

Do Molecular Clocks Run at All? A Critique of Molecular Systematics

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Abstract

Although molecular systematists may use the terminology of cladism, claiming that the reconstruction of phylogenetic relationships is based on shared derived states (synapomorphies), the latter is not the case. Rather, molecular systematics is (largely) based on the assumption, first clearly articulated by Zuckerkandl and Pauling (1962), that degree of overall similarity reflects degree of relatedness. This assumption derives from interpreting molecular similarity (or dissimilarity) between taxa in the context of a Darwinian model of continual and gradual change. Review of the history of molecular systematics and its claims in the context of molecular biology reveals that there is no basis for the “molecular assumption.”

Keywords

cladism, Darwinism, dissimilarity, similarity, systematics

Claims that humans and chimpanzees are essentially identical molecularly, and therefore the most closely related large-bodied hominoids (humans/hominids and great apes), are now commonplace. Indeed, in a science in which philosophers (Popper 1962, 1968, 1976; Wiley 1975; Patterson 1978) have long argued that nothing can be proven, only falsified, this hypothesis is so entrenched that any explanation of inconsistency in the data is accepted without question. Witness, for example, the recent scenario that for some millions of years after their lineages split, hominids and chimpanzees continually interbred and produced reproductively viable hybrids (Patterson et al. 2006).

For historians and philosophers of science the questions that arise are how belief in the infallibility of molecular data for reconstructing evolutionary relationships emerged, and how this belief became so central, especially to paleoanthropology, which as a paleontological enterprise can only rely on morphology. Part of the answer comes from the history of human paleontology itself.

A Brief History of Human Paleontology

When Pilgrim (1915) described the first specimen of the Miocene hominoid, *Sivapithecus*, he thought it was a hominid ancestor. Gregory (1915), however, allied *Sivapithecus* with the orangutan, with which he later also aligned the australopithecids (Gregory and Hellman 1926). With more specimens of Siwalik hominoids to work with, Lewis (1934, 1937) named *Ramapithecus* and declared it a human ancestor. Gregory and Lewis (1938) subsequently incorrectly allocated other specimens to *Ramapithecus*, which led to years of systematic confusion. In 1964, Simons correctly identified specimens of *Ramapithecus* and reaffirmed its hominid status.

Features that Simons suggested united *Ramapithecus* with hominids and which survived scrutiny by other paleoanthropologists were the development of low-cusped cheek teeth and thick molar enamel. Since *Sivapithecus* and other fossil hominoids could be similarly described, they were grouped with *Ramapithecus* (Pilbeam 1986), and it was from some member of this group that the ancestry of proper hominids was sought. Among living apes, only orangutans had thick molar enamel (Schwartz 2005b), but since the preferred theory of hominoid relationship was that humans were related to a great ape group, various scenarios were proposed to explain how orangutans evolved thick molar enamel in parallel with a ramapithecid–hominid clade (ibid.). Adherents of the less popular human–African ape theory of relatedness also failed to entertain an orangutan–ramapithecid–hominid theory of relatedness, although, they admitted, their theory was based on scanty morphology (Andrews and Cronin 1982). But for an increasing number of paleoanthropologists, morphology need not assume a prominent role in reconstructing phyloge-

netic relationships because they embraced the human–African ape theory advocated by molecular systematists (reviewed in Schwartz 2005b).

The “straw that broke the morphologists’ back” came with the discovery of facial skeletons of *Sivapithecus*, which possessed many features otherwise seen only in orangutans (Andrews and Tekkaya 1980; Pilbeam 1982). However, rather than seeing this new information as leading to a theory of orangutan–ramapithecid–hominid relatedness and falsifying the human–African ape theory, most paleoanthropologists clung to the latter. Consequently, they rejected a ramapithecid–hominid relationship and, via *Sivapithecus*, allied all ramapithecids with the orangutan.

By rejecting a ramapithecid–hominid relationship, and the antiquity of the human lineage inferred from it, another facet of interpreting molecular data came into play: namely, the calculation of much younger dates (between 4 and 6 mya) for the presumed split between human and African ape lineages. Elimination of a Miocene human ancestor thus appeared further justified. This presumption had numerous effects, not least of which was loss of faith by most paleoanthropologists in the reliability of morphological data in phylogenetic reconstruction (Pilbeam 1986). Yet, while this was clearly not the only available conclusion, its widespread acceptance had the effect of imbuing molecular data with an aura of infallibility and thus unfalsifiability. The contradiction, of course, is still that the systematics of extinct organisms can only be approached through study of morphology, and the only way in which one can calculate presumed rates of molecular change, and thus estimate dates of lineage splitting, is by reference to the fossil record.

In light of this history and the contradictions that emerged during it, it might be instructive to revisit the history of “molecular systematics” in order to better understand its theoretical and methodological underpinnings.

The Molecular Assumption (MA)

In 1962 Emil Zuckerkandl and Linus Pauling contributed to a volume on the biochemistry of blood in which they focused primarily on describing different kinds of hemoglobin. In the latter part of their contribution, they investigated degrees of similarity and difference in adult hemoglobin among a small number of taxa and attempted to interpret their results in an evolutionary context.

The premise of their interpretation was first articulated by Nuttall (1904), who sought to determine phylogenetic relationships by analogy with the concept of “blood relationships.” That is, the more similar taxa were in “their blood”—as determined by the degree of intensity of whole blood serum–antiserum reactivity—the more closely related they were. On one level this analogy seems reasonable: one is more closely

related to one's kin than to one's friends, and, among one's kin, there is a hierarchy of relatedness. And Nuttall's arrangement of taxa based on "blood relatedness" did mirror the generally accepted, morphologically based pattern of relatedness between and among invertebrates and vertebrates.

Zuckerkindl and Pauling (1962) compared human, gorilla, horse, chicken, and, although it accesses oxygen differently than tetrapods (and would thus have very different hemoglobin), fish hemoglobins. They discovered that there was less difference between human and gorilla than between them and horse, but more between these taxa and chicken, and more again between these vertebrates and fish. Since their arrangement of taxa mirrored a generally accepted, morphologically based scheme of relatedness—(((human–gorilla)–horse)–chicken)–fish—they felt justified in proposing a model of molecular change that explained varying degrees of similarity in terms of the Darwinian notion that organisms are continually and gradually changing. From this perspective, one envisions lineages with one species gradually transforming into another. Diversification occurs when one lineage splits into two or more that become increasingly different from one another as they gradually change over time.

Zuckerkindl and Pauling (p. 223) suggested that their observations "can be understood at once if it is *assumed* [emphasis added] that in the course of time the hemoglobin-chain genes duplicate, [and] that the descendants of the duplicate genes 'mutate away' from each other." As such, they argued, "over-all similarity must be an expression of evolutionary history," with descendants "mutating away" and becoming "gradually more different from each other." The more ancient the divergence of lineages from a common ancestral lineage, the more time there will be for molecular changes to accumulate in descendant lineages; the more recent the divergence, the less time there will be for molecular changes to accrue. They then assumed that the taxa that are most similar in the molecule being compared are the most closely related while those that are less similar are more distantly related. Crucial to this model is the assumption that lineages exist and that they are in constant states of gradual change.

Zuckerkindl and Pauling's assumption of continual molecular change quickly became central to a phylogenetic interpretation of degrees of immunoreactivity in studies involving other blood serum proteins (Goodman 1962; Sarich and Wilson 1966, 1967a, 1967b).

Early Applications of the MA

From study of immunoreactivity of the protein albumin in a small sampling of various Old World monkeys and hominoids, Sarich and Wilson (1966) devised a test of reciprocity (= the relative rate test), whereby albumin of taxon A was combined with antialbumin of taxon B and then albumin of taxon B was combined with antialbumin of taxon A. If the degree

of reactivity was similar in both tests, they assumed that the amount and rate of change in each lineage had been the same and thus that the taxa were equidistantly related to each other. They also argued that if the rate of molecular change was the same and constant in all lineages, then molecular change must occur in a clocklike fashion. They "calibrated" the resultant "molecular clock" with a paleontologically derived date for the divergence of Old World monkey and hominoid lineages and inferred times at which lineages within each diverged from one another. Inevitably, calculated times of divergence, particularly of the hominoids, came into conflict with paleontologically derived dates for these events (Sarich 1971; Sarich and Wilson 1967a).

Goodman (1962, 1981; Goodman et al. 1983) also studied immunoreactivity of albumin among a limited number of primate taxa. But while he interpreted degrees of similarity in the context of Zuckerkindl and Pauling's assumption of continual molecular change, he rejected Sarich and Wilson's molecular clock because he used paleontologically derived dates for the emergence of *each* primate lineage. Since, however, molecular clock and paleontologically derived dates of hypothetical lineage splitting did not coincide, Goodman proposed a model in which molecular change could slow down or speed up, which meant that he had to explain each episode of change. For example, he suggested that the mutation rate decreased in the hominoid clade because of these primates' hemochorial mode of placentation, which presents an increased opportunity for antigenic reactivity to occur between mother and fetus, would make rapid molecular change disadvantageous. Sarich and Wilson (Sarich and Wilson 1967a; also Sarich 1971) rejected Goodman's unconstant rates of molecular change.

Forgotten Alternatives to the MA

Rather than determine degrees of molecular similarity on the basis of degrees of immunoreactivity, or by comparing electrophoretically produced starch gel patterns (another popular approach), Britten and Kohne (1968) turned to DNA hybridization. In this approach, DNA helices are split using heat to produce single-copy DNA. Single-copy DNA from two different taxa are then allowed to combine (anneal) and the resultant partial helices dissociated using heat. The rationale was that the more heat it took to melt a hybrid helix, the greater was the amount of sequence homology between the annealed DNA strands. In the context of the MA, greater apparent similarity in DNA sequences meant closer evolutionary relatedness. And since DNA was portrayed as the "blueprint of life," it seemed reasonable to imbue even surrogate representations of nucleotide sequences with considerable phylogenetic valence.

Upon unexpectedly discovering that genomes comprise a vast amount of repetitive DNA compared to single-copy DNA, Britten and Kohne suggested that there might be different mechanisms of molecular change. Gradual change, they

thought, was relevant only to preexisting families of DNA sequences. But new families of DNA nucleotide sequences resulted from infrequent but sudden replicatory events (“saltatory replications”), which would have more profound evolutionary effects than a process of gradual change. It would thus seem that Britten and Kohne were distinguishing between molecular changes that had no significant effect on the organism’s phenotype and those that did.

Unfortunately, the implications of Britten and Kohne’s “saltatory replications” had no effect on other biochemists’ interpretation of molecular data for reconstructing phylogenetic relationships. No doubt because it was completely Darwinian, the MA continued to dominate the increasingly influential field of what was now often called molecular systematics.

In 1975, King and Wilson (1975) approached, but from a different direction, the essence of Britten and Kohne’s suggestion that something other than gradual molecular change was relevant to evolutionary questions, especially if there was a one-to-one correspondence between molecules—in particular DNA sequences—and an organism’s morphology. After reviewing the available data on human and chimpanzee blood serum proteins and hybrid DNA, King and Wilson (p. 107) concluded: “all the biochemical methods agree in showing that the genetic distance between humans and the chimpanzee is probably too small to account for their substantial organismal differences.” The contradiction between genetic similarity and morphological dissimilarity—as between human and chimpanzee—could, they suggested, best be understood in terms of differences in genes that regulate development rather than between genes that are orchestrated by regulatory genes and yield structure. And it was the latter—structural genes—that were being assessed indirectly in molecular analyses.

Reaffirmation of the MA

King and Wilson’s insight should have provoked reconsideration of the MA. First, Zuckerkandl and Pauling’s model was based on a structural protein, not the nucleotide sequence coding for it. Second, the phylogenetic relevance of molecular similarity now appeared questionable because, third, it seemed that regulatory rather than structural genes were the more evolutionary significant. Nevertheless, Zuckerkandl and Pauling’s model of continual molecular change at the protein level was applied to DNA sequences primarily, we suggest, because of three factors: Kimura’s (1968, 1985) neutral mutation theory; the importance postsynthesis evolutionists attributed to selection in shaping phenotypes; and the weight of the central dogma, which portrayed DNA as the “blueprint of life.”

One cannot underestimate the impact the discovery of DNA had on the biological sciences, especially evolutionary biology. Prior to Watson and Crick’s (1953) publication of the structure of DNA, Morgan (1916) and other population geneti-

cists, who believed that there were specific genes *for* specific structures, concentrated on identifying at specific chromosomal loci the genes that underlay phenotypic traits. Subsequent to Watson and Crick’s publication, with the “genes for” notion intact, the goal was to reveal the molecular basis of these genes. Because of difficulties in determining DNA sequences and belief in a consistent relationship between one gene and one amino acid, proteins (sequences of amino acids) were the surrogate molecules of investigation. Consequently, evolutionary significance was sought through the analysis of hemoglobin, albumin, and other proteins of physiological significance. In the context of the stultifying effect, the evolutionary synthesis had on alternative thinking by constraining thought to a blend of Darwinism and fruit fly population genetics (Schwartz 2006 and in press), a neo-Darwinian interpretation of differences or similarities between taxa in their proteins seemed reasonable. For as it was assumed that organismal evolution occurred via the gradual accumulation of infinitesimally minute changes, so, too, could it be hypothesized that differences between taxa in their proteins occurred via the gradual accumulation of small molecular changes. DNA hybridization made it seem that biologists were closer to the molecular backbone of evolution.

There was a problem, though, with the application of the MA to proteins as well as to DNA. And Britten and Kohn and King and Wilson recognized it: if DNA nucleotide sequences comprised genes, which in a one-to-one ratio produced amino acids, and amino acids comprised proteins, and proteins somehow orchestrated the phenotype of an organism, then continual, gradual change in nucleotide sequences should be reflected in a process of continual and gradual change in amino acids, proteins, and ultimately the phenotypes of organisms. But, as King and Wilson pointed out for humans and chimpanzees, molecular similarity did not reflect morphological similarity.

One solution to this contradiction came from studies on bacteria that indicated that the same amino acid could be produced by codons that differed at the third base position. Because, in bacteria, the linear sequence of amino acids is critical to the identity and function of a specific protein, it seemed reasonable to extrapolate to eukaryotes a similar one-to-one relationship between nucleotide and amino acid sequences. Consequently, Kimura (1968, 1985) could suggest for all organisms that differences in nucleotide sequences could accrue without affecting the organism if change occurred in codons at the third base position. Since most evolutionary biologists were wedded to the role of Darwinian selection in continually fine tuning an organism’s phenotype to an ever-changing environment, they could embrace Kimura’s theory of unexpressed or neutral change because changes (point mutations) at the third-base position were invisible to selection. The combination of an unyielding adherence to a Darwinian model of evolution that emphasized the plasticity of an organism’s

phenotype and the apparent discovery of an ever-changing aspect of an organism's biology that was selectively neutral widened the schism between morphological and molecular systematists.

By distinguishing between aspects of an organism that are molded by selection and those that are not, the notions of "phenotype" and "selection" regained a meaning more similar to Darwin's (1859, 1868): the ability of selection to actively modify an organism's features. Consequently, although an organism's phenotype indeed reflected its evolutionary history—because it represented the organism's sustainability—"the phenotype" was branded an unfaithful mirror of an organism's phylogeny precisely because it was supposedly so easily modified by selection. Only ongoing changes at the molecular level to which selection was blind were regarded as "true" indicators of an organism's phylogeny.

Molecular systematics thus came to decipher an organism's evolutionary history in terms not of its biology, but of relative molecular similarity between it and other organisms, from which theories of relatedness were generated and times of lineage divergence inferred. Molecular systematists also began to express the belief that even if taxa clearly shared uniquely derived morphologies, if they were molecularly dissimilar, they could not be closely related (Sarich 1971). Curiously, the purported dichotomy between selectively neutral versus selectively malleable aspects of an organism's biology was that the molecular systematists' emphasis on selection as the provocateur of phenotypic change reversed the process of development. Now selection-induced changes in the adult must induce molecular changes in its gametes that will affect the development of offspring. This scenario is reminiscent of Lamarckian use–disuse arguments invoked by Darwin and others, i.e. an organism's desires engender its morphological change. But, then Lamarck, Darwin, and others did not understand inheritance.

Because the only presumably credible theory of evolution was Darwinism, many evolutionary biologists thought that in his neutral mutation theory Kimura rejected evolution (Gould 2002). Nevertheless, the underlying concept of the neutral mutation theory could be made compatible with the phylogenetic interpretation imposed on protein similarity (as determined by immunoreactivity) via the MA. As such, the neutral mutation theory was incorporated into the interpretation of taxic similarity in protein sequences (Goodman 1981), DNA hybridization comparisons (Sibley and Ahlquist 1983, 1984), restriction enzyme polymorphism analysis (Ferris et al. 1981), and ultimately similarity in DNA sequences and genes (Ruvolo 1997; Wildman et al. 2003).

Absent, however, from these analyses was consideration of the function of the molecule under study or the effects of nucleotide change on gene products. Although contradictory then, but especially now in light of present-day molecular

biology, molecular systematists of the 1980s and 1990s presumed that proteins and nucleotide sequences could always be changing without consequence to the organism. Indeed, one still sees publications contrasting "gene" versus "organism" phylogenies, as if these aspects of an organism's biology were actually unconnected (Patterson et al. 2006). But since the Darwinian perspective envisioned an organism's phenotype, but not its molecules, as easily manipulated by selection, such a dichotomy seemed viable.

The MA and DNA Hybridization, Part 1

It is significant that decades after Zuckerkandl and Pauling articulated the MA, this assumption remained untested. In a paper on DNA hybridization and human–ape relationships, Caccone and Powell (1989: 936) acknowledged that the MA was only an assumption:

Virtually all molecular phylogenetic studies, including these, have a major underlying *assumption*: the genetic similarity or difference among taxa is an indication of phylogenetic relatedness. Lineages that diverged more recently will be genetically more similar to one another than will be lineages with more ancient splits.

This *assumption* cannot be tested with absolute rigor, as we can never be absolutely certain about the correct phylogeny of any group, just as we cannot be certain about any historical event. However, two facts lend powerful credence to the assumption. First, virtually all molecular studies are in perfect accord with phylogenies deduced by other methodologies. For example, the molecular data are in excellent agreement with all other evidence indicating that humans and African apes are each other's relatives, followed by orangutans, gibbons, and the Old World monkeys (emphasis added).

First among the inconsistencies is the admission that the accepted model of molecular change and its phylogenetic interpretation were assumptions, and then the declaration that, since these assumptions were widely accepted, one could embrace them as truths. But they are assumptions and testable. From the perspective of the philosophy of science, since the interpretation of differing degrees of molecular similarity among taxa derived from apparent compatibility with a morphologically based scheme of phylogenetic relationship, when there are discrepancies between "gene" and "morphology" phylogenies, the latter should falsify the former. But molecular phylogenies did not consistently depict the same phylogenetic relationships of taxa studied (see Schwartz 1987, 2005b; also Ruvolo 1997), which should not be so if continuous change, in whatever molecule, always paralleled the evolutionary history of organisms.

Also at issue is how, from Zuckerkandl and Pauling on, similarity was interpreted: as always reflecting the amount of change accrued over the "life" of a lineage before it splits into other ever-changing lineages. Although this model may derive from Darwinian ideas of continual morphological change, the

reality is that organisms' features are not in a state of perpetual alteration. In addition, relatively few features are unique to an organism's "morphology," which is largely comprised of primitive retentions. As such, when morphologists make comparisons, particularly between individuals of closely related species, a demonstration of similarity is often between shared primitive features, which do not reflect closeness of relatedness, rather than between shared derived features, which do. Since an organism's "molecules" are part of its biology, demonstration of similarity between taxa will also largely be between shared primitive rather than shared derived characters. Thus, delineation of undifferentiated, overall molecular similarity is not a demonstration of relatedness (Schwartz 1987, 2005b). But while morphologists understand the distinction between primitive and derived character states, and that they can be sorted out only via a broad comparison of taxa, these systematically crucial elements are not part of molecular analysis.

To return to Caccone and Powell, after accepting the MA because most molecular systematists had done so, they then declared that they had definitively demonstrated a close evolutionary relationship between humans and chimpanzees, which, they stated, was corroborated by "other methodologies." This, however, is an illusion. For the majority of studies that claimed a close relationship between humans and one or both African apes were molecularly based and thus based on the MA (Schwartz 2005a). The few papers published by morphologists around that time favoring a human–African ape over the human–great ape theory of relatedness derived not from rigorous morphological analysis, but from accepting the human–African ape relationship promoted by molecular systematists (*ibid.*). Nevertheless, Caccone and Powell's belief in their interpretation bolstered the claimed viability of molecular systematics and the model on which it was based.

The MA and DNA Hybridization, Part 2

The appeal of DNA hybridization as a mirror of evolutionary relationship is seen in Sibley and Ahlquist's (1984) work on hominoids, the group to which they turned after developing their models on the phylogeny of birds (Sibley and Ahlquist 1983). Besides emphasizing that DNA hybridization studies should be based on single-copy, not repeated DNA, they promoted two concepts: the "law of large numbers" and a "uniform average rate of genomic change" (UAR).

Since an organism's genome is composed of millions (= large numbers) of nucleotides, Sibley and Ahlquist argued that by sampling it virtually in its entirety, the "law of large numbers" provided the checks and balances necessary to rule out "false" similarities, including parallelisms in base substitutions. Since DNA hybrid strands only anneal along complementary stretches of nucleotide sequences, homology is not at issue: stretches of DNA that do not anneal are not

homologous. Since such a large number of elements (i.e., millions of bases) was being compared, degree of similarity must reflect degree of relatedness.

The UAR, which Sibley and Ahlquist argued characterized molecular change across taxa, paralleled Sarich and Wilson's molecular clock. Because degrees of similarity were interpreted in the context of the MA, UARs could be used to calculate times of lineage splitting by calibrating them with fossils. As Sibley and Ahlquist saw it, the advantage of the UAR model was that, while different molecules supposedly changed at different rates, averaging rates of change across the entire genome eliminated the problem.

Because the technique of DNA hybridization compared entire genomes—at least the single-copy DNA—it had great appeal, no doubt because DNA was supposed to be the "blueprint of life." However, while this idea may obtain to bacteria, in which ~98% of their DNA is coding and the integrity of nucleotide sequences is functionally significant, it does not obtain to metazoans, in which only a small fraction of their DNA is coding. Also because the interpretation of degree of similarity as derived from DNA hybridization studies was based on the MA, one might ask: Why does not degree of similarity reflect primitive retention rather than a shared history of change?

But the interpretation of DNA hybridization analyses raises questions beyond the MA. First is the matter of similarity, but, in addition to the issue of shared primitive retention, there is also the assumption that when hybrids are formed between various taxa, the annealed DNA strands always reflect pairing or nonpairing between the same nucleotides at the same loci.

For example, suppose that the nucleotide sequences of taxa A and B are 95% alike while those of taxa A and C are 80% alike. Are the segments of DNA in which taxa A and B and then A and C are similar the same ones? Or does one array of nucleotide sequence segments produce similarity and dissimilarity between taxa A and B while a totally different array produces a different arrangement of similarity and dissimilarity between taxa A and C? Since DNA hybridization cannot identify nucleotide sequences or those segments where hybrids do or do not anneal, one cannot know the answer, which makes this and the question of whether similarity is actually reflecting primitive retention even more significant.

But there is also the rationale behind the notion of a UAR: that averaging rates of change across the entire genome obviates concern about different rates of change in different molecules. The problem lies in conflating mutation rates in proteins with changes in regions of DNA that code for these molecules. And this raises two other questions. Are different aspects of DNA (e.g., coding versus noncoding regions or introns versus promoter regions) more or less susceptible to base changes? And does it matter?

The MA and DNA Sequences

As nucleotide sequencing became more commonplace, molecular systematists focused on coding and some of the noncoding (introns) regions of genes (see, e.g., reviews in Ruvolo [1997], Goodman et al. [1998], Goldberg et al. [2003], Wildman et al. [2003]). Analyzing introns makes sense in terms of supposed selective versus nonselective aspects of an organism's biology: with regard to postembryonic development, introns are nonfunctional (do not code for proteins), and thus selection would be "blind" to changes in intron nucleotide sequences (Goldberg et al. 2003); notions of change in intron sequences also seem to be compatible with Kimura's neutral mutation theory. Nevertheless, comparing sequences of coding regions of genes might reflect phylogenetically significant changes that would be realized in morphological (also physiological) differences between organisms (Wildman et al. 2003). On the contrary, however, while the comparison of sequences of coding regions is believed to reveal the phylogenetic relationships of taxa (e.g., Ruvolo 1997), molecular systematists using this approach often ignore the fact that if a process of continual change does lead to nucleotide differences or similarities in these regions, expected phenotypic differences or similarities are lacking (Yi et al. 2002).

This inconsistency aside, we might benefit by scrutinizing some of the publications that claim to have demonstrated a close relationship between humans and chimpanzees since this will elucidate assumptions beyond Zuckerkandl and Pauling's that underlie the interpretation of the data.

Although Caccone and Powell (1989) declared that they proved a close relationship between humans and chimpanzees, their conclusion was not accepted by all molecular systematists: some believed that a trichotomous relationship between humans, chimpanzees, and gorillas had not been resolved, while others maintained that the African apes were sister taxa and humans their equidistant relative. But in 1997, Ruvolo analyzed the available DNA sequence data using a multiple-locus test and concluded (p. 248) that she had actually demonstrated a close relationship between humans and chimpanzees:

The implication of the multiple-locus test is that existing DNA sequence data sets provide overwhelming and sufficient support for a human–chimpanzee clade: no additional DNA data sets need to be generated for the purpose of estimating hominoid phylogeny. Because DNA hybridization evidence (Caccone and Powell 1989) also supports a *Homo–Pan* clade, the problem of hominoid phylogeny can be confidently considered solved.

But is it? The data sets that Ruvolo isolated as supporting a human–chimpanzee relationship were: mitochondrial (mt) DNA, glycophorin A, *c-myc*, carbonic anhydrase I (*CAI*), immunoglobulin C ϵ 3-pseudogene, α 1,3-galactosyl-transferase, δ - β -globin intergenic region, γ globin, ψ - η globin, B cell

growth factor, *HOX2B*, glucose-6-phosphate dehydrogenase (*G6PD*), and the Y-specific regions of PABY, ZFY, and SRY (the latter three Ruvolo treated as a single data set). Analysis of involucrin and protamine supported an African ape sister group; immunoglobulin C α 1 yielded a human–gorilla sister group. Other data sets were rejected as uninformative because they did not yield schema that conformed to the "accepted" view of the evolutionary relationships among New World monkeys, Old World monkeys, and hominoids: i.e., ϵ -globin, tyrosinase (*TYR*), X-specific pseudoautosomal boundary region sequences, X/Y pseudoautosomal regions, prion protein gene (*PRNP*), dopamine D4 receptor (*DRD4*), cytochrome P450/complement C4, 28S rRNA and spacers, red and green opsin pigment genes, *HLA-DQA1*, *DRB1*, and Y-encoded testis-specific protein (*TSPY*).

First, we might wonder about the comparability of the data sets. Are, for instance, "X-specific pseudoautosomal boundary region sequences," "28S rRNA and spacers," and "*HOX2B*" equivalents? Here, the "law of large numbers" would not necessarily support a human–chimpanzee relationship. As for absolute numbers of data sets, however, Ruvolo invoked 11 as her proof of this relationship (mt DNA is discussed separately below), even though 16 did not support it. Thus of only 30 (or 27, depending on how one counts them) genes or portions of DNA in the analysis, Ruvolo rejected more than half as being "uninformative" because they either led to a theory of human–ape relationship that was not human–chimpanzee, or differed from the "expected" scheme of relationships among anthropoid primates.

A question one should immediately consider is whether demonstrated degree of similarity actually reflects closeness of relatedness. Like other molecular systematists, Ruvolo did not consider this possibility. But unlike them, she used the language of cladism in describing degrees of similarity, which is deceiving.

Molecular Analysis Is Not Cladistic Analysis

In cladistic analysis, primitive versus derived character states are hypothesized on the basis of their relative distributions (Hennig 1966; Eldredge and Cracraft 1980). The more widespread and commonly shared a feature is, the more likely it is a primitive feature (plesiomorphy) retained from a distant common ancestor. The more uniquely and restrictively shared the feature, the more likely it is a derived feature (apomorphy), inherited from a recent common ancestor. But primitiveness and derivedness are relative concepts. A feature can be derived at one level, say at the level of a common ancestor of a clade, but within the clade it is a shared primitive retention (symplesiomorphy). In cladistic analysis, one must continually test one hypothesis of derivedness with another in order to test theories not only of homology—for shared primitive features are also homologous—but also of derivedness. Since cladism

is based on the hypothetico-deductive approach, in which hypothesis testing and falsification are paramount, the more a hypothesis of shared derivedness (synapomorphy) and thus of closeness of relatedness is contradicted by other theories of synapomorphy, the less viable it becomes.

In light of this procedure of hypothesis testing, it is obvious that any molecular analysis based on the MA is not cladistic. In molecular systematics, relative derivedness equates with degrees of overall similarity, and this assumption derives from the notion that lineages are in states of continual molecular change and thus the most similar are those that diverged most recently from a common lineage. Here, the notion of derivedness emerges from the belief that a lineage is constantly accumulating derived changes and these are retained in descendant lineages.

Paradoxically, while cladistic analysis is based on the fact that most of an organism's biology is retained from a succession of distant ancestors, and thus only a small fraction of an organism's biology is uniquely derived for it or for it and its closest relatives, the reverse is imbedded in the MA: similarity reflects a history of change in a shared ancestral lineage. But even if this assumption were true, the interpretation of molecular similarity does not constitute a cladistic analysis. Yet, because cladism is the dominant methodology in systematics because of its emphasis on the hypothetico-deductive approach, Ruvolo's and other molecular systematists' use of the language of cladism gives the false impression that the same theoretical and methodological concerns pertain to molecular systematics. In a cladistic analysis, however, one of the first questions one asks is whether shared similarity is a reflection of shared primitive retention. This question has not yet become part of molecular analyses.

Although this problem should be sufficient to question the theoretical and methodological soundness of molecular systematics, other questions must also be addressed. The first relates to the software used to analyze molecular data. Whether parsimony, maximum likelihood, or nearest-neighbor joining, the algorithms are based on the assumption that overall similarity equates with closeness of evolutionary relatedness (Czelusniak et al. 1990; Goodman et al. 1998; Chen and Li 2001). When the particular program arranges taxa by first uniting the two that are most similar, and then uniting with these taxa with the next most similar taxon, and so on, the MA is not violated. A contradiction arises, however, if the program needs to be "rooted," which means that a taxon is chosen as the primitive outgroup and its sequence used to contrast and then group other taxa on the basis of their overall similarity (reviewed in Schwartz 2005a). According to the MA, an earlier divergent taxon is different from more recently emergent taxa because the former had more time over which to accumulate difference. Yet, by selecting a presumed earlier divergent taxon as the outgroup in which to root a phylogenetic tree-building pro-

gram, that taxon is defined as primitive in its entirety relative to other taxa, which became different from it. Clearly, the same taxon cannot be primitive because it presumably diverged earlier than others and yet also derived in its own right through its lineage's unique history of accumulating of molecular change (Schwartz 2005a).

The MA, DNA Sequence Alignment, and Sample Size

Beginning with comparative studies using immunoreactivity and electrophoresis, Goodman (1962, 1981) has been a central figure in molecular systematics. As techniques for inferring and then demonstrating DNA sequences became viable, he was among the first to use them (Goodman et al. 1998).

In a recent analysis, Goodman and colleagues (Wildman et al. 2003) compared ~90 kb of coding DNA for 97 human genes with their (presumed) corresponding sequences in chimpanzees, more often *Pan troglodytes* than *P. paniscus*. Only 67 sequences were available for the gorilla, 69 for the orangutan, 58 for a composite of various Old World monkeys, and 49 for the mouse, the defined primitive outgroup. Of note are two comments the authors made in passing: one on sequence alignment and another regarding the corresponding coded proteins.

Sequence alignment, while not often discussed in detail, is, however, of great importance because it involves the sets of assumptions in the analysis that precede the application of any phylogenetic algorithm to the data (Lake 1991; Marks 2003). The methodological problem is that not all presumed homologous (orthologous) sequences, say from start codon A to stop codon B, are the same length in all taxa. Consequently, one must subdivide shorter sequences into segments that one thinks are homologous with segments of longer sequences. But assumptions underlie the subdivision of shorter sequences into segments that appear to align with portions of longer sequences: e.g., are shorter sequences shorter because they lost nucleotides (deletion), or are longer sequences longer because they gained nucleotides (insertion)?

The assumptions one makes affect the way in which one aligns segments of shorter sequences with presumed counterparts (orthologues) of longer sequences (Lake 1991). For instance, Marks (2003) demonstrated how one could align in three totally different ways a nucleotide sequence from an orangutan with a shorter, presumably orthologous sequence from a human. Although a comparative study of nucleotide sequences cannot begin without first aligning them, rarely is the reader informed of the criteria underlying the alignment.

The second issue is Wildman et al.'s (2003) comment that not all loci compared were verified by the actual presence of translated proteins in a given tissue. Although seemingly innocuous, this comment has greater implications than most systematists might expect because, without knowledge of whether a region of DNA actually codes for a specific protein either in the adult organism or during embryogenesis, one

cannot know if one of the regions being compared represents a pseudogene or whether sequences are nonhomologous (paralogous) (*ibid.*). Clearly, this has implications beyond those of alignment for deriving patterns of similarity from sequence data. Yet, Wildman et al. admit that transcriptional data were lacking for “many” of the loci they compared. Consequently, they were obliged to refer to the loci in their study as “inferred.”

These concerns aside, Wildman et al. compared all 97 inferred loci only between the chimpanzee and humans; the number of comparisons between them and other taxa was far less. But this does not mean that at least 49 loci, the number representing the mouse, could be compared across all taxa. Or that 58 loci, the number representing the collective Old World monkey sample, could be compared among the anthropoids. As Wildman et al. explain, the number and identity of loci that could be compared was dependent on the taxa being compared. Yet, in spite of these problems, Wildman et al. stated in the paper’s title and elsewhere, that humans and chimpanzees exhibited 99.4% identity in nonsynonymous (amino-acid changing, therefore potentially functionally significant) DNA.

Although they did not conceal the fact that they only compared ~90 kb, and that only humans and chimpanzees could be compared for this entire stretch of DNA, their language highlights a larger issue in the communication and understanding of molecular data: how details become submerged in the summation of actual results. No doubt the general public and most scientists would imbue Wildman et al.’s claim with the broadest possible meaning: humans and chimpanzees are almost 100% identical in their DNA. Yet, if one took these authors’ conclusion at face value, the only inference one could make is that humans and chimpanzees are 99.4% identical in a ~90 kb stretch of presumably orthologous coding DNA. Considering that only ca. 2% of the entire metazoan genome is coding, the significance of this comparison, and of all DNA sequence comparisons, diminishes considerably. Nonetheless, one can understand how much psychological effect the “law of large numbers” has on scientists and lay people alike. For it seems impressive that humans and chimpanzees share 99.4% of 90,000 nucleotide bases in contrast to their sharing only a few hundred bones and teeth. But if most of the 99.4% similarity is primitive retention, the comparison is phylogenetically meaningless.

The inequality of comparisons and other issues aside, we must point out that the sample in Wildman et al.’s study was very limited. Gibbons and siamangs, the small-bodied hominoids, were not included, and the sampling of Old World monkeys was small. If a morphological systematic analysis were based on such a restricted comparative sample, it would be faulted for being seriously inadequate. Yet, Wildman et al.’s study is characteristic of the majority of molecular analyses. The rationale is that, if molecules are continually changing throughout lineages, then regardless of the constancy or un-

evenness of molecular change, it is not necessary to sample many taxa because ongoing change is ubiquitous across taxa, and the model internally consistent. Nevertheless, from a methodological standpoint, any study based on a small sample that claims to demonstrate a close evolutionary relationship between two taxa (e.g., human and chimpanzee) only demonstrates similarity between two of only a handful of taxa.

The MA and the Application of Data to Assumed Phylogenetic Relationships

Although many molecular analyses attempt to generate theories of relationship from distance measures—even with small and taxically underrepresented sample sizes—it is not uncommon to find studies claiming to have determined a close relationship between two taxa when, in reality, they only applied their data to an already assumed arrangement of phylogenetic relationship. An example is Yunis and Prakash’s (1982) paper on chromosomes, in which they claimed to have demonstrated a close relationship between humans and chimpanzees. This paper continues to be cited in this regard (e.g., Ruvolo 1997). Yet, the authors admit in their text that they first accepted the orangutan as the primitive outgroup of a human–chimpanzee–gorilla clade, which by necessity predisposed the analysis to finding similarities between the latter three hominoids to the exclusion of the former.

A recent example of the application of one’s data to an accepted theory of relationship is found in Patterson et al. (2006). Although their analysis of 20 million base pairs sounds impressive, the abstract makes clear that the authors had already decided that African apes, and chimpanzees in particular, were more closely related to humans than the other primates in their small sample. When the authors discovered that some of their data grouped humans with orangutans or other primates in unexpected, nontraditional ways, they modified their analysis to “correct for recurrent mutation,” which, in the context of their assumed scheme of relationships, explained away the contradictory results. It would have been scientifically more rigorous had they used their unexpected results to test their assumptions.

The MA and Mitochondrial DNA

For over two decades, mitochondrial (mt) DNA has been favored in reconstructing evolutionary relationships (Brown et al. 1979, 1982; Ruvolo et al. 1991). Although closeness of relatedness is still based on the MA, other assumptions are specific to the interpretation of mt DNA: it is clonal (maternally transmitted), and thus is not as problematic as combinatorial nuclear DNA is; it possesses control regions with high mutation rates (hypervariable sites or zones) that can be used in phylogenetic reconstruction; and it “evolves” 5 to 10 times more rapidly than nuclear DNA and thus can be used to reconstruct relationships among recently divergent taxa.

The latter “aspect” of mt DNA is, of course, determined in comparison with assumed rates of change in nuclear DNA; in both cases, rates of change are calibrated to the fossil record. But in order to decide if taxa are candidates for analysis using mt DNA, one must first have a general notion of how they are related to each other and to extinct taxa. Perhaps more important, however, are the first two assumptions.

As summarized by Hagelberg (2003), neither of these assumptions has been proven. In fact, as has been demonstrated for mice, mt DNA is inherited paternally when some of the sperm’s tail, which carries these organelles, is introduced into an ovum—which is a situation that both Awadella et al. (1999) and Hagelberg calculated as being quite common. As such, the notion of maternal inheritance, uncomplicated by recombination, is too simple.

Hagelberg also questioned the reality of the “hypervariable” sites, which are assumed to be regions of preferentially high mutation rates. But, she points out (p. 85), “there is no direct evidence of hypervariability,” only the belief “that anomalous patterns of DNA substitution are best explained by mutation.” Yet, it is “because the notion of hypervariability fits with the received view of mtDNA clonality [that] anomalies are seldom questioned.” She warns: “The picture is far from simple, and it is clear that extreme care must be taken in the interpretation of mt DNA phylogenetic trees in the face of possible recombination” (p. 85).

Although the evidence of mt DNA recombination is so far circumstantial, there are enough unexplained patterns in mt DNA data to warrant reassessment of the conclusions of many mt DNA studies.

Molecular Biology and Implications for Molecular Systematics

We have so far dealt with the MA primarily from a historical and philosophical perspective. Here, we focus on some of the realities of molecular and cell biology in order to demonstrate further the flaws in the MA as well as the disconnect between molecular systematics and biology.

Comparing Amino Acid Sequences

As we reviewed, the reconstruction of metazoan phylogenetic relationships typically relies on comparison of structural proteins (to infer DNA sequences) or sequences of DNA coding regions. Although the justification may derive from study of bacteria, it is incorrect.

In prokaryotes, as much as 98% of the genome can code for proteins (i.e., represents structural genes) (Eisen 2000). As single-celled organisms, prokaryotes do not regulate gene expression in time and space as multicellular organisms do in different tissues and/or during embryogenesis. This is due to the simplicity of bacterial genomes, which are largely or-

ganized in polycistronic operons with promoter regions consisting of short regulatory regions (up to ca. 100 nt) (Salgado et al. 2000) that can accommodate only a few *trans*-acting regulatory proteins (transcription factors). Thus, these transcription factors positively or negatively modulate the expression of downstream structural genes, but not to the extent of permitting distant interactions and multiple inputs. Mutations occurring in bacterial structural genes allow the corresponding proteins to adapt to a specific environmental condition or situation, while mutations occurring in *cis*- or *trans*-regulatory regions will permanently turn a set of structural genes (e.g., proteins involved in the same metabolic pathway) either on or off.

The oldest bacteria existed ~3.6 billion years ago and they were virtually identical morphologically to present-day prokaryotes (Walsh 1992). Yet, in spite of the vast amount of time over which mutations accumulated since prokaryotes first appeared, they remained single celled and morphologically unchanged. Comparisons of aligned bacterial proteins and their corresponding DNA sequences thus reveal how these sequences *adapted* to environments in which bacteria carrying them thrive; they do not inform how or when these proteins evolved.

“Lower” eukaryotes, metazoans, and plants differ markedly from bacteria in DNA organization and transcriptional regulation (Shapiro 2002). The change from bacteria to lower eukaryotes and then from the latter to multicellular organisms required a profound reorganization of DNA to produce the kind found in plants and metazoa. This includes, for example, the emergence of very long promoter regions (up to 50 kb upstream and downstream), introns, complex multiregulatory proteins (20–30 different proteins per gene), enhancers/silencers, specific nucleotide sequences in nucleosomes, splicing and alternate splicing mechanisms used as physiological and developmental mechanisms, long repetitive stretches of noncoding DNA, and complex RNA polymerases, as well as of mitochondria and chloroplasts with their own coding DNA. In, for example, the human genome, repetitive sequences comprise over 50% of total DNA (Lander 2001).

In metazoans, there is thus not a direct relationship between the number of genes an organism has and its evolutionary “position” relative to other organisms (e.g. humans have ~25,000 genes, but nematodes have upward of 21,000 [Copley et al. 2001]). What “makes” a metazoan is not the number of structural genes, but the presence of regulatory genes (Levine and Tijian 2003) and the location and timing of their expression during embryogenesis or/and in the adult, which is a function of transcription factors (e.g., the yeast genome has ~300, the *Drosophila* genome ~1000, and the human genome probably ~3000 [Wyrick and Young 2002]).

The latter implies that genetic modifications (e.g., mutations in structural proteins, or duplication/deletion events)

are not sufficient to generate the morphological novelties of “higher” eukaryotes. Metazoa (and plants) have the capacity to express in time and space specific transcription factors (such as *hox* genes, which are highly conserved [= primitively retained] across clades) that control the expression of developmentally regulated genes and allow the transition from a fertilized egg to an adult multicellular organism. New morphologies occur by diversifying and modifying regulatory linkages (Gerhart and Kirschner 1997a). Changes in the expression of developmentally regulated genes, either by mutations in the long promoter regions of metazoan genes and/or in transcription factors (or in their expression), are known to alter the pattern of development (Grzeschik 2002) and may be directly responsible for the emergence of novelties (and thus perhaps also for speciation). In contrast, mutations in structural proteins (the basis of molecular clocks) most likely reflect in bacteria adaptation of a specific protein to the environment in which the organism operates. Consequently, it is not appropriate in an analysis of metazoan phylogenetic relatedness to compare DNA or amino acid sequences of structural genes because, in spite of the huge number of mutations that have accumulated over a period of ≥ 3.6 billion years, bacteria have not “evolved” at all.

The inappropriate extrapolation from bacteria to metazoans is also seen in the absence of a direct relationship between the number of genes an organism possesses and its phylogenetic “position.” Indeed, gene duplication (Ohno 1970), with perhaps a fourfold increase in DNA (Holland et al. 1994) followed by the functional divergence of new genes, no doubt played a major role in the emergence of the first metazoans from a unicellular ancestor and the subsequent diversification of this clade. Genome sequencing is now providing evidence that large-scale gene duplication and even complete genome duplication events contributed significantly to both gene family expansion and genome “evolution” (Taylor et al. 2003): e.g., it appears that a whole-genome duplication event occurred early in the evolution of ray-finned fishes, prior to the emergence of teleosts (the most diverse monophyletic group of vertebrates) (Christoffels et al. 2004). Consequently, “evolution” in metazoans may be seen as the result of genomic diversity and organismal complexity that emerged along with more elaborate mechanisms for the regulation of gene expression (Gerhart and Kirschner 1997a) and of mutations in transcriptional factors, which affected their transcriptional regulation (e.g., promoters, repetitive DNA sequences, etc.).

Mutations can occur in any region along a gene and, more generally, along the entire DNA molecule. Some mutations in a protein-coding gene are silent due to the degeneration of the genetic code (recalling Kimura’s mutation theory), others have a different impact depending on the type of amino acid substitution and where it occurs in the amino acid sequence, while in some cases there can be major change in amino acid sequence without changing protein function.

When the same protein is compared among different organisms it is evident that some regions, such as an active site of an enzyme or binding region of a transcription factor, are often retained (conserved) among taxa of disparate evolutionary relatedness. Some proteins, such as actin, tubulin, histone H4, Hsp70, etc., are probably retained because of their critical role in cytoskeleton and chromatin assembly and stress response. For example, there is 100% identity in human and bovine histone H4, which is a component of chromatin, and only 13 differences between *Tetrahymena* and humans.

The human homolog of the *cdc2* gene, which codes for a key cell cycle control protein in yeast, can replace the yeast protein in spite of amino acid sequence differences (Murray and Hunt 1993), and the *Drosophila pax-6* gene, which codes for a protein involved in positioning of the eye, is efficiently replaced by the mouse homolog (Halder et al. 1995). Other DNA segments however, are not as broadly conserved or as strictly involved in the biological activity of a stretch of amino acids of a given protein that are not part of the active site of the protein itself (e.g., in spite of the conservancy of cytoplasmic regions of membrane proteins, the membrane spanning fragments differ markedly). In additions, proteins may be conserved in some sites and yet quite different in others. For instance, Δ^9 -desaturases, which occur in all eukaryotes and in virtually all cells, may appear to be highly conserved when only cytoplasmic regions are compared, but in cell membrane regions they are highly divergent (ProteinDatabase 2004). In any discussion of change in amino acid sequence, one must not, however, forget that although protein sequences can be quite different between individuals or taxa, a protein’s function derives from its three-dimensional structure (e.g., among more than 200 eukaryotic globins only two residues are absolutely conserved in all sequences, while the residue identities of some pairs of sequences are only 16% [Bashford et al. 1987]).

Clearly, uncritical comparisons of structural proteins or the DNA sequences that code for them (from which phylogenetic relationships are inferred) overlook the fact that different regions may reflect different histories of change or nonchange. Given the fact that $\sim 98\%$ of a prokaryote’s genome codes for proteins, but only $\sim 2\%$ does in eukaryotes, it is inappropriate to use a bacterial model to interpret eukaryote genomes (as in the formative years of molecular systematics). “Molecular change” (i.e., in DNA or amino acid sequences) in bacteria is not equivalent to “molecular change” in metazoans, in which there is not a one-to-one relationship between DNA sequences, codons, genes, and structural proteins. But as change in bacterial structural proteins and genes represent adaptation, this might also obtain to metazoans, wherein structural protein change may be deemed “phenotypic.” Similarity among taxa in structural proteins, whether or not due to primitive retention, may thus reflect only similar adaptations and not a shared evolutionary history.

Phylogenetic relationships among metazoans might, therefore, be better revealed through comparing developmentally regulated genes, because changes in their expression can alter phenotypes (Wray et al. 2003). Since novel phenotypes must arise via a process that diversifies, reorganizes, and modifies regulatory linkages (Gerhart and Kirschner 1997a), it follows that identifying and comparing this process is most relevant to phylogenetic reconstruction. As such, the false dichotomy of “molecules” versus “morphology” that molecular systematists promote disappears and the relationship between molecular processes, development, and the emergence of phenotype regains centrality in systematics (Schwartz 2005a, 2005b). “Evolution” at the molecular level in metazoans would thus be not reflected in changes in structural proteins or the regions of DNA that code for them, but in changes that affect mechanisms regulating gene expression, including transcriptional factors and transcriptional regulation (e.g., promoter regions, repetitive DNA sequences, etc.) (Britten and Kohne 1968; Gerhart and Kirschner 1997b; King and Wilson 1975). As Shapiro (2002) put it, “We need to demystify DNA and cease to consider it the complete blueprint of life.”

Comparing Noncoding DNA Sequences

If, according to the MA, DNA is free to mutate, the same frequency of mutation should occur across the entire genome: not just in a select number of protein-coding regions but in noncoding DNA stretches and promoter regions. In addition, the MA allows one to speculate that regions that do not affect the organism (e.g., repetitive DNA, introns) will have higher and/or more constant effective mutation rates than coding regions, in which changes will impact the organism and thus be reflective of relatedness. This is not the case either. Since, however, there is extensive evidence of widespread intertaxic variation of promoter (*cis*-regulatory) sequences (Wray et al. 2003), these regions may be of potential phylogenetic significance.

Every gene is flanked by regulatory sequences that, in cooperation with the expression of proteins coded elsewhere in the genome (*trans*-acting transcription factors), regulate the tempo and mode of expression of a downstream sequence, especially when, where, at what level, and in which tissue (adult or embryonic) it occurs, and whether it is affected by physical or chemical environmental conditions. A metazoan promoter is thus highly complex and very irregular compared to the rules that govern the structure of a protein-coding DNA sequence (Lemon and Tijian 2000).

While up to 15 structurally distinct DNA-binding domains have so far been inferred for metazoan transcription factors, little is known about factors that interact with DNA-binding proteins but not with DNA itself (Taatzjes et al. 2004). Since

transcription factors can bind to more than one sequence, their activity is often modulated by posttranslational covalent modifications (e.g. phosphorylation) (Gu and Roeder 1997). Thus, proper expression of a developmentally regulated gene requires complex interactions between promoter sequences and transcription and cotranscription factors as well as posttranslational modifications and decondensation of chromatin structure, which, in turn, depends on the proper activity of proteins that orchestrate the timing of expression. Large-scale DNA sequence comparison reveals that half of an organism’s functionally or phenotypically relevant nucleotide sequences involve noncoding rather than coding sequences (Wray et al. 2003). This indicates that a significant fraction of metazoan DNA is devoted to *extracting* information (coded proteins) from the DNA itself.

Eukaryotic DNA is packaged into chromatin. The nucleosome, the repeating unit of chromatin, consists of 160–240 base pairs of DNA wrapped around histones. Chromatin prevents the DNA from interacting with most DNA-binding proteins. Recently, Segal et al. (2006) have shown that genomes encode an intrinsic nucleosome organization. This nucleosome positioning code (specified by particular nucleotide sequences) may facilitate specific chromosome functions, including transcription factor binding, transcription initiation, bending flexibility, and remodeling of the nucleosomes themselves. Thus, eukaryotic DNA not only codes in its nucleotide sequences for proteins, but it contains nucleotide sequences that regulate its own readout. These elements enable regulatory factors to assign precise locations in the genome that are essential for their function. Consequently, mutations in nucleosomes can have profound effects on gene expression and thus on development.

As do DNA coding sequences, promoter sequences mutate at different rates (Li 1997). But because promoters are integral to organismal development, changes in them will have phenotypically significant and thus potential evolutionary consequences. For example, in *Drosophila*, changes in promoter sequences can modify bristle patterning (Sucena and Stern 2000), while changes in the promoter region of the *Ubx* gene will greatly alter the pattern of expression that determines a four-winged phenotype (Simon et al. 1990). In humans it has been estimated that all promoter loci show ca. 40% polymorphism (Rockman and Wray 2002). However, it is also true that some promoters can tolerate specific nucleotide substitution without losing activity (Latchman 1998).

According to the MA, if mutation is random, it should affect promoter regions as frequently as other noncoding as well as coding regions. But as a rule metazoans do not change from one generation to the next. As Darwin, Huxley, and others were aware: like tends to reproduce like.

Conclusion

In this review, we discussed the theory and method of molecular systematics from the perspective of the history and philosophy of science. For, unless molecular systematics falls outside the bounds of science, it, like any science, should be subject to objective scrutiny. But unlike some sciences, it is unlikely that in evolutionary science much can be proven. As Popper and others (op. cit.) have argued, it is more likely that evolutionary hypotheses, especially of relatedness, can only be falsified or contradicted. Although Caccone and Powell (1989) believed that the MA could not be tested—because of the internal consistency of that assumption—we believe it can. First, since the MA derives from an interpretation of degrees of similarity that paralleled a morphologically based phylogeny, a molecularly based theory of relationship can be tested with a morphologically based one (Schwartz 1986). Second, the supposed internal consistency of the MA has been and is tested and seemingly falsified by those molecular phylogenies that do not corroborate a “favored” or “expected” phylogenetic scheme (e.g., Ruvolo 1997 and Patterson et al. 2006; also Romero-Herrera et al. 1976). Andrews (2000: 447) characterized the problem thus:

The pattern of change (in DNA analysis) is analyzed stochastically to produce inferred relationships between taxa, and it is generally the case that different DNA sequences, and different parts of the same sequence, provide evidence for differing sets of relationships. When this occurs, the greater number of similarities in the most strongly supported cladogram are defined as homologies, and by definition those supporting other sets or relationships must therefore be homoplasies. Judgements about homology are thus dependent on the phylogeny accepted and do not provide independent support for the phylogeny.

Clearly, this is the case in Ruvolo (1997) and Patterson et al.’s (2006) analyses.

Our review also reveals that, no matter how sophisticated their mathematical models, molecular systematists have not questioned the basic assumptions upon which they are based. Thus, while refining computer programs to analyze molecular data phylogenetically continues apace (Huelsenbeck and Rannala 1997) with statements of certitude about results following suit, no algorithm is more viable than the assumptions that inform it.

But perhaps the most basic question we can ask is whether the MA is actually biologically tenable. For, aside from UV-induced point mutation, there is no other constant source of mutation in the physical world, and spontaneous mutation rates are low (approximately 10^{-8} to 10^{-9}) (Maresca and Schwartz 2006). Indeed, cells contain myriad stress and other proteins that eliminate potential change from becoming established in the genome and maintain DNA homeostasis, which can be de-

railed only by the most extreme environmental stresses (ibid.). The fact that mutation occurs, albeit at extremely low frequencies, does not mean that it always occurs in germ cells. Rather, since mutation is random, it is perhaps more likely that a somatic cell will be affected than a germ cell. But only altered germ cells have potential evolutionary impact. In short, the notion that molecules of germ cells—DNA, RNA, and proteins and transcription factors necessary, e.g., for DNA repair, protein folding, chaperone functions, and control of signal transduction pathways, which are necessary for the survival of cells and their bearers—are in states of perpetual change is not, in our present understanding of cell biology, tenable. This does mean that “molecular change” does not occur; only that mechanisms provoking such change in germ cells are likely instantaneous and stochastic and probably often lethal (Maresca and Schwartz 2006)—which will preclude their persistence into future generations.

But we do not despair. Acknowledging that organismal development is a tightly controlled process lends itself to a melding of “morphology” and “molecules” in a way that can lead to more realistic models of evolutionary change and to methodological approaches to phylogenetic reconstruction. Although this will undermine the assumed hegemony of molecular systematics in determining phylogenetic relationships, it will mend the unnatural schism that has kept morphological and molecular systematics apart.

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