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Journal

Molecular phylogenetics and evolution, 16(3)

ISSN

1055-7903

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Publication Date

2000-09-01

DOI

10.1006/mpev.2000.0813

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Tree Rooting with Outgroups When They Differ in Their Nucleotide Composition from the Ingroup: The *Drosophila saltans* and *willistoni* Groups, a Case Study

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Received August 26, 1999; revised April 4, 2000

Rooting is frequently the most precarious step in any phylogenetic analysis. Outgroups can become useless for rooting if they are too distantly related to the ingroup. Specifically, little attention has been paid to scenarios where outgroups have evolved different nucleotide frequencies from the ingroup. We investigate one empirical example that arose seeking to determine the phylogenetic relationship between the *saltans* and the *willistoni* groups of *Drosophila* (subgenus *Sophophora*). We have analyzed 2085 coding nucleotides from the *xanthine dehydrogenase* (*Xdh*) gene in 14 species, 6 from the *saltans* group and 8 from the *willistoni* group. We adopt a two-step strategy: (1) we investigate the phylogeny without outgroups, rooting the network by the midpoint method; (2) we reinvestigate the rooting of this phylogeny using predefined outgroups in both a parsimony- and a model-based maximum-likelihood framework. A satisfactory description of the substitution process along the *Xdh* region calls for six substitution types and substitution rate variation among codon positions. When the ingroup sequences are considered alone, the phylogeny obtained using this description corroborates the known relationships derived from anatomical criteria. Inclusion of the outgroups makes the root unstable, apparently because of differences between ingroups and outgroups in the substitution processes; these differences are better accounted for by a simplified model of evolution than by more complex, realistic descriptions of the substitution process. © 2000 Academic Press

Key Words: outgroup and midpoint rooting; *Drosophila saltans* and *willistoni* groups; *Xdh*; nucleotide composition; GC content; maximum-likelihood, among-site rate variation.

Given a set of aligned sequences from four or more taxa, most tree-building methods yield unrooted trees.

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Unrooted trees, or networks, inform us about the relationships among the taxa, but if we are to distinguish plesiomorphic from synapomorphic changes and decide, accordingly, the path of evolution, the network must be rooted. Usually the only feasible way of determining the polarity of nucleotide character evolution is using one or more outgroups (i.e., taxa that are assumed to lie cladistically outside of a putative monophyletic group), such that a character state shared within the ingroup is assumed to be ancestral if it is also present in the outgroup. Still, outgroup rooting is frequently recognized as the most precarious step in phylogenetic reconstruction (Wheeler, 1990; Smith, 1994; Swofford *et al.*, 1996).

The topic of rooting using outgroups has been addressed in a series of papers (Lundberg, 1972; Watrous and Wheeler, 1981; Maddison *et al.*, 1984; Wheeler, 1990; Smith, 1994) focused on situations where the outgroup(s) show similar nucleotide frequencies to the ingroup. In such a case, the most likely source of topological bias at the time of rooting is long-branch attraction (Felsenstein, 1978). This phenomenon arises when the outgroups are distantly related (either because of large divergence time, or increased rates of evolution) to the ingroup, so that homoplastic changes at rapidly evolving sites can lead to artifactual rooting (termed “random rooting” by Wheeler, 1990) along longer ingroup branches (Felsenstein, 1978; Hendy and Penny, 1989; Wheeler, 1990; Maddison *et al.*, 1992). Long-branch-attraction biases are confronted by employing tree-building methods that account for variation in the substitution rate among sites present in the sequences. In this way, the phylogenetic value of each site is given a weight which is inversely related to the rate of evolution of the site, so that rare events are emphasized relative to fast evolving, putatively homoplastic positions. Eventually, the ingroup taxa are analyzed separately, and the outgroup(s) is connected to the resulting network secondarily (Lundberg, 1972; Nixon and Carpenter, 1993).

However, no attention has been paid to scenarios

FIG. 1. Unrooted ML tree of the *Drosophila saltans* and *willistoni* groups based on *Xdh* nucleotide sequences. The corresponding subgroup names are shown besides the species labels. Predefined outgroups are represented on the left. GC content in 1st, 2nd, and 3rd codon positions is given in parentheses. The tree is obtained with PAUP* 4.0b2 (Swofford, 1999) using the general reversible Markov process model (Yang, 1994), which allows different rate parameters for codon positions (REV + C model). Quartet-puzzling support values (based on 1000 puzzling steps) are given on the nodes. Support estimates for internal branches produced by this method can be interpreted as bootstrap scores (Strimmer and Haeseler 1996). Branch lengths are proportional to the scale given in substitutions per nucleotide. The question mark alludes to alternative rootings (encircled numbers) produced by: (1) midpoint rooting using ML under the REV + C model, NJ based on the REV + dG distance transformation, and weighed parsimony; and rooting with outgroups using NJ based on the LogDet (with and without the INV% component) distance, and ML and NJ under the REV model; (2) outgroup rooting using ML under the REV + C model, and parsimony (weighed and unweighed); and (3) outgroup rooting using NJ based on the REV + dG model.

where the outgroups have diverged in their nucleotide composition from the ingroup. Yet, there is growing evidence that base composition differences among taxa are quite common in nature (e.g., Sueoka, 1962; Bernardi *et al.*, 1988; Rodríguez-Trelles *et al.*, 1999a). It can therefore be expected that, at least for some species groups, the only suitable outgroups for phylogenetic reconstruction are taxa with very different nucleotide frequencies to the ingroup. Here we investigate one example that arose while trying to determine the phylogenetic relationships between the *saltans* and the *willistoni* species groups of *Drosophila*. The *saltans*-*willistoni* lineage has evolved highly distinctive nucleotide frequencies (Rodríguez-Trelles *et al.*, 1999a, 2000a) to the extent that the only potential rooting outgroups are compositionally very different.

Locating the Root of a Compositionally Diverged Ingroup: The Case of the *saltans* and *willistoni* Species Groups of *Drosophila*

The *saltans* and the *willistoni* groups represent the New World radiation of the *Sophophora* subgenus

(Throckmorton, 1975; Wotjas *et al.*, 1992; Tatarenkov *et al.*, 1999). Currently they consist, respectively, of five (*cordata*, *elliptica*, *parasaltans*, *saltans*, and *sturtevantii*) (Magalhães, 1962), and two (*willistoni* and *bocainensis*) (see Val *et al.*, 1981) species subgroups (Fig. 1). The two species groups are considered monophyletic because they can unambiguously be distinguished from each other morphologically (Throckmorton, 1975), and because of the deletion of an intron of the *Adh* gene specific to the *willistoni* group (Anderson *et al.*, 1993; our unpublished results). Evolutionary relationships have been investigated separately for each group using nucleotide variation (Gleason *et al.*, 1998; O'Grady *et al.*, 1998; Rodríguez-Trelles *et al.*, 1999b). None of these studies attempted to combine the two species groups on the same rooted tree. Here we address this issue using already published sequences (Tarrio *et al.*, 1998; Rodríguez-Trelles *et al.*, 1999b, 2000b). The data set consists of 2085 aligned coding nucleotides from the *xanthine dehydrogenase* (*Xdh*) gene in 17 species: 6 *saltans* and 8 *willistoni* represen-

TABLE 1

Results of the Likelihood-Ratio Test Carried Out on the *Xdh* Data for the Species Analyzed in This Study

Assumptions	H_0	:	H_1	df	Ingroup		Ingroup + outgroups	
					$-2 \log \Lambda$	P	$-2 \log \Lambda$	P
Equal base frequencies	JC69	:	F81	3	14.08	$<10^{-2}$	6.28	0.09
Transition rate equals transversion rate	F81	:	HKY85	1	530.44	$<10^{-6}$	667.16	$<10^{-6}$
Equal transitional rates	HKY85	:	TN93	1	48.32	$<10^{-6}$	93.36	$<10^{-6}$
Equal transversional rates	TN93	:	REV	3	22.08	$<10^{-5}$	27.36	5×10^{-6}
One category of rates for sites	REV	:	REV + %INV	1	908.72	$<10^{-6}$	1726.46	$<10^{-6}$
Uniform rates among sites	REV	:	REV + dG	1	916.72	$<10^{-6}$	1798.72	$<10^{-6}$
Uniform rates among codon positions	REV	:	REV + C	2	1765.04	$<10^{-6}$	2685.02	$<10^{-6}$

Note. In each row, the null hypothesis (H_0) is compared with a hypothesis (H_1) that removes the assumption indicated on the left column. Log-likelihood values are obtained assuming the topology shown in the Fig. 1. P represents the probability of obtaining the observed value of the likelihood-ratio test statistic ($-2 \log \Lambda$) if the null hypothesis were true, with degrees of freedom (df) indicated, and $\alpha = 0.01$. JC69, Jukes-Cantor, 1969; F81, Felsenstein, 1981; HKY85, Hasegawa-Kishino-Yano, 1985; TN93, Tamura-Nei, 1993; REV, general reversible model; REV + C, assuming different rate parameters for codon positions; REV + dG, assuming discrete gamma rates at sites; and REV + %INV, assuming a proportion of invariable sites.

tatives, plus 3 outgroups, namely *D. melanogaster* and *D. pseudoobscura*, of the *Sophophora* subgenus, and *D. virilis*, of the subgenus *Drosophila* (Fig. 1).

We test the homogeneity of the base composition with the method of Rzhetsky and Nei (1995). Because 20 tests are performed (i.e., for 1st, 2nd, 3rd, 1st plus 2nd, and the three codon positions, separately for the *saltans* group, the *willistoni* group, the *saltans* and *willistoni* groups together, and the entire data set including the outgroups), the significance value for rejection of the null hypothesis is adjusted using a Bonferroni correction (i.e., $\alpha = 0.01/20 = 5 \times 10^{-4}$) (Rice, 1989). Figure 1 shows that the outgroups exhibit very different nucleotide frequencies from the *saltans* and *willistoni* species in the *Xdh* region ($P < 10^{-6}$; 48 df); differences are accounted for by the 3rd (45.5% vs 69.7%, for the average GC content across the *saltans*-*willistoni* species and the outgroups, respectively; $P < 10^{-5}$; 48 df) and, to a lesser extent, by the 1st (57.6% vs 60.7%; $P < 10^{-4}$; 48 df) codon positions. Within the *saltans*-*willistoni*, clade heterogeneity is only detected for 3rd positions ($P < 10^{-4}$; 39 df), ascribable to differences between the *saltans* (GC = 41.4%) and *willistoni* (48.6%) groups; the differences are small, but significant due to the large sequence length (2085 bp).

In order to avoid nucleotide composition biases introduced by the outgroups, we first address the relationship between the *saltans* and the *willistoni* groups using the midpoint-rooting method (as implemented in PAUP* 4.0b2); this method assumes that the most divergent lineages in the phylogeny evolve at the same rate, and so it does not require the use of external reference taxa. To be safe, one could disregard the more-diverged 3rd codon positions, but the 1st and 2nd positions alone contain insufficient phylogenetic information to resolve the short-time-scale relationships involved in the diversification of the *saltans* and *will-*

istoni species. We follow a statistical model-fitting approach within the maximum-likelihood (ML) framework of phylogenetic inference (e.g., Ritland and Clegg, 1985; Yang *et al.*, 1995; Cunningham *et al.*, 1998; Rodríguez-Trelles *et al.*, 1999b). As a working topology for model fitting we use a topology that remains stable after applying the computer programs DNAML, DNADIST, and DNAPARS from the PHYLIP package (Felsenstein, 1993), using the default options. This topology coincides with that shown in the Fig. 1; use of alternative topologies is not expected to affect parameter estimates (Yang, 1994). We use three approaches to fit the among-site rate variation into the substitution models: the invariable sites model of Hasegawa *et al.* (1985) (referred to as INV% model), the discrete gamma distribution method of Yang (1996a) setting eight equal-probability categories of rates (dG models), and the codon-position-specific rate parameter model of Yang (1996b) (C models). In addition to ML, we use distance-based neighbor-joining (NJ), and parsimony criteria for tree making. The estimates of the among-site rate variation and transition bias that we use in distance computation and weighting for parsimony are those obtained by ML in the model-fitting stage, which can be considered the most reliable (Yang, 1996a).

The best description so far of the substitution process along *Xdh* is attained with the REV + C model (see Table 1), which allows six substitution types (two C $\leftrightarrow$ T and A $\leftrightarrow$ G transitions, and four C $\leftrightarrow$ A, C $\leftrightarrow$ G, T $\leftrightarrow$ A, and T $\leftrightarrow$ G transversions), and substitution rate differences between codon positions. Figure 1 shows the ML tree obtained for the *Xdh* data set using the REV + C model, with the position of the root (branch labeled 1) inferred by the midpoint rooting method; as noted earlier, this method assumes that the most divergent taxa in the phylogeny evolve at equal rates. To check this premise we tested the somewhat more re-

strictive assumption that the rate of evolution is the same for all taxa (i.e., the molecular clock assumption). Likelihood-ratio tests were performed under two reasonable topologies: one representing *saltans* and *willistoni* as monophyletic sister groups, and a similar one except for placing *D. subsaltans* as an outgroup; in both cases the clock assumption is rejected ($-2 \log \Lambda = 59.10$; 12 df, $P < 10^{-6}$, and $-2 \log \Lambda = 67.70$; 12 df, $P < 10^{-6}$, for each topology respectively); however, after removing the relatively slowly evolving *D. emarginata*, *D. sucinea*, and *D. capricorni* (see Fig. 1), the data set meets the clock assumption ($-2 \log \Lambda = 9.00$; 9 df, $P \sim 0.44$, and $-2 \log \Lambda = 21.90$; 9 df, $P \sim 0.01$). The ML topology shown in the figure clusters the *saltans* and *willistoni* species as separate lineages. If we use the estimates of the rate variation among-sites and transition bias obtained by ML for tree building with distance and maximum parsimony, this result remains unchanged (not shown). So far, analysis of the ingroup alone indicates that *saltans* and *willistoni* are monophyletic groups, which agrees with previous studies (Throckmorton, 1975; Anderson *et al.*, 1993); in the following, we will assume this to represent the best corroborated phylogeny.

Table 1 shows the results of the likelihood-ratio tests for the data set combining the *saltans*-*willistoni* species and the outgroups. Log-likelihood values are obtained assuming the topology shown in Fig. 1. As it is the case for the ingroup sequences (see Table 1), the best depiction of the substitution process in *Xdh* is provided by the REV + C model; inclusion of the outgroups has little effect on parameter estimates (results not shown). When inferring the tree, however, the position of the root is unstable, depending on the tree-building criterion. Of the methods assayed, only the NJ, based upon the LogDet + %INV distance correction, places the root along the branch joining the *saltans* and *willistoni* groups (branch 1 in Fig. 1); ML assuming the REV + C model and weighted parsimony situate the root in the branch leading to *D. subsaltans* (branch 2), whereas the distance criterion based on the REV + dG model places the root along the branch leading to *D. nebulosa* (branch 3).

The reasons why ML, generally more robust to assumption violations than maximum-parsimony and distance methods (see Felsenstein, 1988; Huelsenbeck, 1995; Swofford *et al.*, 1996), fails to find the putatively true location of the root are unclear; more so given the relatively "realistic" model of evolution used to infer the tree (Table 1). Other confounding factors, besides base composition differences among sequences, may be at work. Recent stimulation studies have shown that tree shape, as determined by the length of the branches, can affect the efficiency of ML for recovering the true tree, so that overly simple models often outperform more realistic models of evolution (Yang, 1997b; Bruno and Halpern, 1999). In order to explore

this possibility, we repeated the analyses above but ignored the among-site rate variation (i.e., components C, dG, and %INV are dropped from the models; see Table 1). In this case, only unweighted maximum parsimony miss-places the root (it locates it again on the branch leading to *D. subsaltans*; branch 2 in Fig. 1), whereas the ML, assuming the REV model, and the distance methods (either based upon the LogDet or the REV transformation) root the tree at the junction between the *saltans* and the *willistoni* species groups (branch 1).

Phylogenetic Implications

Outgroup sequences can become useless for determining the polarity of ingroup characters if they (i) are distantly related, or (ii) have evolved too fast, so that their sequences have become effectively randomized with respect to the ingroup. Here we provide an empirical example that differences in nucleotide composition between the outgroup(s) and the ingroup can also be a significant source of bias for plesiomorphy assessment. We draw two conclusions: (i) simple models recover the best corroborated topology in most cases, as well as or better than a variety of fairly realistic models of *Xdh* evolution; and (ii) among the complex models, solely the LogDet distance transformation (either ignoring the among-site rate variation or using the INV% model) succeeds in accurately rooting the tree.

Since the LogDet correction is the only one that relaxes the stationarity assumption (i.e., constant nucleotide frequencies) (Lockhart *et al.*, 1994), our second conclusion points to the large base composition differences between ingroup taxa and outgroup taxa as the factor responsible for the inability of the ML method to identify the root. Compositional differences, however, do not appear to be the only determinant, because ML succeeds when a simple model of evolution is assumed. Long-branch attraction (Felsenstein, 1978) can also be at work. This would explain that ML and parsimony locate the root along the branch leading to *D. subsaltans* (see Fig. 1). This scenario might seem unlikely, inasmuch as our best-fit model accommodates rate differences among sites, so that fast-changing sites, mainly 3rd codon positions, should have little influence on the phylogeny (Yang, 1996a; Cunningham *et al.*, 1998). In our case, however, the amount of among-site rate variation accounted for by the models could be insufficient for phylogenetic methods to overcome long-branch attraction.

This interpretation need not be at odds with the observation that, when the among-site rate variation is ignored, all methods correctly identify the root (except parsimony; see Fig. 1). As we have noted earlier, the *Xdh* sequences from the *saltans* group species have a somewhat lower GC content than the *willistoni* sequences in 3rd codon positions; the latter species are more similar to the outgroups in this respect. Account-

ing for substitution-rate differences among sites would thus reduce the phylogenetic signal contained in the nucleotide composition of 3rd codon positions, which place *D. subsaltans* within the *saltans* group. However, the signal would emerge after removal of the among-site rate variation from the model. Long-branch attraction and nucleotide composition differences would thus be exerting antagonistic effects on the phylogeny, their relative importance depending on the parameters included in the model.

Simplified models were already known that can outperform more realistic models when long branches are adjacent in the true phylogeny (Yang, 1997b; Bruno and Halpern, 1999). The present study illustrates that fairly simple models can also perform best when long-branches are not contiguous (the outgroups and *D. subsaltans* are at opposite ends of an internal branch; see Fig. 1). Our results challenge conclusions from simulation studies about the importance of accommodating among-site rate variation into the substitution models to overcome long-branch attraction problems (Cunningham *et al.*, 1998). Our case may rather add to the list of examples unveiling nonlinearities in the performance of tree-building methods.

Our results corroborate the phylogenetic relationships among the *saltans* species previously obtained in a ML study combining data from several nuclear (*Xdh*, *Adh*, *28SrRNA*, and *ITS1*) and mitochondrial (*COI* and *COII*) regions (Rodríguez-Trelles *et al.*, 1999b). The study used outgroups from the compositionally highly dissimilar *melanogaster* and *obscura* groups. Our inferences, based as they are on the most closely related outgroups of the *willistoni* group, are expected to be more reliable. This apparent insensitivity of the *saltans* topology to the base composition of outgroups may happen because of the relatively homogeneous base composition across the species of the group (Fig. 1); accordingly all species would equally be influenced by the composition of the reference taxa. Regarding the *willistoni* group, the *Xdh* phylogeny unambiguously places *bocainensis* as the earliest derived subgroup and shows it to be paraphyletic, with *D. nebulosa* being more closely related to the *willistoni* subgroup siblings than to *D. sucinea* and *D. capricorni* (Ayala *et al.*, 1975). The relationship among the *willistoni* siblings are fully consistent with those derived previously from a combined analyses of the *per*, *Adh*, and *COI* loci (Gleason *et al.*, 1998).

ACKNOWLEDGMENTS

We are indebted to Victor DeFilippis for help with the PCR analyses of the *Adh* intron, and to Rob DeSalle for critical comments. F.R.T. has received support from Consejo Superior de Investigaciones Científicas (Spain) (Contrato Temporal de Investigación) and Grant XUGA 400001B98 to A. M. Vieitez. Research supported by NIH Grant GM42397 to F.J.A.

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