stable population units of species, detected in analysis of spatial genetic differentiation of particular generations or their mixtures [18]. In further text, we will consider the question whether and to what extent these data are associated with speciation genetics.

Introgression of mitochondrial DNA. Investigation of mtDNA genotypes, in combinatio0n with nuclear DNA markers or isozyme loci, may allow establishing introgression mtDNA from one species to the nuclear background of the other species, if the hybrids between these species and their progeny are fertile. This introgressive hybridization requires successful backcrosses of the ancestral hybrid female with males of the parental species or other taxa. This introgression is independent of recombination and segregation events, occurring in the nuclear genome, if natural selection, maintaining nuclear–cytoplasmic compatibility, is absent [7, 69]. Evidence of this kind appears increasingly often, indicating operation of fine mechanisms that maintain the interaction of nuclear and, for example, mitochondrial genes [70]. A high number of cases of mtDNA introgression (see below) show that this selection, if it exists at all, in nature is not sufficiently strong to prevent hybridization and introgression. Thus, the cases of possessing foreign mtDNA in natural species hybrids, identified by other methods, may be a proof of hybridization of closely related species (taxa). Such interspecies mtDNA transfer was found in species of invertebrates (*Drosophila*) and vertebrates (*Mus* and *Rana*) [71–75]. Literature on the topic was already considered with regard to comparative analysis [9, 75, 76]. Based on an analytical approach for analysis of nuclear–cytoplasmic equilibrium [77, 78], an original method has been developed for testing hybridization and direction and intensity of introgression [79]. In this section, we only briefly touch upon the issue of mtDNA introgression, to elucidate its relationship with the species status.

Let us consider some examples. In southern Denmark, there is a hybrid zone between two house mouse species, *Mus musculus* and *M. domesticus* (previously assigned to two distinct subspecies). Northern Denmark is inhabited mainly by *M. musculus*. Examination of mtDNA of these species have shown [72] that, in contrast to the Eastern European form of *M. musculus*, in Denmark *M. musculus* to the north of the hybrid zone also possesses mtDNA of *M. domesticus*, which shows a 5% divergence of mtDNA nucleotide sequence from *M. musculus.* This part of *M. domesticus* is restricted to northern Denmark and some Swedish regions. Because of this, mtDNA introgression from *M. domesticus* to *M. musculus* seems to have appeared relatively recently. Interestingly, the nuclear genes did not show evidence of introgression. It may well be that introgression at nuclear genes in these mammalian species is prevented by sterility or nonviability of hybrids, which is caused by the nuclear genes, whereas mtDNA, which does not affect fitness, can be inherited and transmitted independently.

MtDNA has been used for investigation of natural hybridization in fish and marine invertebrates since the mid-1980s [80–82]. Avise and Saunders [80] used mtDNA combined with allozymes to study hybridization rate among nine sunfish species (the genus *Lepomis*) from two localities from the southeastern United States. The results of this study can be summarized in four following items. (1) Hybridization occurs at a relatively low rate, but involves five out of nine species examined. (2) no mtDNA or allozyme evidence of gene introgression among the *Lepomis* species was found; all hybrids proved to be F_1 progeny. (3) Each of the hybrids was produced by a cross between the most common and the rarest species. (4) In six out of seven possible hybrid combinations, the maternal parent was from the rare species, as shown by the mtDNA genotype. This was explained by intense mating competition among males and general promiscuity of females. In many fish groups, hybridization and introgression are quite common [76], although in many cases introgression occurs sporadically, as a result of a past climatic shift, which, in particular, was demonstrated for two char species of the genus *Salvelinus* [83]. The list of examples of mtDNA analysis can be easily extended. We will consider as an example the mussel species of the *Mytilus* spp. complex.

Among 12 samples of mussel collected in southwestern British Columbia and in Vancouver Island, the distribution of alien alleles at two marker loci (*PL*IIa and *ITS*) differ in different sampling sites, which implies differential introgression [84]. The wide distribution of alien alleles, combined with the evidence for intense hybridization between the native and the introduced (alien) species indicate that the introduced alleles may have existed for some time in the mussel population of British Columbia [84]. One of the markers used in [84] (*ITS*) is a nuclear gene. Other nuclear DNA markers also provide strict proofs for hybridization in marine organisms [85]. In mussels in the Peter the Great Bay, Sea of Japan, the proportion of hybrid animals, estimated using DNA marker *Me-5* and allozyme locus *MPI**, varied from 1.6 ± 0.9 to $8.9 \pm 1.7\%$, indicating the ongoing process of the hybrid zone formation [86–88]. Examining hybridization by a single marker does not permit to clearly distinguish F_1 hybrids from the individuals produced in backcrosses [76, 87]. For the Peter the Great Bay, Sea of Japan, there is a set of data, testifying for gene introgression between two mussel forms [87, 88]. Here, introgression occurs from the more ancient form, *M. trossulus*, to a younger form, *M. galloprovincialis* [87, 88]. Investigation of the common mussel, based on a set of marker genes (allozyme, mitochondrial and nuclear) in another part of the range, in England, yielded interesting results [89–92]. The latter authors, using an enzyme gene and two nuclear DNA markers, confirmed the presence of a reported earlier large hybrid zone, occupied by hybrid animals (F_1, F_2, and different F_b) and a patchy distribution of *M. edulis* and *M. galloprovincialis* [89–91]. In addition, it was shown that hybrid mussels from Whitsand Bay, United Kingdom, carry alleles that had appeared as a result of intragenic recombination [92]. A high (10%) frequency of these recombinant alleles within the hybrid population suggest either frequent recombination at this gene or significant hybridization between *M. edulis* and *M. galloprovincialis* that has occurred during a long period of time in the evolutionary history of the taxa. Facts of a cline presence, as in [92], for instance, may be interpreted as evidence of selection. Though such evidence is usually problematic to obtain under normal conditions, the above association, together with other evidence from the hybrid zones of this species group [89–91], testifies for an increase in natural selection intensity in these zones, including selection against the hybrids. An assessment of introgression among all of the three forms of the *Mytilus* complex showed that it is maximal between *M. edulis* and *M. galloprovincialis* and minimal between *M. trossulus* and *M. galloprovincialis*. Restricted introgression between the latter species pair was found along the Pacific coast of the United States [85]. The gene introgression in the Sea of Japan is also low and asymmetric [88]. Asymmetric introgression *edulis* $\longrightarrow$ *galloprovincialis* was found using RFLP markers of the mitochondrial genome and a sequenced DNA fragment [93]. Laboratory crosses showed that *M. trossulus* $\times$ *M. galloprovincialis* hybrids have considerably deteriorated morphology in the development than hybrids between *M. trossulus* and *M. edulis* [94]. This evidence suggests that in this mussel species complex, *M. trossulus* is closest to completing the RIB (reproductive isolating barriers) formation and its species status is the most definite among the three forms. The remaining two forms are likely semispecies [88].

In all, the above evidence indicates that mtDNA crosses species boundaries and stably exists over many generations in the gene pools of species, whose repro-

ductive and biological integrity is confirmed by means of other molecular markers or phenotypic traits. This is illustrated by the data on mice [75], frogs, fish, mussels (see above), and other organisms [79]. Asymmetry of introgression, as was shown for two frog species from the genus *Hyla,* is particularly clearly demonstrated by data on nuclear–cytoplasmic disequilibrium, being a common phenomenon in nature [79]. Thus, surpassing interspecies barriers by mtDNA and probably some mobile elements from alien genomes, as such does not necessarily leads to disintegration of the species, and in some cases, as predicted by BSC, may play a role in subsequent RIB formation. The appearance of RIBs depends on the establishment of further nuclear–cytoplasmic relationships and on other biological and climatic events, which probably currently occurs in the mussel species complex. To date, monitoring of hybridization and introgression in various biological species seems to be among most relevant tasks of general and evolutionary genetics.

Divergence of DNA nucleotide sequences on the interspecies level. As measures for comparison, we employ non-corrected *p*-distance, *p*-distance of the two-parameter Kimura model (K2P), or other indices, used in literature for genes *Cyt-b* and *Co-1* (table, Appendix). The possibility of their use follows from the theory and from numerical simulation, as noted in Section 1.1. The optimistic expectations at the preliminary stage of comparison were generated by similarity of *K2P* and $\hat{p}$ at the *Co-1* gene in butterflies (Lepidoptera) in two studies [15, 16]. However, below we will examine actual comparability of the *p*-distances for the four comparison groups and for the two genes.

Variation series of pair-wise K2P comparisons for sequences of the *Cyt-b* gene, presented, for instance, in a review of data on vertebrate animals, are far from normal distribution [13]. This creates additional problems of analyzing this and other genes, in which the distance distributions also seem to deviate from normality. In such cases, means of the estimates generally provide more satisfactory variation distributions.

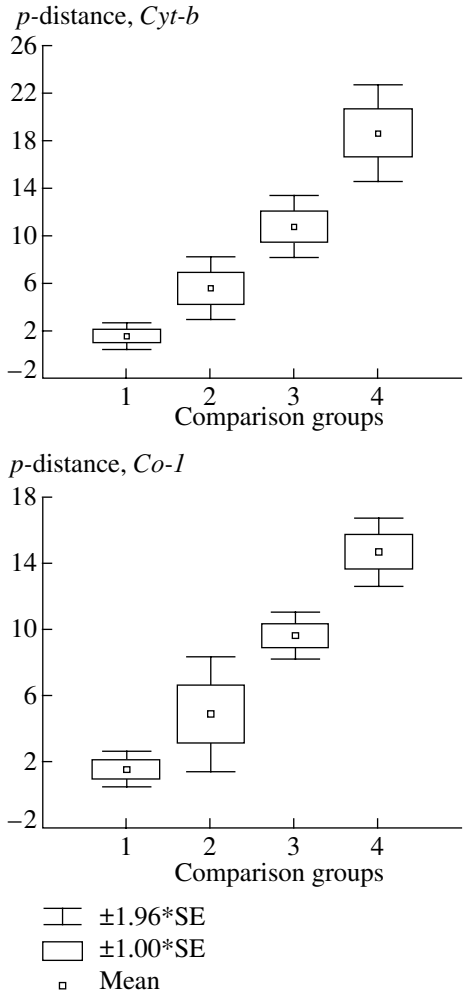

Fig. 1. Graph illustrating variation in mean *p*-distances for nucleotide sequences of genes *Cyt-b* and *Co-1* in various animal groups, depending on the rank of these groups. Groups of comparison: 1, populations within species; 2, sibling species; 3, species within a genus; 4, species of different genera within a family. Significance of variation for ANOVAs: $F = 14.26$, $d.f. = 3; 45$, $P < 0.000001$ (*Cyt-b*); $F = 21.17$, $d.f. = 3; 52$, $P < 0.000001$ (*Co-1*). SE, standard error.

We have analyzed their distribution, based on the data table (Appendix) for all of the four comparison groups (1–4) and for genes *Cyt-b* and *Co-1,* respectively. The distribution of the mean *p*-distances in fact did not differ from normality, except one series, based on the *W* statistics in the Shapiro–Wilk test: gene *Cyt-b* (1) $W = 0.933$, $P < 0.156$; (2) $W = 0.692$, $P < 0.006$; (3) $W = 0.824$, $P < 0.070$; (4) $W = 0.909$, $P < 0.909$; gene *Co-1*: (1) $W = 0.824$, $P < 0.070$; (2) $W = 0.971$, $P < 0.848$; (3) $W = 0.983$, $P < 0.892$; (4) $W = 0.909$, $P < 0.154$.

According to Kolmogorov–Smirnov test, all the distributions were normal at $P > 0.2$. A one-way ANOVA (model with random effects for groups of the same size) showed that mean distances in the four groups analyzed were significantly different for genes *Cyt-b* and *Co-1:* $F = 14.26$, $d.f. = 3; 45$, $P < 0.000001$; $F = 21.17$, $d.f. = 3; 52$, $P < 0.000001$. Accordingly, pooling of the data for the two genes produced a statistically significant increase in the *p*-distances in the hierarchy of the comparison groups. However, this pooling is not quite correct for all of the DNA sequences compared, because it includes heterogeneous groups of different size. Consequently, categorized representation of mean values for two genes is more correct. It shows that the divergence for both genes increases with the rank (Fig. 1). Mean unweighted distances for the four groups were as follows: *Cyt-b*: (1) 1.55 ± 0.56, (2) 5.52 ± 1.34, (3) 10.69 ± 1.34, (4) 18.51 ± 2.09; *Co-1:* (1) 0.55 ± 0.19, (2) 4.91 ± 0.83, (3) 9.66 ± 0.72, (4) 14.69 ± 1.02 (table, Appendix).

Taking in account variation in sample size for each *i*th *p*-distance in comparison group (*n*) (table, Appendix), we performed a two-way ANOVA with *p*-distances weighted by *n* (factor 1, comparison groups: 1, populations within species; 2, sibling species; 3, spe-

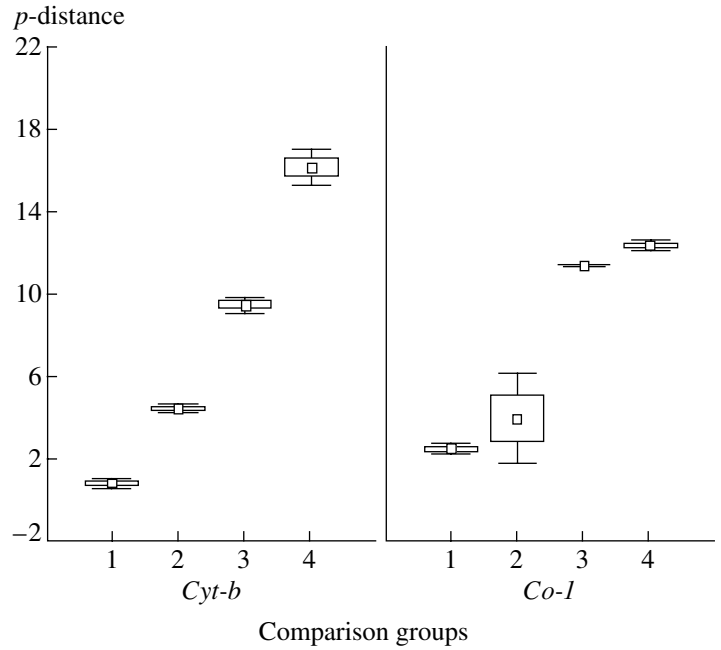

Fig. 2. Categorized distributions of mean *p*-distances in four comparison groups for genes *Cyt-b* and *Co-1* (see Fig. 1 for designation of abbreviations).

cies within genera; 4, genera within a family; factor 2, genes: *Cyt-b* and *Co-1*; also, a model with random effects of the factors was applied). In this ANOVA, the effect of factor 1 (i.e., comparison group) was significant $F = 1010.0$, *d.f.* = 3, 14894; $P < 0.000001$. The effect of factor 2 (mean *p*-distance differences for two genes) proved to be nonsignificant: $F = 0.341$, *d.f.* = 1, 14 894; $P < 0.559$. The interaction between factors 1 and 2 was significant: $F = 175.9$, *d.f.* = 3, 14 894; $P < 0.000001$. The categorized graph of the distribution of mean weighted *p*-distance values supported the earlier conclusion on the increase of distances with the rank of the groups compared (Fig. 2). Figure 2 clearly shows the meaning of the factor interaction: the *p*-distance values of the two genes differ between three out of four comparison groups (see Fig. 2).

We are primarily interested in comparative series of taxa of different rank. The data presented in Figs. 1 and 2 demonstrate that both genes show a trend of increasing mean *p*-distances with increasing rank of the groups compared, from populations to families. Because of the importance of this conclusion, the data presented in Figs. 1 and 2 were additionally tested using nonparametric Kruskall–Wallis ANOVA. For gene *Cyt-b,* $H = 27.85$, *d.f.* = 3, $N = 49$, $P = 0.0001$. For gene *Co-1,* $H = 29.54$, *d.f.* = 3, $N = 56$, $P = 0.0001$. Thus, the comparative analysis of the data for virtually complete nucleotide sequences of genes *Cyt-b* and *Co-1,* performed for groups with increasing the rank for each of the genes separately, demonstrates (with a probability of error $P < 0.0001$) that in animals, genetic divergence increases with the taxon rank. Heterogeneity of gene evolution rate, also significant in our data for the two genes (Fig. 2), is widely known in literature (see, for example, [95]), which we noted previously.

Let us take a deeper look on the essence of the detected differences. The differences in *p*-distance estimates between the two genes can have three explanations. Firstly, the rate may be in fact different in the two genes. Secondly, the data on taxonomic groups from the most representative sources [13, 15, 16], which can differ in the divergence level, may be differently represented in our database. For instance, heterogeneity of *K2P* values at gene *Cyt-b* was found for the vertebrate groups examined: amphibians and reptiles have the highest, and birds, the lowest variability [13]. Interspecies heterogeneity of nucleotide diversity estimates at *Cyt-b* can be found even within a fish genus [96]. Thirdly and finally, in two most representative works on *Co-1* [15, 16] used several different measures. In addition, instead of *K2P* and other similar measures (expected distance), non-corrected *p*-distance (observed distance) was employed in many studies. In general, a shortcoming of analysis of such data array id high biological heterogeneity of the material and some unknown or not identifiable components of the *p*-distance estimates. For instance, non-weighted *p*-distances in the most numerous comparison group (species within a genus) did not statistically significantly differ between two groups—(1) non-corrected distance and (2) other *p*-distance estimates (*K2P, GTR, TrN*; see table, Appendix). The results of ANOVA were as follows: *Cyt-b*: $F = 0.84$; *d.f.* = 1, 19; $P < 0.372$; *Co-1*: $F = 1.98$; *d.f.* = 1, 29; $P < 0.169$. The differences between these groups are also nonsignificant, when *n* is used as covariance in ANOVA of the *p*-distances. However, the differences between the groups are significant, if the *p*-distances

are weighted by *n*: *Cyt-b*: $F = 142.46$; *d.f.* = 1, 455; $P < 0.0001$; *Co-1:* $F = 207.47$; *d.f.* = 1, 13 436; $P < 0.001$. However, the latter differences apparently are partly caused by the unequal representation of the taxa in different groups,. For the *Cyt-b* gene, the group 1 consists almost exclusively of fishes, which on average have smaller *p*-distances (see table, Appendix). For gene *Co-1*, 50% of group 2 constitute less variable butterflies (see table, Appendix, Lepidoptera). Note also different directions of differences of the mean *p*-distances at two genes in these two groups. Theoretically, unmodified *p*-distance must undergo homoplasy faster, i.e., be smaller than the expected values of *K2P, GTR, TrN*.

2. BIOLOGICAL SPECIES: GENETIC VARIABILITY AND DIVERGENCE

In this part of the review, we briefly present a concept of the species (Subsection 2.1), compare molecular genetic and biochemical genetics data (Subsection 2.2), and draw conclusions from this evidence (Subsection 2.3).

2.1. Species Examined

Let us clarify, what is usually considered as species in most studies.

According to the biological species concept (BSC), the definition of the species is as follows. A species is a biological group, consisting of one or several crossbreeding individuals that are reproductively isolated from other such groups, are stable in nature, and occupy a particular area. This is the definition by the authors of the present article, but it is very close to the one given earlier in the monograph by Timofeeff-Ressovsky, Vorontsov, and Yablokov [97]. In principle, this is a definition typical for the BSC. For instance, one of the BSC definitions is formulated by Mayr as follows: "A species is a reproductive community of individuals (reproductively isolated from others), occupying in nature a certain habitat" ([98, p. 273]). In what follows, we will take this definition as a basis for discussing the BSC (which is largely limited to higher bisexual organisms) [97, 99, 100]. As the BSC is the concept closest to the population genetic theory, it seems expedient to use it as the foundation of the discussion, despite the above limitation. Several other concepts of the species, with their advantages and restrictions, have been critically analyzed in [100–104]. Conceptual analysis of BSC and its contraposition to the typological species concept were provided by Altukhov [3, 103, 105]. We did not set a task to compare all of the species concepts. Most authors, in spite of criticisms, accept the BSC as the main modern paradigm. We confine ourselves to listing the existing concepts of the species: (1) Linnaean species, (2) biological species concept; (3) biological species concept modified by Mayr (BSC) [99]; (4) BSC, modification II [98]; (5) concept of species recognition [106, 107]; (6) concept of species cohesion [100]; (7) evolutionary concept of the species; (8) Simpson's evolutionary concept of the species [108]; (9) Wiley's evolutionary concept of the species [109]; (10) ecological concept of the species [110]; (11) phylogenetic concept of the species [111], and others (see, e.g., [28, 112]).

2.2. Brief Analysis of Biochemical Genetics Data and Their Comparison to Nucleotide Divergence

Let us briefly consider the evidence on variability of structural protein-coding genes. The mean heterozygosity per individual (locus) has been recognized as the best measure of variability [7, 113, 114]. Many statistics have been used to measure taxon divergence during evolution [114–116], but the most popular among them is standard Nei's distance D_n and the inverse measure, similarity I [117]. To assess differentiation at the intraspecies level, minimal distance and standardized variance of allele frequencies are more convenient (these measures are not considered in this review). Examination of genetic diversity of natural species requires analysis of heterozygosity (diversity) and distances (differences), assessing different aspects of variability, which is not always taken into account. Heterozygosity (and its equivalent, nucleotide diversity) estimates weighted variability of individuals in a population (species), while distance similarity measures the pairwise differences between populations (species) in marker genes or molecular sequences. Note, however, that *p*-distance and π can be used both as a measure of variability and a measure of distance. Comparing individuals from one or several populations of a species, one can estimate intraspecies diversity (heterozygosity), while comparison of individuals of different species provides an estimate of their divergence (distance).

Brief results of comparing H and I. Mean heterozygosity per individual H widely varies in plant and animal taxa. The total mean $H = 0.076$; in vertebrates, $H = 0.054$, for invertebrates, $H = 0.100$ [118]. A number of other surveys give similar data [87, 119–122]. The H value underestimates the actual genetic diversity approximately by one-third, owing to technical restrictions of protein electrophoresis, which is used to estimate variability at that level ([4, 113, 123] and others).

Coefficients of genetic distance or similarity at enzyme loci show in comparable scale genetic divergence in taxa of various ranks, from subspecies to families [7, 113, 114]. Comparison of higher-rank taxa at this level is hindered by high probability of synonymous substitutions increasing nonlinearity of genetic similarity (distance) and divergence time [7, 113]. Coefficients of intraspecies genetic similarity of difference were estimated in many groups of animals. The mean genetic difference at this level is $I = 0.95$ [3, 4, 7, 87, 113]. According to our database, which comprises more than 300 populations of 80 animal species, $I = 0.94 \pm 0.01$. In the hierarchy of animal taxa, subspecies have coefficients of similarity (normalized Nei's identity) I ranging from 0.6 to 1.0, with a mode of approxi-

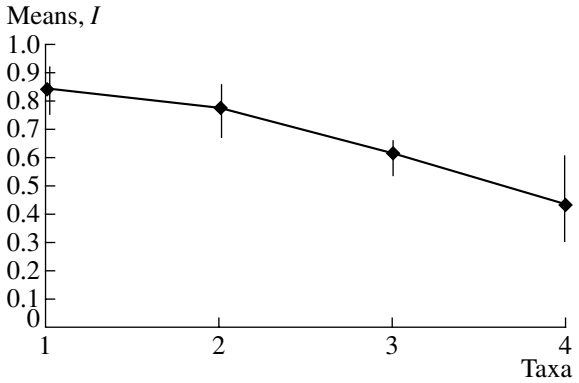

Fig. 3. Genetic similarity in taxa of different ranks: group means. 1, subspecies ($I = 0.84$); 2, semi-species and sibling species ($I = 0.78$); 3, species ($I = 0.63$); 4, genera ($I = 0.47$). Lines show confidence interval for means, 95% (our data, after [87] with supplements).

mately 0.9; the variation range is 0.5–1.0 (mode about 0.7) for species within a genus and 0.0–1.0 (mode 0.4) for genera within a family [7, 87, 124] (see also Fig. 3). This means that genetic similarity significantly decreases with increasing rank of the taxon (see confidence intervals in Fig. 3) and conversely, it increases with decreasing taxon rank.

Thus, the current molecular genetic evidence (Subsection 1.2) and the results of analysis of protein marker genes support, first, the basic BSC idea that taxon formation necessarily requires isolation of gene pools and, second, that the geographic (divergent) speciation mode prevails in nature, implying gradual accumulation of small genetic differences. Yet, there are facts warning against simplified conclusions on ways of speciation. For instance, it has long been known that the genetic "weight" of the species, say, on the D_n scale, may be different for different animal taxa. For example, D_n is on average 1.1 in amphibians, which is an order of magnitude higher than the corresponding value in birds ($D_n = 0.1$) [124]. Other examples of this trend can be found [9]. The range of nucleotide diversity also shows that some animal taxa display a high divergence level among the species, while others are characterized by a very low value of this parameter. As already noted above, avian taxa are substantially less differentiated at *Cyt-b* than amphibians and reptiles [13]. For three main geographic phyletic groups of *Orizias latipes*, the nucleotide diversity of *Cyt-b* was found to be comparable to the within-genus divergence: p = 11.3–11.8% [125]. For the other gene, *Co-1,* the species within the genus *Cnidaria* have p = 1%, while in crustaceans p = 15.4% [16].

Some studies show that the concept of natural selection is necessary to explain joint variation of H and environmental variability [118], an association of individual heterozygosity at enzyme genes (H_o) with physiological, morphological, and other components of phenotypic variation in population–environment gradient [4, 5, 119–134]. The data on genetic similarity may be interpreted in the same way. For instance, frequencies of genetic similarity coefficients for enzyme loci, estimated for various species, follow a U-shaped distribution, whereas neutrality implies a reverse association with the expected differentiation [135], i.e., a distribution close to normal. Nevertheless, a nearly normal distribution of coefficients of similarity have been found for some protein loci, e.g., duplicated hemoglobin loci of salmonid fishes ([87, Fig. 8.3.5]). Thus, the observed temporal differentiation at many loci is consistent with the neutral process of drift [7, 136–139]. On the other hand, as stressed at the end of Section 1, the role of natural selection in determining molecular diversity of various genes and their different regions has been conclusively demonstrated. Thus, the early expectations of predominantly selective neutrality of variation in DNA sequences and other markers, including mitochondrial DNA markers, have not been supported by observations. The problems of selectivity/neutrality of mtDNA markers were considered in special reviews [70, 139, 140]. In particular, it was pointed out that assessments of genotype expression in different nuclear backgrounds in many cases reveal differential fitness, caused by coevolution. Experimental manipulations also showed that particular haplotypes are selectively advantageous [70]. Based on (1) variability of the ratio of intergenic nucleotide diversity and between-species differences and (2) sequence differences in polymorphism and divergence at silent sites, mutations in the analyzed mitochondrial *Cyt-b* gene are likely moderately deleterious, while their polymorphism is maintained by selection–drift balance [140]. For protein sequences of the copepod *Tigriopus californicus*, encoded by three mitochondrial genes (*Cyt-b, Cyt-c*, and *RISP*), a selective signal was detected only for the *Cyt-c* gene [141]. However, this situation is complicated and ambiguous. First, as known since early studies by Mukai [142], it is virtually impossible to experimentally assess weak effects of molecular markers on fitness and second, there are a multitude of factors disrupting stochastic processes, but these factors are not necessarily adaptive ones. In particular, the gene bank data show that a half of the species pairs examined do not substantially deviate from neutrality expectations, while the other half exhibit a significant excess of amino acid polymorphism in structural genes [140]. Gillespie [143] has offered his view on the ratio of stochastic and selective processes, expressed as the genetic draft model. Some novel ideas on using molecular data for proving the role of natural selection [144] received strong criticism [145, 146].

2.3. Applicability of Molecular Evolution Data to Speciation Genetics

It is of interest to comprehend whether the obtained evidence is relevant to genetic aspects of speciation? As shown in the previous sections of this review, genetic differences are acquired gradually, in formed isolated

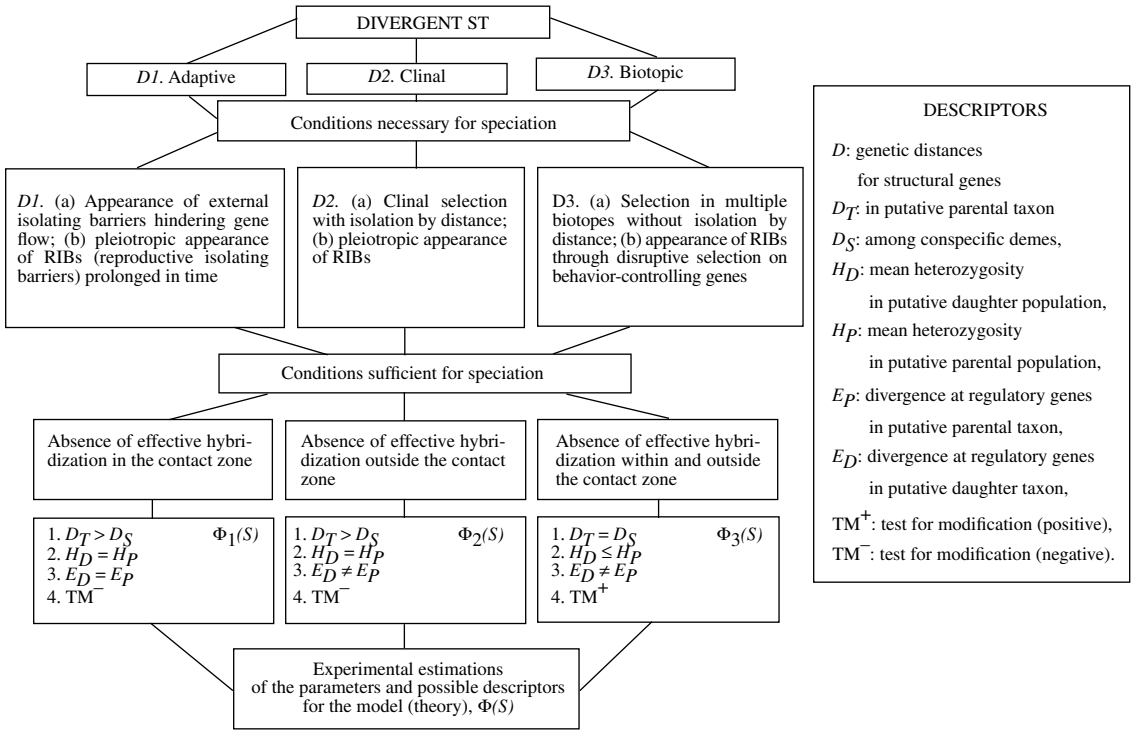

Fig. 4. Schematic representation of the divergent speciation type (ST), based on population genetic principles (after [150], simplified to three model types).

populations or their groups. The process of divergence further proceeds to diversify semi-species and sibling species, genera, and so on. The presented data on nucleotide sequences of genes *Cyt-b, Co-1,* and protein markers conclusively demonstrate that this process is implemented to the family level (see Figs. 1–3), although other molecular markers present good evidence in favor of phyletic evolution as the main process of divergence, also for higher rank taxa [7, 11]. Evidently, we cannot cover all aspects of speciation in a short paper. This issue was addressed to different extents by a number of authors [8, 147, 148]. We present our view on these processes. It is important to emphasize that evolutionary genetics lacks a speciation theory in the strict scientific sense, implying a formal, analytic model and prediction of the future events on its basis. In a particular case, such model must predict the formation of a species or at least distinguish different speciation modes on the basis of quantitatively estimated parameters and their empirical estimates. The steps taken in this direction [8, 28, 100] do not meet the above requirements. In this connection, a scheme and an algorithmic approach have been developed [87, 149, 150] to distinguish speciation modes (models) on the basis of key population genetic parameters and their estimates available in literature. This approach may lay foundation for a future theory, a genetic theory of speciation. As a basis for the evolutionary genetic concept of speciation, descriptions by Templeton [148] were used. As a result, a classification scheme for seven known modes of speciation was developed [87, 150]. Here, we present for illustration three elements of this scheme for types *D1–D3* (divergent speciation) (Fig. 4). This approach lead to a relatively simple experimental scheme, which allows to (1) organize further investigation of speciation in various groups of organisms, based on a verified genetic approach and (2) obtain analytic expressions (equations) for each of the speciation modes (Fig. 5). Using the proposed scheme [87, Fig. 7.4.1; 150], one can determine the conditions required for speciation and sufficient for the formation of a species. Importantly, in addition to the general definition of the sufficient conditions, four (1–4) experimentally measured descriptors are introduced (their number can be increased, if necessary) to clarify, how and in which form these conditions are manifested in a particular case of speciation or in a potential model. For instance, the divergent type of speciation *D1* explains classic geographic (or allopatric) speciation (see Fig. 4).

According to the BSC, this model implies that large populations are isolated (disruption of the gene flow) and evolve separately, accumulating mutations, while reproductive isolating barriers (RIBs) are caused by pleiotropic effects. The longer the time elapsed from the isolation event, the greater the distances between the corresponding taxa. Accordingly, in our notation we introduce a descriptor: (1) $D_T > D_S$ (where subscripts T and S indicate genetic distances in the putative parental taxon and in conspecific populations or at the higher and lower levels of taxomonic hierarchy). Likewise,

$$\Phi_1(S) \in \{(D_T > D_S) \subset (E_D = E_P) \subset (H_D = H_P) \subset TM^-\} \quad (D1)$$

$$\Phi_2(S) \in \{(D_T > D_S) \subset (E_D \neq E_P) \subset (H_D = H_P) \subset TM^-\} \quad (D2)$$

$$\Phi_3(S) \in \{(D_T = D_S) \subset (E_D \neq E_P) \subset (H_D <= H_P) \subset TM^+\} \quad (D3)$$

$$\Phi_4(S) \in \{(D_T = D_D) \subset (E_D \neq E_P) \subset (H_D <= H_P) \subset TM^-\} \quad (T1)$$

$$\Phi_5(S) \in \{(D_T = D_D) \subset (E_D = E_P) \subset (H_D > H_P) \subset TM^-\} \quad (T2)$$

$$\Phi_6(S) \in \{(D_T > D_D) \subset (E_D \neq E_P) \subset (H_D > H_P) \subset TM^-\} \quad (T3)$$

$$\Phi_7(S) \in \{(D_T > D_S) \subset (E_D \neq E_P) \subset (H_D < H_P) \subset TM^-\} \quad (T4)$$

Fig. 5. Analytic representation of seven speciation modes. *D1–D3*, divergent speciation modes; *T1–T4*, transformative speciation modes. Descriptors: *D,* genetic distances for structural gene; D_T: in putative parental taxon; D_S : among conspecific demes; D_D: among subspecies or sibling species; H_D: mean heterozygosity in putative daughter population; H_P: mean heterozygosity in putative parental population; E_P: divergence at regulatory genes in putative parental taxon; E_D: divergence at regulatory genes in putative daughter taxon; TM^+: test for modification (positive); TM^-: test for modification (negative).

since upon implementation of the *D1* mode, no significant differences appear in either structural genes or the regulatory part of the genome (the initial and derived taxa are large), we introduce parameters (2) $H_D = H_P$ and (3) $E_D = E_P$ (differences in heterozygosity and gene expression between the daughter and the parental taxon are absent). Finally, upon some types of speciation, not only variability and genetic distances, but also some quantitative loci (polygenes) are of importance, which cannot be distinguished at the molecular level, but lead to the RIB formation. Hence, we introduce *TM* (experimental test for modification), which also allows to distinguish between epigenetic variation and taxonomic differences.

Do all these data imply that speciation always corresponds to the *D1* type? Apparently not. Here is an example supporting this answer. In one of mountain Swedish lakes, two trout forms were known. It was unclear whether their gene pools were isolated. A genetic examination [151] revealed in these forms two different fixed alleles, which unambiguously proved total reproductive isolation of these sympatric trout forms. After the advent of a method permitting to exactly distinguish all individuals, the gene pools of these taxa were found to differ by five out of seven polymorphic loci examined [151]. There are other examples of bursts of fish evolution, documented by molecular markers [152, 153]. These, as well as other data, for instance from our data base of coefficients of similarity, indicate that sometimes very small differences in structural genes may result in the appearance of RIBs (and thus reproductively isolated biological entities).In the case of trout mentioned above, the genetic differences between the two forms $D_n = 0.02$ [151], which corresponds to the level of intraspecies genetic differentiation. There are many examples for salmonid fishes [87], supporting the view that in these fishes, small changes can generate biological species during a short period of time. This evidence also suggests an alternative speciation model, such as the transformational (*T1*) or other type (Fig. 5), though in general, *D1* speciation mode prevails in this group.

Thus, we can now accept that speciation does not necessarily involve changes in structural genes that can be very small (at the level typical for populations of the species). Conversely, in some cases of speciation we can expect substantial rearrangements of regulatory genes [154], chromosomal or other reorganizations of the genome. Data on regulatory changes upon speciation are scarce in literature, because exact investigation of regulatory shifts or changes in gene expression is very labor-consuming. Moreover, the classification of genes into structural and regulatory ones is rather arbitrary [52, 154]. However, apart from the task of precise estimation of differences in the expression, very valuable comparative information for speciation studies can be obtained. In particular, considerable regulatory differences (in the expression level of enzyme genes) were found for two sibling char species, in which up to 32% of loci diverged in this respect, whereas distance D_n = 0.08, i.e., nearly at the level, characteristic of populations within a species [155]. Similar results were obtained for a group of species in status nascendi, in the family of white-fish and graylings in Baikal Lake. In this case, genetic differences D_n between these fish forms ranged from 0.01 to 0.03, whereas the divergence in the expression level reached 9–27% [156]. These and other similar data [150, 157–159] suggest that correct judgment on the mode of speciation should be based not only on distances, but also on heterozygosity and variability of other genomic elements. Other authors reached similar conclusions on the basis of independent analysis [160, 161]. In particular, the latter authors emphasize the idea on diffuse character of the species concept and species boundaries and, consequently, the necessity of a multiple approach and employment of different methods for their identification [161], which is also emphasized in out approach.

APPENDIX

Mean *p*-distances between species within a genus for two genes (*Cyt-b* and *Co-1*) and four comparison groups

p-Distance	Model of distance assessment	Species number *n*	Taxon		Reference
		Cyt-b			
		1. Population within species			
0.96	K2P	9	Mammalia	Apodemus	[162]
1.54	K2P	9	″	″	[162]
3.2	TrN	1	″	Martes	[163]
0.4	TrN	5	″	″	[163]
0.4	K2P	7	″	Microtus	[164]
4	GTR	2	Amphibia	Rana	[165]
0.32	K2P	20	Pisces	Mormiridae	[166]
		$M = 1.55$; $SE = 0.56$; $k = 7$; total $n = 53$			
		2. Sibling species			
12	*p*	2	Mammalia	Rhabdomys	[167]
4.8	HKY	2	″	Peromiscus	[168]
5.5	K2P	87	″	–	[13]
3.5	K2P	94	Aves	–	[13]
3.8	HKY	12	″	Motacillidae	[169]
3.5	K2P	96	Pisces	–	[13]
		$M = 5.52$; $SE = 1.34$; $k = 6$; total $n = 237$			
		3. Species within a genus			
9.4	K2P	7	Mammalia	Microtus	[164]
12.5	GTR	6	″	Scuridae	[170]
14	K2P	2	″	Apodemus	[171]
11.4	K2P	92	″	–	[13]
6.2	TrN	5	″	Martes	[163]
22	TrN	2	″	Mustella	[163]
13.5	HKY	2	″	Peromiscus	[168]
7.8	K2P	88	Aves	–	[13]
11	K2P	15	″	Pollimirus	[172]
7.4	K2P	7	″	Alectoris	[172]
12.5	TrN	6	Pisces	Clupeidae	[173]
1.8	K2P	13	″	Pollimyrus	[166]
3.5	*p*	19	″	Zoarcidae	[174]
1.43	*p*	15	″	Sebastomus	[175]
2.3	*p*	15	″	″	[176]
9	*p*	45	″	Sebastes	[176]
11.8	K2P	81	″	–	[13]
12	K2P	11	Reptilia	–	[13]
14	K2P	16	Amphibia	–	[13]
14.8	K2P	8	″	Rana	[177]
26.2	*p*	2	″	″	[165]
		$M = 10.69$; $SE = 1.34$; $k = 21$; total $n = 457$			
		4. Species of different genera within a family			
32.8	*p*	5	Mammalia	Scuridae	[170]
14.7	K2P	2	″	Murinae	[171]

(Contd.)

p-Distance	Model of distance assessment	Species number *n*	Taxon		Reference
15.5	K2P	48	Mammalia	–	[13]
16.7	TrN	9	″	Scuridae	[163]
12.5	K2P	37	Aves	–	[13]
31.3	K2P	25	″	Phasianinae	[172]
14.5	*p*	15	″	Falconidae	[178]
24.8	TrN	6	Pisces	Clupeidae	[173]
13.2	K2P	19	″	Mormiridae	[166]
9.5	*p*	19	″	Zoarcidae	[174]
6.6	*p*	32	″	Cottidae	[179]
14.5	K2P	28	″	–	[13]
20.5	K2P	18	Reptilia	–	[13]
19.5	K2P	3	Amphibia	–	[13]
31	K2P	8	″	Rana/Xenopus	[177]
$M = 18.51$; $SE = 2.09$; $k = 15$; total $n = 274$					
Co-1					
1. Population within species					
0.2	TrN	5	Hymenoptera	Bombus	[180]
0.33	K2P	13	Lepidoptera	Arctidae	[15]
0.23	K2P	30	″	Geometri	[15]
0.17	K2P	42	″	Noctuida	[15]
0.36	K2P	14	″	Notodont	[15]
0.17	K2P	8	″	Sphingid	[15]
1.4	*p*	2	Agnata	Letentheron	[181]
1.5	*p*	6	Arthrapoda	Theridiidae	[182]
$M = 0.55$; $SE = 0.19$; $k = 8$; total $n = 119$					
2. Sibling species					
9.1	*p*	2	Agnata	Letentheron	[181]
4.75	HKY	2	Tunicata	Ascidiacea	[183]
5.4	*p*	2	Arthrapoda	Theridiidae	[182]
0.4	K2P	4	Mollusca	Dressana	[184]
$M = 4.91$; $SE = 0.83$; $k = 4$; total $n = 10$					
3. Species within a genus					
7	K2P	4	Lepidoptera	Arctidae	[15]
9.1	K2P	10	″	Geometri	[15]
5.8	K2P	12	″	Noctuida	[15]
5.9	K2P	4	″	Notodont	[15]
6.4	K2P	3	″	Sphingid	[15]
5.5	*p*	4	″	2 genera	[185]
6.3	GTR	14	Coleoptera	7 genera	[36]
9.2	*p*	15	″	3 genera	[186]
4	TrN	3	Hymenoptera	Bombus	[180]
5.6	K2P	12	Diptera	Drosophila	[187]
13.7	HKY	4	Ascidiacea	Clavelina	[36]
18.3	K2P	2	Mollusca	Dressena	[184]
11.2	*p*	7	Arthrapoda	Lactrodectus	[182]

(Contd.)

p-Distance	Model of distance assessment	Species number *n*	Taxon		Reference
13	*p*	19	Arthrapoda	Lactrodectus	[182]
3.9	*p*	2	″	Chlorina	[188]
9.1	*p*	3	″	″	[188]
15.7	*p*	128	Annelida	Annelida	[16]
14.4	*p*	1249	Arthrapoda	Chelicerata	[16]
15.4	*p*	1781	″	Crustacea	[16]
11.2	*p*	891	″	Coleoptera	[16]
9.3	*p*	1429	″	Diptera	[16]
11.5	*p*	2993	″	Hymenoptera	[16]
6.6	*p*	882	″	Lepidoptera	[16]
10.1	*p*	1458	″	Other orders	[16]
9.6	*p*	964	Chordata	–	[16]
1	*p*	17	Cnidaria	–	[16]
10.9	*p*	86	Echinodermata	–	[16]
11.1	*p*	1155	Mollusca	–	[16]
11	*p*	49	Namatoda	–	[16]
14.4	*p*	84	Platyhelmintes	–	[16]
13.3	*p*	154	Other taxa	–	[16]
$M = 9.66$; $SE = 0.72$; $k = 31$; total $n = 13438$					
4. Species of different genera within a family					
10	K2P	18	Lepidoptera	Arctidae	[15]
12.5	K2P	61	″	Geometri	[15]
10.4	K2P	90	″	Noctuida	[15]
12.4	K2P	20	″	Notodont	[15]
10.5	K2P	11	″	Sphingid	[15]
14	*p*	2	″	2 genera	[185]
12.8	GTR	59	Coleoptera	Carabidae	[36]
17.1	*p*	18	″	2 genera	[186]
22.7	K2P	4	Mollusca	3 genera	[184]
13.8	*p*	23	Arthrapoda	Dressenidae	[182]
13.3	*p*	2	″	Delphacini	[188]
20.1	*p*	3	″	″	[188]
16.1	*p*	2	″	Delphaeidae	[188]
19.9	*p*	2	″	″	[188]
10	K2P	18	Lepidoptera	Arctidae	[185]
$M = 14.69$; $SE = 1.02$; $k = 15$; total $n = 315$					

Note: Absence of information is indicated by a dash. *M* and *SE* are respectively the mean and standard error of the arithmetic mean for the groups compared; *k* is the number of the groups. Distance models: *p*, *p*-distance (observed or non-corrected); K2P, two-parameter Kimura distance; GTR, General time reversible distance model; HKY, Hasigawa–Kishino–Yano distance; TrN, Tamura–Nei.

CONCLUSIONS

(1) The theory and the algorithms of calculation of genetic distances from nucleotide DNA sequences suggest that a suitable model should be thoughtfully selected for analysis of empirical data. However, the observed data for more than 24 000 species confirm the realistic character and interpretability of the data sets, analyzed for *p*-distance or its estimate. This testifies to the possibility of using this measure for most of interspecies and intraspecies comparisons of genetic divergence up to the family level.

(2) The data on *p*-distances show different levels of genetic divergence of sequences of the compared *Cyt-b* and *Co-1* genes in the four comparison groups exam-

ined. Differences between genes themselves were also found. This is in good agreement with ample data on different evolution rates of genes and their regions.

(3) The results of our analysis of the nucleotide and allozyme divergence within animal species and taxa of different ranks, first, are in good agreement with other similar data, including protein gene markers [9, 13, 16, 79] and, second, these data allow a generalization that phyletic evolution prevails in the animal kingdom at the molecular level, while speciation mainly follows the *D1* type (the geographic model).

(4) The prevalence of the type *D1* speciation does not preclude other speciation modes. There are at least seven such modes. Recognition of different speciation modes is a task, resolving of which implies construction of a quantitative genetic model (theory) of speciation. Although, in view of vast diversity of the possible causes of RIBs and speciation initiation, some of the newly appearing questions might remain unanswered. Their solution is likely to lay in an increase of the number of descriptors and members of the equations (*D1–T4*, Figs. 4, 5) on the basis of DNA markers and other genomic characteristics.

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