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Review Article

Methodological Aspects of Molecular Phylogeny of Fishes

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ABSTRACT

Guillaume Lecointre (1996) Methodological aspects of molecular phylogeny of fishes. *Zoological Studies* **35**(3): 161-177. Fish phylogeny has seen 2 major methodological changes during the last 30 years. First, the introduction of cladistics in Ichthyology in the late 1960s led to dramatic progress in fish classification and phylogeny. Second, molecular methods and especially DNA sequence data offered new collections of discrete characters useful for phylogenetic investigations, especially for phylogenetic problems left unresolved by morphological characters. But until now, their impact on fish phylogeny has remained limited. Whatever the aim of a study, fundamental or applied, different kinds of molecular methods exist, among which those allowing identification of molecular structures (such as sequencing) should be preferred, in order to avoid "experimental screens" which are described herein. The choice of genes, species, and tree-construction methods presents pitfalls that one should avoid. Robustness of phylogenetic trees should be considered. Differences exist between molecularists (geneticists) and morphologists as to their respective conceptions of phylogenetic trees. Distance-matrix methods are widely used in the 1st group, and a naive essentialist way to consider sequence alignment and trees is often encountered. Cladistics was born in the world of morphologists. Most molecularists have not yet reached the corresponding "phylogenetic maturity". Only parsimony methods allow researchers to identify in fine homologuous characters, and are, therefore, really phylogenetic. A phylogeny, as an inference on the history of life, must be performed with hypotheticodeductive methods. Parsimony methods should therefore be preferred over distance-matrix methods.

Key words: Fish molecular phylogeny, Phylogenetic reconstruction, Robustness, Systematics, Trees.

INTRODUCTION

Systematics, sometimes seen by reductionist biologists as an "obsolete" science, is the science of integration that generates fundamental data for all fields of Biology. The richness of debate within this discipline since Hennig shows that to consider Systematics as "obsolete" is to ignore the history of this science. Molecular phylogenies are extensively used in applied sciences as well as in fundamental sciences. Obtaining trees from molecular data has become easier since the development of spectacular technological advances such as polymerase chain reaction, or simplification of DNA sequencing techniques. A huge number of publications shows the intense interest in the theory and practice of molecular systematics. The choice of genes, species, and tree-construction methods presents pitfalls that one should avoid. Because technical aspects of molecular systematics are well described elsewhere (Hillis and Moritz 1990, Meyer 1994a), I will focus on opinions beyond the techniques rather than the techniques themselves. The aim of this paper is to briefly introduce the beginner to some fundamental problems and current debates in molecular systematics. As an ichtyologist, I will choose examples in major fish lineage relationships (Stock et al. 1991a, Lecointre 1994a, Meyer 1995). For a wider scope on advances in phylogenetic knowledge from molecular systematics, see Patterson et al. (1993).

FISH PHYLOGENY: HISTORICAL ASPECTS

The term "fishes" refers to a paraphyletic group, and is therefore not valid in scientific classifications. Hennig (1950 1966) provided concepts and tools to satisfy Darwin's aim concerning classifications: that classifications should be genealogies (Darwin 1859 1871), or more appropriately, "phylogenies", a term introduced by Haeckel (1866). According to both Darwin and Hennig, modern classifications must reflect phylogenies, i.e., must be cladograms. But this aim was imperfectly achieved by Darwin, because Hennig's tools (character polarization, grouping exclusively on the basis of shared derived characters, choice of the most parsimonious tree) were not yet available to him. Early attempts to classify vertebrates according to their phylogeny, i.e., to consider tetrapods as sarcopterygian fishes and to reject the taxon "Fish", are found in writings of Garstang (1931) and Säve-Soderbergh (1934 1935). Ichthyology was the 2nd zoological discipline (after entomology, the discipline of Hennig himself) to incorporate cladistics. The phylogeny of fishes, and particularly that of teleosts was rapidly and dramatically improved by the introduction of cladistics in ichthyology, in the late 1960s (see Dupuis 1978 1986 1992, and Hull 1988 for the history of cladistics; and Rosen 1982, or Lecointre 1994a for consequences for knowledge of fish phylogeny). To get a measure of this methodological impact, one may compare the classifications of Bertin and Arambourg (1958), Greenwood et al. (1966), Greenwood et al. (1973), and finally, Stiassny et al. (1996). As advocated by Nelson (1972), Tassy (1991), and Lecointre (1994a), phylogenetic classification (in Hennig's sense) is a modern expression of the Darwinian tradition. Systematic ichthyology has been conducted for 25 years within this Darwinian-Hennigian tradition. The increasing importance of methodological debates in systematics since the 1950s led to changes in the way systematists defined themselves. As a cladist ichthyologist, it is easier to speak with cladist ornithologists than with classical (Mayrian or Romerian) ichthyologists. I share with the 1st group the same conception of science and how to improve phylogenetic knowledge. Common methods allow me to understand their problems with birds. I share with the 2nd the taxon on which I work. We do not agree (among many other things) about the validity of a paraphyletic group. Communication is so difficult that it would be impossible to work with them on what is most interesting to me, phylogenetic reconstruction. Taxa do not define fields of research in systematics anymore; methods do.

EMERGENCE OF MOLECULAR PHYLOGENIES

Since Zuckerkandl and Pauling (1965a,b), molecules have provided characters for phylogenetic reconstruction. The most commonly used molecular tools can be divided into 2 families. In the first, a measure of similarity between organisms is extracted from physical and chemical interactions between molecules by such methods as isoenzymes electrophoresis (Avise 1974, Murphy et al. 1990), DNA-DNA hybridization (Sibley and Ahlquist 1981 1987 1990, Werman et al. 1990), immunological distances (Maxson and Maxson 1990), restriction fragment length polymorphism (R.F.L.P., Dowling et al. 1990), and random amplification of

polymorphic DNA (RAPD, Smith et al. 1994). Methodological implications of such approaches have been discussed by Swofford and Olsen (1990), Meyer (1994a), and some are discussed below. In the 2nd family, the structure of the molecule is identified, from which a comparative approach allows phylogenetic investigations. Here one finds nucleic and amino-acid sequences (Hillis et al. 1990), and, to a certain extent, karyological data (Sessions 1990). Considering relationships of major fish lineages, most of the early molecular phylogenetic studies were based on sequence data and investigated (1) the relationships of the craniate lineages, i.e., relationships between Myxinoids, Petromyzontids, Chondrichthyans, Cladistians, Chondrosteans, Ginglymods, Halecomorphs, Teleosteans, and Sarcopterygians (Goodman 1981b, Goodman et al. 1987a,b, Lê et al. 1989 1993, Joss et al. 1991, Stock et al. 1991a,b), and (2) the interrelationships of dipnoans, the coelacanth, and the tetrapods (Maeda et al. 1984, Hillis and Dixon 1989, Meyer and Wilson 1990, Gorr et al. 1991, Hillis et al. 1991b, Joss et al. 1991, Normark et al. 1991, Stock et al. 1991b, Meyer and Dolven 1992, Hedges et al. 1993, Lê et al. 1993, Noso et al. 1993, Yokobori et al. 1994; see also Forey 1988, Lecointre 1994a, Meyer 1995).

In the 1st set of studies, the relationships of hagfishes, lampreys, and gnathostomes were investigated to determine whether lampreys were the sister-group of hagfishes (monophyletic cyclostomes) or the sister-group of gnathostomes (monophyletic vertebrates) (Goodman 1981a, Lecointre 1989, Smith and Doolittle 1992, Stock and Whitt 1992a,b). Molecular data did not convincingly resolve this problem, despite the claim of Stock and Whitt (1992a) to have found monophyletic cyclostomes on the basis of 18S rRNA sequences. The results of this study depend to a significant degree on the outgroup chosen. A higher rate of 18S rRNA evolution in the hagfish leads to a long-branch attraction artifact, the position of the hagfish depending on the branch length of the outgroup. Molecular investigations on the interrelationships within gnathostomes have failed to resolve relationships between Chondrichthyans, Actinopterygians, and Sarcopterygians (Lê et al. 1989 1993, Normark et al. 1991, Stock et al. 1991b). This tends to support the idea of a rapid diversification of gnathostomes 420 my BP. If relationships of these groups are known and supported by reliable morphological synapomorphies (Maisey 1986), these relationships cannot be recovered through various molecular studies and lead to a multifurcation involving monophyletic chondrichthyans, monophyletic actinopterygians and polyphyletic sarcopterygians (Lê et al. 1989 1993, Stock et al. 1991a). A rapid diversification of early gnathostome lineages could have taken place in the Late Silurian, perhaps concomitant to the appearance of the jaw, that could be considered as a "key evolutionary innovation" (Hall 1992, p. 140; Liem 1974 1990). This would have left insufficient time to accumulate molecular synapomorphies compared to the longer elapsed time during which, later on, they could disappear.

The 2nd set of studies, following controversial discussions, ended with convincing data for a dipnoan-tetrapod sister-group relationship. The coelacanth is the sister-group of the clade (dipnoans + tetrapods) (Meyer and Wilson 1990, Forey 1991, Meyer and Dolven 1992, Hedges et al. 1993). Molecular phylogenies within Actinopterygians (Lê et al. 1989 1993, Normark et al. 1991, Bernardi et al. 1993, Müller-schmidt et al. 1993) provided robust nodes for Neopterygians from 28S rRNA (Lê et al. 1993). Neognaths, Percomorphs, Otophysans, and Salmonids are significantly supported on the basis of amino-acid sequences of growth hormone (Bernardi et al. 1993). Within teleosteans, a new clade grouping Clupeomorphs and Ostariophysans was found to be robust from 28S rRNA data of Lê et al. (1993) and from 18S rRNA data (unpublished work of Littlewood, Smith and Patterson 1994, Patterson's communication at the fish phylogeny workshop of the Thirteenth Willi Hennig Society meeting). This new clade contradicts the poorly defined concept of Euteleosteans (Rosen 1973). Morphological synapomorphies that supported the euteleostean clade (Patterson and Rosen 1977, Lauder and Liem 1983) can easily be rejected (Rosen 1985, Lecointre and Nelson 1996); and 4 morphological synapomorphies supporting the Clupeomorpha-Ostariophysi clade were proposed (Lecointre 1995, Lecointre and Nelson 1996). During the last few years the number of molecular phylogenetic studies dealing with relationships within a fish order or suborder has increased (Block et al. 1993, Meyer and Lydeard 1993, Müller-Schmid et al. 1993, Bargelloni et al. 1994, Cantatore et al. 1994, Alves-Gomes et al. 1995). Phylogenies at this taxonomic rank, or beneath it, are not in the scope of this paper (see for instance Meyer et al. (1990); Sturmbauer and Meyer (1993) on cichlids; Stock and Whitt (1992a) on lamprey lactate dehydrogenase; and Meyer et al. (1994) on the genus *Xiphophorus*). Numerous other studies concern "micro-phylogeny" (i.e.,

within a genus or species) of fishes of commercial interest (salmonids, gadids, pleuronectids, etc.) using various molecular tools.

SOME PROBLEMS WITH MOLECULAR TOOLS

Experimental screens

Some molecular tools lead to specific tree-construction methods. DNA-DNA hybridization and immunological data lead to distance-matrix methods. Such similarity measurements involve physical interactions at a level of integration that is higher than that of the strict sequence structures. Nucleotide and/or amino-acid sequences determine the complex interactions that are measured, but these interactions may not be the exact reflection of differences in linear sequences. First, a huge amount of structural information is lost. For instance, it is well known that amino-acid residues can be modified without affecting the electrophoretic properties of the protein. Second, such experimental evaluations of global sequence similarity suffer from specific artifacts. DNA-DNA hybridization and immunological data can provide asymmetric distances (dAB is not equal to dBA), while symmetric distances are always obtained from structural data. DNA-DNA hybridization can distort the relationships that could have been found through structural molecular synapomorphies (Sibley and Ahlquist 1990, Harshman 1994). The reason is that factors affecting the additivity of experimental distance matrices (differences in genome size, differences in rates of change among species, measurement error, paralogous sequences, intraspecific variations, Werman et al. 1990) are not detected at the same scale (and may not necessarily be the same) as those that would affect site composition in a given stretch of aligned sequences. These factors could have a similar role in the analysis of DNA sequences (i.e., increasing homoplasy) but could lead to differences in resulting trees. In other words, with these molecular tools, structures are detected through an "experimental screen" (Lecointre 1994b) that does not favor detection of a "phylogenetic signal" (Hillis and Huelsenbeck 1992, Hillis and Bull 1993).

Artificial homoplasy

Some molecular techniques provide discrete character data that can be analyzed through distance-matrix methods as well as parsimony methods. According to Swofford and Olsen (1990) discrete characters have to be homologous, independent and variable. These conditions are not always met. Restriction endonuclease data (Restriction Fragment Length Polymorphism) should always lead to restriction map reconstruction. When restriction maps are used, losses and gains of particular restriction sites can be coded as absence/presence and analyzed through parsimony procedures (Hillis et al. 1992). Some authors have directly coded the presence/absence of restriction fragments. Swofford and Olsen (1990) gave correct arguments to reject this. Their main argument is that the assumption of character independence is violated. Indeed, the size of a DNA restriction fragment strongly depends on the size of all other fragments. Throughout the 1970s and 1980s, data from isozyme electrophoresis were predominantly analyzed through matrices of pairwise similarities. But as discrete characters, methods have been proposed to analyze them cladistically (Patton and Avise 1983, Swofford and Olsen 1990). Data from Random Amplification of Polymorphic DNA are discrete and can be analyzed through parsimony (Smith et al. 1994), but they are not independent and may not be homologous. DNAs from various species are hybridized with a given mix of oligonucleotide probes. Similarity in amplification patterns does not mean homology in sequences contained in the bands observed. Phylogenetic reconstruction is therefore difficult to carry out from these data (Smith et al. 1994). Data from random amplification of DNA are useful for typing but should be used with caution for phylogenetic purposes.

In each case, the structures of sequences are not directly observed but revealed by a factor (electric field, restriction enzyme, mix of probes) that is more integrated. This factor can produce artifacts that could be called "artificial homoplasy", a kind of homoplasy that originates from our tools and methods, specifically when the nucleotide or amino acid sequence is not identified.

Homoplasy in sequence data

Immunological and DNA-DNA hybridization data have been widely used but are now increasingly being replaced by sequencing techniques, that have become more and more accessible, especially these last 10 years, since the spread of utilization of the polymerase chain reaction. The distinction between artificial homoplasy and

homoplasy in sequence data is somewaht arbitrary, because homoplasy is always due to a failure in observing or detecting characters as homologuous when, in fact, they are not. Homoplasy derived from experimental screens can be distinguished from homoplasy contained in the data, just because in the latter case sequence data are obtained, therefore amino acids or nucleotides are identified. In this case, in a given DNA sequence, a G is a G and there is no written pedigree to indicate if this common G in 2 species was inherited from a common ancestor (homology as synapomorphy, Patterson 1982, de Pinna 1991) or if it was gained 2 times independently (a case of homoplasy). This is determined a posteriori by the most parsimonious tree. Alignment of sequences is a hypothesis of homology (primary homology of de Pinna 1991). At some sites, for some nucleotide shared by a subset of species, this hypothesis is confirmed by the most parsimonious tree, identifying secondary homologies (nucleotides gained from common ancestry, i.e., synapomorphies). For some other shared nucleotide, this hypothesis is refuted by the most parsimonious tree, identifying convergences or reversions.

Homoplasy (convergences, reversions) is present in every biological data base (morphological or molecular) and can only be managed. Morphologists discard a priori an important part of homoplasy from their data, because the complexity of morphological characters allows them, in certain cases (for instance studying the ontogeny of the character), to identify convergences, reversions, etc. Molecularists use huge amounts of characters (sites in the aligned sequences) but cannot identify homoplasy a priori. A nucleotide at a given position is the ultimate structure. This difference explains why statistical approaches for measuring the robustness of phylogenies (for instance the bootstrap, Felsenstein 1985) are so commonly used with molecular data and so rarely used with morphological data. Statistical approaches for distinguishing "phylogenetic signal" from "noise" (Hillis and Huelsenbeck 1992) are easier to consider when using numerous and potentially homoplastic characters, but not at all when using only a few meticulously chosen characters. Morphologists therefore prefer to consider the number (and the quality) of synapomorphies at a given node as the indicator of its robustness. For both types of data, only the most parsimonious tree can reveal a posteriori which characters are homoplastic and which ones are homologous (synapomorphies).

Homoplasy in sequence data is generated by convergences, reversions, superimposed substitutions at a given site resulting from differences in rates of change among lineages, or by inadequate rates of change compared to the phylogeny inferred. For example, to limit the impact of homoplasy in coding genes, mutational saturation should be studied at each position of the codon separately, for transitions and transversions, respectively, by plotting the pairwise numbers of differences against the pairwise numbers of substitutions inferred from the most parsimonious tree. This method allows one to propose weighting schemes for codon positions, for instance the removal of 3rd positions of codons (for instance see the study of Kornegay et al. 1993 on cytochrome b sequences, and Meyer 1994a,b). Another way to limit the impact of homoplasy is to sequence the maximum number of taxa, because theoretically, superimposed substitutions should be identified and artifactual effects of long branches weakened. Some molecular studies have been carried out with the minimal number of species, i.e., 4 species (Meyer and Wilson 1990, Miyamoto et al. 1990 Graur et al. 1991). Analyses with too few taxa have been shown to create positively misleading situations (Lecointre et al. 1993, Philippe and Douzery 1994). In particular, Philippe and Douzery (1994) have shown that 4-species trees are excessively sensitive to long-branch attraction artifacts (Felsenstein 1978). A small difference in rates of sequence evolution tends to cluster the 2 species having close rates, and this artifactual grouping is highly supported by bootstrap proportions. Consequently, contradictory topologies can be significantly supported (using the bootstrap test) according to the species chosen to represent each of the 4 taxa. Four-species trees can be robust (in terms of bootstrap proportions or in terms of synapomorphies) but they cannot be reliable. Lecointre et al. (1993) had already reported that 4-species trees were too sensitive to species sampling. To a certain extent, so were trees with more species: the impact on variations in bootstrap proportions of species sampling (i.e., choosing a given species to represent a pre-defined group) decreases as the number of species increases. This impact of species sampling has to be related to the level of mutational saturation. The higher the mutational saturation level, the higher will be the perturbations generated by species sampling (changing 1 species for another to represent a given group) as detected by Lecointre et al. (1993). It

is therefore important to discard sites saturated with changes when they can be detected, for instance 3rd positions of codons from coding genes (Kornegay et al. 1993, Meyer 1994a,b).

Different properties of tree-construction methods

Tree-construction methods do not all have the same properties and do not imply the same assumptions. This question will not be developed in full details in this paper and all tree-reconstruction methods will not be cited (see Felsenstein 1983 1988, Swofford and Olsen 1990, Darlu and Tassy 1993). In the context of this general paper, only 2 important points will be briefly considered. First, one must not consider the tree-construction method as a black box. In other words, one must be aware of the assumptions in the method used. For instance, it is surprising to find in some papers (most of them in applied sciences) molecular phylogenies built with UPGMA (Sokal and Michener 1958) with no indications about the rate of evolution of the sequences used. UPGMA postulates that sequences evolve at the same rate in each branch, i.e., all lineages have diverged equal amounts. This is far from being always observed (see Li 1993), and the method is misleading if this assumption is not met in the data. If a distance method has to be used, it is better to use a method that makes no assumptions about rates of changes, such as Neighbor-Joining (Saitou and Nei 1987) (better yet, one must use parsimony methods, see below). Molecular clocks are sparsely observed, depending on the gene and the taxa. A molecular clock must not be a general assumption, but just a heuristical tool: it is the null hypothesis to measure gene deviations from a clockwise behavior.

The 2nd point concerns different sensitivities of parsimony methods and distance-matrix methods towards homoplasy. Generally, when a huge number of taxa are used, topological effects of homoplasy tend to be diluted throughout the tree from distance data (for instance with the Fitch and Margoliash (1967) method), while topological perturbations are more concentrated in some restricted area(s) of a parsimonious tree (for instance with PAUP, Swofford 1990). Consequently, differences in trees obtained with the 2 methods from the same data set can be found. The distance-matrix method that provides the closest results to parsimony is Neighbor-Joining(NJ), perhaps because a criterion of minimal evolution is used in NJ. Another source of incongruence between parsimony and distance-matrix trees is the clustering of taxa on symplesiomorphies (as well as synapomorphies) by distance-matrix methods. The root of the general UPGMA-tree of birds of Sibley and Ahlquist (Sibley and Ahlquist, 1990) is a classical example where Paleognathes, Anseriformes and Galliformes are clustered on the basis of shared primitive characters. From sequence data, NJ can cluster two species together within the ingroup while there is not a single synapomorphy for the 2 species in the sequence data (Leclerc et al., in prep).

These differences come from the concept of global similarity as used when distances are calculated, which constitutes a real problem in phylogenetic reconstruction. Distances were rejected by cladists a long time ago (see Hull 1988). Morphologists do not analyze their data with distances anymore, while molecularists continue to do so. This difference may partly be linked to the intrinsic interest devoted to individual characters. Morphological characters are highly integrated structures resulting from complex epigenetic phenomena and combinations of various ontogenetic factors. Consequently, it is interesting to infer, through the nodes of the most parsimonious tree obtained, the way in which a given character has evolved. Such an inference of ancestral states of the character at each node can be performed only through parsimony procedures (Lecointre 1994b; for instance using PAUP, Swofford 1990). But the simplicity of molecular characters (a nucleotide at a given position), in the absence of any other functional or structural information, weakens the interest that could be devoted to the inference of ancestral states, i.e., to the history of individual sites. This may be the reason why most molecularists do not concern themselves at all about using distances versus using parsimony. They do not feel the need to produce analytical feedback to reveal evolutionary pathways of the characters they have used. Every paleontologist I know working on a molecular data base uses parsimony methods for tree reconstruction. A paleontologist or any morphologist instinctively pays attention to the way individual characters have evolved. Only parsimony procedures allow one to follow character changes at each node.

Reading this, one might think that it is technically possible to perform molecular character mapping on distance-matrix trees. In this case, the history of a given site is inferred from nodes resulting from the distance matrix method. I do not recommend this. First, using a distance matrix

method to perform this inference is just introducing useless complications. It would be far simpler to use PAUP. Second, depending on the properties of the data, parsimony methods and distance matrix methods can provide different trees (Hillis et al. 1994, see above). A character (a site in a sequence) in this case would have to be read vertically (as a site) in a tree for which sequences have been read horizontally for pairwise distance calculations. Some nodes may be suspected of having been obtained because of distance-matrix method artifacts (for instance, groupings based on symplesiomorphies). This way of working violates the basic philosophy of inferring ancestral states. Ancestral states to be inferred at a node must result from the most parsimonious changes in the most parsimonious tree(s), not in a tree containing artifactual nodes given by the global similarity concept. In the practice of character mapping, the nature of characters does not matter. Morphological characters can be mapped on a molecular cladogram, which makes sense because sets of independent characters are supposed to provide the same evolutionary history (problems of sampling and homoplasy apart), given the same tree-construction method: maximum parsimony. The philosophy underlying the reference tree is more important than differences in the nature of the characters. I consider the Hennigian method of analyzing characters to represent undeniable progress over global similarity that was used before (and modern distance-matrix methods are computerized ways to deal with the same old global similarity concept).

Intellectual attitudes towards "true phylogeny"

Belief in a knowable "true phylogeny" is one of the factors which makes communication difficult between those working on molecular data, often analyzed with statistical tools, and those working on morphological characters, with most researchers defending parsimony (cladistic) procedures. Everyone agrees that species have evolved, and that their relationships can be inferred. The problem comes from what one projects on this inference. There are 2 different intellectual attitudes towards homology and trees: many molecularists exhibit essentialist behavior, while cladists utilize hypothetico-deductive approach. Essentialists see homology as revealing the essence of characters, and therefore, to a certain extent, the concept of parsimony is not easily used in the alignment of sequences. The tree obtained, which robustness is measured with statistical tools (such as the bootstrap), is supposed to be the "true tree". Consequently, bootstrap proportions are often erroneously seen as the probability for the node to be true, as an idea criticized by Hillis and Bull (1993). Essentialists have a vision of their results with the idea that "true" historical links can be recovered, an intellectual attitude that does not favor the acceptance of tests and refutations. The hypothetico-deductive approach of cladist morphologists, on the contrary, considers that homology is an inference by itself. Sequence alignment can be (and must be) processed through a parsimony procedure. The tree is a hypothesis on relationships open to refutation, and this heuristic property is far more important than the problem of knowing whether the tree obtained is "true" or not (which is unknowable). I will try to evaluate each point of view.

In the choice of a tree-construction method, many molecularists are most interested in the efficiency of the method. If there is a "true tree" to be found, the best tree-construction method is the one having the best scores in recovering the "true tree". They are not much concerned about philosophical grounds and assumptions of the method. All existing methods are even sometimes seen as grossly equally pertinent (see Meyer 1994a). If Neighbor-Joining (Saitou and Nei 1987) is found to be a fast and efficient distance-matrix method (with best scores), they will just consider it as the best one, in spite of the underlying global similarity concept. Meyer (1994a) seems to regret that studies that test the "power" of various commonly used methods of phylogenetic reconstruction are rare and have "failed to clearly identify a particular methodology as the best". Here, this enigmatic "power" of a method is the criterion one should focus on, not historical or philosophical grounds. The accuracy of methods of phylogenetic analysis can be assessed by the examination of numerical simulations in which evolution of virtual sequences occurs according to a model of evolution (including rates of changes, transition versus transversion frequencies, etc.) and a virtual "true tree". For example, Hillis's group has produced such analyses (Huelsenbeck and Hillis 1993). But Hillis et al. (1994) stress that models are far from satisfactory: "the primary limitation of numerical simulations is that they always include gross simplifications of biological processes". Pheneticists should consider that even when using such simulations, the weighted parsimony method was found by Hillis et al. (1994; Figs. 1 and 2 of Hillis et al.

1994) to be the most efficient (i.e., in recovering the "true tree" under various conditions of unequal rates among branches). However, until now, tests of tree-construction methods depended on models of sequence evolution including a true tree. Because of the unreliability of models of molecular character evolution, Hillis et al. (1992) have produced experimental phylogenies of viruses derived from bacteriophage T7 in which the shape of the phylogeny (order of branching events and time between events) and some details, such as population size and mutagenic environment are controlled by the investigator. The evolutionary changes incorporated depend on the constraints imposed by the experimental organisms. For the 1st time, the true tree can be claimed to be completely known and experimental phylogenies can provide a "reality check on simulation studies and a test of the fallibility of tree-construction methods" (Hillis et al. 1994). All the methods recovered the actual tree from restriction site maps but differed in their ability to recover branch lengths of the phylogeny (Hillis et al. 1992 1994). From the complete sequence data set, only parsimony and weighted parsimony found the correct tree (Hillis et al. 1994). The authors advocated combining numerical simulations and experimental phylogenies, one of the aims being to improve simulations, because ". . . the experiments suggest additional complexities that need to be added to simulations". Experimental phylogenies could be considered the best way to test tree-construction methods, at least because there are evolutionary constrainsts in the experimental organism that are never taken into account in simulations.

But one should be very careful with extending conclusions to situations in the wild. Sober (1993) criticized ". . . one needs a model of the evolutionary process to assess whether laboratory success strongly supports the idea that a method will succeed in the wild. . . . It is difficult to determine whether natural processes and laboratory processes are relevantly the same without knowing which points of similarity and difference actually influence the performances characteristics of various phylogenetic inference methods. . . . The problem of determining whether an inference method will probably retrieve true phylogenies from natural data sets has two parts. First, there is the empirical task of finding a realistic model of natural processes. Second, there is the mathematical problem of discovering whether the model makes it probable that the method will retrieve the true phylogeny when supplied with enough data. Neither of these problems is solved by observing that the method happens to retrieve the true phylogeny in an experiment". Hillis et al. (1993) answered that they "did not consider that the methods would retrieve true phylogenies in nature when the methods all agree. . . . The difference between experimental and simulated phylogenies is like the difference between experimental and simulated bombs: the explosion of an experimental bomb does not indicate what will happen every time a bomb explodes, but it does provide information on one actual explosion. Simulations, on the other hand, provide exhaustive information on an idealized set of conditions that never actually exist in nature. Combining simulations with experiments is likely to result in the refinement of both, and the theory of phylogenetic estimation can thereby advance."

Another problem with the naive essentialist way to consider phylogenies is many confusions about the nature of parsimonious trees. Meyer (1994a: p. 224) in writing about the parsimony method stated that "evolution is believed to proceed by the shortest, simplest pathway". This is a typical misunderstanding of parsimony procedures. History of taxa definitively belongs to the past. The only thing we can do is to infer this history through phylogenetic reconstruction. The parsimony procedure is only a heuristical mean to perform this inference, and has nothing to do with the underlying evolutionary processes. The use of parsimony follows Ockham's principle according to which, when facts have to be explained, one must choose the theory implying the minimum number of hypotheses, simply because there's nothing else we can do. Otherwise, everything can be proposed and nothing explained. Parsimony is just this general principle of every science transferred to comparative biology, and intrinsically makes no assumption on evolutionary pathways (i.e., if they were parsimonious or not. . .). Another common mistake is the confusion between the cladogram and a scenario where processes are shown. For example, there is a typical belief that nodes of a cladogram mean that speciations were all dichotomic. It is true that, in principle, 2 taxa diverged in the past through a speciation. But the way it occurred (i.e. number of populations separated, etc.) is unknowable. Dichotomic nodes are just the result of the maximum resolution of a tree indicating sister-group relationships. It makes no a priori assumption on evolutionary processes.

Morphologists and, usually cladists, prefer to focus on the assumptions of the tree construction method, rather than on its efficiency, because

the true tree is considered as unknowable. The most important point is to keep a hypothetico-deductive approach that leads one to consider the tree as a temporary hypothesis, open to further tests. In other words, epistemological factors are more important than technical ones in the choice of a tree-construction method. What is important is to introduce knowledge about the process of character changes into weighting schemes. Patterson (1994) writes: "Where models are introduced in phylogenetic reconstruction, we should prefer models dictated by features of the data to models derived from explanatory theories", and, more precisely "If (phylogenetic) knowledge is claimed, how was that knowledge gained? If assumptions are held to be merely provisional or approximate, how will they be tested by phylogenies that depend on them? I am therefore interested in methods that demand either no model at all, or the most minimal. . . . In molecular systematics . . ., I argue that efficient models are dictated by properties of the data, not by assumptions about or knowledge of phylogeny". For cladists, the method used must only be compatible with phylogenetic aims (character polarization that makes operative the concept of descent with modification, and no use of global similarity) and must incorporate what we know about the processes of molecular evolution. The aim is to recover a hierarchy, whatever the result that will be obtained.

Molecules versus morphology: robustness analysis to avoid false conflicts and total evidence as the issue of data conflicts

Because molecular and morphological characters are different in essence, i.e., they are not in the same integration level, we often tend to focus on phylogenetic conflicts between molecules and morphology. But Hillis (1987) correctly wrote that "conflicts among morphological or among molecular studies are probably as common as real conflicts between morphological and molecular studies". Patterson et al. (1993) concluded: "Congruence between molecular phylogenies is as elusive as it is in morphology and as it is between molecules and morphology." Conflicts between molecules and morphology can be due to various factors discussed by Hillis (1987), Patterson (1987), and Patterson et al. (1993), but do not merit a special status. These factors can be divided in 3 families: first, the sampling factors; second, the homoplasy factors; and third, the methodological factors. In the first we find taxon extinction (Huelsenbeck 1991, Wheeler 1992); the sampling of different terminals as representatives of taxa in 2 different data sets (Lecointre et al. 1993, Patterson et al. 1993, Philippe and Douzery 1994); and differences in size of taxonomic samples (Graur et al. 1991, Lecointre et al. 1993, Lecointre 1994a). In the second family we find unequal rates of molecular evolution among lineages; saturation in molecular changes; base composition bias within molecules; problems of homology assessment in molecules as well as in morphology, as listed by Patterson et al. (1993) (within molecules: problems of sequence alignment, of paralogy or xenology; within morphology: convergence, weight of tradition, errors in polarity assessment); and different abilities between morphological and molecular characters to retain character states (important in case of rapid evolutionary radiations, Lê et al. 1993). The methodological factors are various artifacts cited above (assumptions of the tree-construction method violated in 1 of the 2 studies, etc.), and some others such as different methods of analysis (for example phenetics versus cladistics); and differences in the species concept (mixiological concept in molecular biology versus typological concept in paleontology, see Philippe et al. 1994a).

If 2 (or more) conflicting trees have to be considered, one must check first whether both conflicting nodes are robust. A method to measure robustness is therefore needed. In the great majority of studies in applied sciences, no information is given about the robustness, and more generally, the reliability of the trees produced. Penny and Hendy (1986) wrote: "In our opinion, it is unreasonable to publish an evolutionary tree derived from sequence data without giving an idea of the reliability of the tree." This is also true for other types of data (electrophoretic data, Restriction Fragment Length Polymorphism data, etc.). The most commonly used statistical tool to measure robustness of trees is the bootstrap (Felsenstein 1985, Hillis and Bull 1993). Possible causes for most of the bootstrap analyses being performed by molecularists have already been discussed above: the numerous and simple molecular characters are easy to analyze statistically. Morphologists often consider that the sole number of synapomorphies supporting a node is, by itself, a measure of its robustness. Kluge and Wolf (1993) have provided arguments to reject the bootstrap. Their extreme position rests on the fact that the bootstrap "makes several assumptions, and that most, if not all, of those premises appear to be violated". However, the relationship between bootstrap proportions and

the number of synapomorphies at a node remains to be elucidated experimentally.

Whichever way one considers the robustness of nodes, only conflicts generated by 2 contradicting robust nodes must be considered. If the conflict is real, one may use 1 of the consensus methods that have been proposed: Adams consensus (Adams 1972), strict consensus (Nelson 1979), or majority consensus (Margush and McMorris 1981). But Barrett et al. (1991) developed convincing arguments to reject the use of consensus trees from different data sets. They recommended the practice of the principle of "total evidence" (Kluge 1989, Barrett et al. 1991, Kluge and Wolf 1993): "But why should the consensus tree be constructed from trees based on different data sets? Instead, why not pool the observations and find the most parsimonious tree for all of the data? . . . When there are many molecular characters but few morphological ones, the result of pooling may be to "swamp" the morphological characters. Of course, characters can be pooled and a weighting scheme imposed (Miyamoto 1985), but the worry has been that the weighting scheme cannot be objectively defended . . . Consensus methods seem to possess the virtue of allowing biologists to avoid apparently unresolvable weighting problems" (Barrett et al. 1991). If pooling all the data in the same data set lightens problems of character weighting, consensus trees do not solve or avoid this problem, because they generate implicit character weighting: "if one has many molecular characters and few molecular ones, for example, the method of consensus appears to imply an equal weighting of data sets and hence an unequal weighting of the constituent characters. If the two data sets count equally, then each molecular character would receive a lower weight than any morphological one" (Barrett et al. 1991). These authors demonstrate that the consensus tree from 2 data sets can contradict the most parsimonious tree obtained from all the data. The best phylogenetic hypothesis is the one obtained from all the available data, pooled in a single set whatever their nature. One of the reasons is that character congruence is more important than tree congruence. Even if some biologists feel uneasy combining morphological and molecular data in the same data set, there is no objective and/or theoretical biological reason to reject the principle of "total evidence". So, management of phylogenetic conflicts can become an issue through application of this principle, i.e., through obtaining the most parsimonious tree from all the data. This requires an effort to provide arguments for a weighting scheme.

SAMPLING PROBLEMS IN MOLECULAR PHYLOGENETICS

When a molecular phylogeny is investigated from sequence data, 2 sampling parameters are to be considered: the taxonomic (vertical) and the character (horizontal) samplings. The impact of these 2 parameters on the robustness of the phylogenetic inference were studied by Lecointre et al. (1993) and Lecointre et al. (1994) using a rich data base (Lê et al. 1993) of 28S rRNA sequences from Gnathostomes (jawed vertebrates, most of the sequences from fishes), and by subsequent studies of Philippe and Douzery (1994) and Philippe et al. (1994b).

Species sampling and robustness of molecular phylogenies

Problems of homoplasy can theoretically be solved by increasing taxonomic sampling. This rests on the idea that if all the organisms that existed were available and analyzed, character changes would be unambiguous. Taxon sampling is a major component of the practice of comparative biology, but only relatively rarely evoked (Gould 1985a, Swofford and Olsen 1990, Dupuis 1992); and has only recently been analyzed (Lanyon 1985, Smouse et al. 1991, Wheeler 1992, Lecointre et al. 1993, Philippe and Douzery 1994). There is neither an "objective" nor "absolute" taxa sample, i.e., none which would "perfectly" represent a given idea of the hierarchy of life. Indeed, the pertinence of a taxonomic sample cannot be tested (the whole biodiversity of the group being inaccessible, such a test would require another sample. . .). This pertinence actually depends on the way a given knowledge (the author's idea of the hierarchy of life: his mental classification) fits the rules of the practice of phylogenetics. Lecointre (1994a) argued that these rules impose constraints to taxonomic sampling prior to any phylogenetic analysis (molecular or whatever), and that there are a priori classifications that are not compatible with such a practice. The pitfall of reductionism of species samples is a weakness of the young science of molecular systematics, and its results are reliable only (1) when they are based on a sufficiently large taxonomic sample, and (2) when some sampling rules are followed. In particular, when an

investigation begins and no data are already available, pilot studies are required (Baverstock and Moritz 1990, Lecointre 1994a, Meyer 1994b).

Using a data base of gnathostome 28S rRNA (Lê et al. 1993), Lecointre et al. (1993) explored the impact of species sampling (changing 1 species for another to represent a given group) on the robustness of fish phylogenies. The richness of this taxonomic sample allowed the precise study of the amplitude and distribution of the variations in bootstrap proportions provoked by variations in species sampling. Results showed that 4-species trees are not reliable because they strongly support contradictory relationships depending on the species sample chosen to represent the same taxa. This feature is more convincingly and completely demonstrated by Philippe and Douzery (1994, see above) using various molecules. An original procedure of exhaustively sampling all the combinations of a single species per presumed monophyletic group (without any a priori assumption about relationships between these groups) showed precisely the impact of each species on the bootstrap proportion of each node of the resulting multiple bootstrap trees. This showed that the impact of species sampling of a given group is strongly localized, limited to its branching node in the tree and its 2 neighboring nodes. These conclusions can be generalized to various other molecular data sets. The impact of choosing a given species to represent a given group on the robustness of the resulting tree will be greater if sequences are saturated with changes. In Lecointre et al. (1993) this impact is stronger than previously thought, perhaps because of the mutational saturation in transitions. Robustness does not mean reliability: 4-species trees can be robust but not reliable. The more species there are to investigate a phylogenetic problem, the more reliable molecular phylogenies are.

Sequence length and robustness of molecular phylogenies

The impact of variations in sequence length on bootstrap proportions was studied from the same data base by Lecointre et al. (1994). In the aligned sequences, informative sites were jackknifed (= sampled without replacement) several times to constitute several new data bases ("subsamples") of various sequence length. Then, bootstrapping (= sampling with replacement) was performed on each of these subsamples. For the numerous bootstrap trees so obtained, bootstrap proportions (BPs) of all the nodes appearing more than 10 times over 1 000 were recorded. For each node, BPs so obtained were plotted against sequence length, showing the evolution of the robustness with increasing number of informative sites. For robust nodes, robustness was an exponential function of sequence length. The pattern of BPs was unvarying and described by the function $BP = 100\,(1 - e^{-b(x - x')})$, where x is the number of informative sites, and b and x' are 2 parameters estimated using a non-linear regression procedure. When a node had a $BP < 100\%$ and the pattern of BPs fitted this function, it was possible to estimate the number of informative sites required to obtain a given average BP. The method also identified nonrobust nodes, for which it would be more cost effective and fruitful to turn to other species and/or genes rather than to continue sequencing longer gene lengths from the same species to reach a BP of 95%. Lecointre et al. (1994) therefore proposed a tool to manage sequencing effort. Indeed, molecular systematists, in their conclusions, often express the hope of solving their remaining unresolved nodes with longer sequences. Until now, however, very few of them have used tools to decide how much extra sequence is needed. Bootstrapping subsamples of sites obtained through site jackknifing constitutes an informative new tool for a better evaluation of the robustness of nodes. The study of Lecointre et al. (1994) led to the conclusion that proving robustness of a given node may often require very long sequences, and that reliability of a tree increases with the number of species (Lecointre et al. 1993). In practice, this leads one to perform a huge sequencing effort for each phylogeny. However, robustness must not be expected for every node of a given tree, especially when there are numerous species. One must admit that some of the nodes will remain unresolved and it is pointless to search for the complete resolution.

So, how should the number of species and sequence length be managed to perform a phylogenetic analysis? The following guidelines may reduce the sequencing effort. First, after the pilot study (Baverstock and Moritz 1990, Lecointre 1994a, Meyer 1994) indicating that the gene is an appropriate one to use to answer the phylogenetic question, a sequencing effort is produced, and a tree is obtained using as many species as possible. A significant number of nodes may be unresolved. Second, one must select the promising nodes of phylogenetic interest, and add supplementary sequences as appropriate for these nodes. To do

this, and to know what resolution can be expected from sequencing a given additional length of sequence with the same informative properties (the same ratio of informative nucleotides/sequenced nucleotides), bootstrapping from diverse subsets of informative sites (obtained through site jackknifing) and construction of BP/sequence length diagrams (as in Lecointre et al. 1994) can be recommended. Obtaining diagrams for each node would require more computing operations and calculation time to carry out the numerous bootstrap analyses; but these operations are easily done using specific programs included in the MUST package (Philippe 1993). The cost of these bootstrap analyses is far less than that of sequencing blindly, without knowing how much sequence length is needed. If nodes of particular phylogenetic interest do not present a diagram with a pattern of resolved nodes, then the sequencing effort to reach a high average BP will be too great. The node should be left unresolved and the sequencing strategy should be modified, i.e., change the species sample, and/or the gene.

An interesting outcome from these tools is provided in the study by Philippe et al. (1994b). Starting from the above formula linking bootstrap proportion and sequence length, and assuming a molecular clock (using palaeontological dates for calibration), these authors have solved for the 1st time the crucial problem of measuring the resolution power of a given molecule: they established a relationship between the number of sites contained in a given data set and the time interval between 2 cladogeneses that this data set can confidently resolve (with 95% bootstrap support). They inferred that their data base of complete 18S rRNA aligned sequences of metazoans cannot confidently resolve cladogenetic events separated by less than about 40 my. The conclusion was that the 18S rRNA molecular approach cannot resolve the cladogenetic events of metazoan diversification (the "Cambrian explosion"), that took place during a relatively short time interval, 20 my (540-520 my BP).

CONCLUSION: WHAT IS A PHYLOGENY?

It is common to say that all trees resemble each other. To understand a tree, it is imperative to know what method is behind its construction. As previously argued by Lecointre (1994c), classifications of biological entities should be produced with tools that produce not only trees, but trees that are phylogenies, i.e., cladograms. The phylogenetic dimension of a tree cannot be considered to come only from the data themselves. There is one major condition: the method used must explicitly and operationally assume that characters have evolved. This condition is manifested in cladistics through character polarization.

The biological entities compared, whatever they are (strains, populations, species, genera, families, etc.), have historical links between them. The comparison of the characters studied (molecular, morphological, or whatever) makes sense only in the light of the concept of common descent (of organisms through generations) with modifications (of characters): there is no phylogeny that is outside of this concept. Though note here that this concept is very different from that of classifying living organisms (where this concept can be used) and classifying, for instance, toys or cartoon characters. In this latter case, groupings are based on non-historical criteria: in absence of descent of cartoon characters from ancestors, this concept cannot be used. These considerations imply that every tree built from biological entities should be cladograms, whatever the field of research.

In applied science symposia, phenograms are sometimes shown pretending that they are not a phylogeny but only a "classification", while the conclusions drawn from these trees are explicitly historical. The problem comes from the fact that some tree-construction methods (phenetics) do not explicitly incorporate in their procedure the concept of descent with modification. Thus one should not be allowed to infer historical conclusions from a phenogram. Cladistics explicitly introduces the concept through character polarization, legitimizing historical conclusions. Phenetics clusters on the basis of synapomorphies as well as symplesiomorphies: a phenogram is not a cladogram. Phenetics can work equally well on non-biological things. At this point, the problem is to know whether this concept must be present and operative inside the tree-construction method itself or can just be outside it, i.e., just relying on how the investigators consider the characters examined and how they speak about the tree obtained.

Darlu (1994) developed the argument that a tree-construction method and data themselves cannot produce the phylogenetic dimension of a tree. A tree becomes a phylogeny with elements that are external to the tree construction; for example, the evolutionary hypotheses underlying the choice of characters, weighting schemes incorporating features of processes of character evolu-

tion, the position of the root, etc. If by "method", Darlu understands the algorithm used, we agree with Darlu concerning cladistics because character polarization is outside the strict tree-construction algorithm. But Darlu's considerations are too general and could lead one to consider that distance-matrix trees could become phylogenies when the investigator speaks about it as such. In my opinion, the concept of descent with modification cannot be facultative, it must be more explicitly incorporated in the tree-construction method sènsu lato. Cladistics explicitly introduces descent with modification when the data matrix is produced (before tree construction itself) and characters polarized (before or after tree construction), whereas distance-matrix methods do not.

The most rigorous definition of a phylogenetic tree has 2 components. The 1st component is that the method, to be phylogenetic, must explicitly incorporate at one step or another the concept of descent with modification (like in Hennig's character polarization). The 2nd component is that the method must allow in fine to reveal homoplastic characters (character convergences, reversions, etc.) and "true" homologous characters (homogenies of Lankester, 1870; synapomorphies of Patterson, 1982; de Pinna, 1991; Nelson, 1994). This last point of view was correctly developed by Tassy and Barriel (1995). This is possible only with parsimony methods (see above) and, to a certain extent, with maximum likelihood method (Felsenstein 1981). Phenetics cannot lead to character mapping (see above). As argued by Lecointre (1994b), character mapping makes sense only on parsimony trees, just because mapping characters (that is revealing on a tree orientation and position of character changes) cannot hold if the method underlying the tree clusters taxa on symplesiomorphies. In other words, if a parsimony tree had been obtained on the basis of characters underlying the distance tree, one would have obtained nodes that are not present in the distance matrix tree, just because of distance methods artifacts. According to this definition of the phylogenetic tree, distance matrix methods cannot pretend to be phylogenetic, and are considered by Tassy and Barriel (1995) as "pseudo-phylogenies".

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魚類分子親緣關係之方法論

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過去三十年以來，在探討魚類親緣關係的方法上發生二個重大的改變。首先，在60年代的末期將支序分類學的觀念引入魚類學，使魚類的系統分類有長足的進展。其次是分子的方法特別是DNA的資料的累積，提供了探討親緣關係上一個有用的特徵，尤其是那些用形態的方法尚未解決的問題。但是時至今日，這些對於整體魚類系統分類的衝擊卻十分有限。

無論其研究的目的爲何，爲了避免爲實驗所遮蔽(experimental screens)，在不同的分子方法中應該要選擇可以確認的分子結構的方式，例如求其序列(sequencing)。在基因、物種、甚至系統樹的建構上，都要避免可能的陷阱。如此才能考慮正確的親緣關係。分子學家(遺傳學家)與形態學家對於親緣樹的概念有許多的差異。前者最常運用距離矩陣，並且很自然的認爲這是在序列排列與系統樹的建構上的一種必然方法。支序學者則大部份都是形態學家。多數的分子學家還無法完成整體的親緣關係。只有最大簡約法(parsimony)能夠鑑定同緣的特徵，也就是眞正的親緣關係。親緣關係是一部生命的歷史的總結，必須用假說演繹法來得到。最大簡約法在這方面應該優於距離矩陣法。

關鍵詞：魚類之分子親緣關係，親緣關係之重構，穩定性，系統分類，系統樹。

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