cell division is disturbed by breakage, fusion and pyknosis. It is clear that these changes in the chromosomes result from alterations in the synthesis of DNA and RNA. Inhibition of DNA synthesis leads to pyknosis while chromosomal breaks and acentric fragments are caused by RNA metabolism due to Orange II feeding. Actually, protein synthesis is first affected by this chemical, in turn altering the DNA synthesis of the chromosomes.

Although at low concentrations this food color does not produce any significant chromosomal abnormalities, at the high concentration of 3.0 g/kg b.wt, many chromosomal abnormalities were induced. Other pathological studies including histopathology and hematology and further tests to evaluate the mutagenicity of this chemical are in progress.

1 Grateful acknowledgment is made to Prof. U.S. Srivastava, Zoology Department for providing necessary laboratory facilities.
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Reticulate evolution and cladism: Tests for the direction of evolution

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Summary. Reticulate evolution between ancestral-descendant lineages can be critically tested for by investigating the relationship between out-group and mid-point roots on numerically derived cladograms. The western grass-snake provides a worked example for the theoretical test.

Numerical cladistic techniques are used to study supraspecific affinities but it could be argued that reticulate evolution renders a cladistic approach to intraspecific affinities inappropriate. Wagner cladograms[1] are a widely used numerical cladistic method. Initially a Wagner network is obtained by computing the most parsimonious connection between taxa. Conversion to a tree or cladogram is then achieved by mid-point or outgroup rooting[1].

This paper suggests tests for detecting reticulate evolution based on statistical investigations of the patristic distance between the mid-point and out-group roots of Wagner trees to decide the correct direction of evolution. Analysis of a test case indicates that reticulate evolution can be detected by these tests and does not perturb Wagner networks. It does, however, perturb the root required for conversion to a tree. Consequently, prior to this conversion, one should test for reticulate evolution and if necessary find the correct roots.

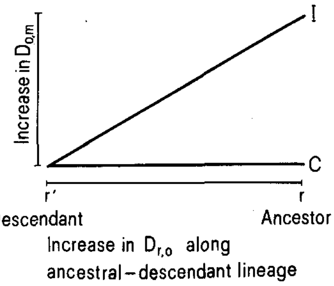

Figure 1. Theoretical expectations of the relationship between $D_{o,m}$ and $D_{r,o}$ at any given cladistic level in an ancestral-descendant lineage if time of divergence is well correlated to extent of divergence. C is the expection if the putative root (r) is correct whilst I is the expectation if evolution is in the opposite direction and r′ is the correct ancestral root. See text for explanation.

When these tests on the direction of evolution are clearly decisive one can confirm that the cause of the pattern of population affinities is phylogenesis rather than adaption to current environmental gradients. How then can one test for reticulate evolution?

If the extent of patristic divergence is well correlated to the time of divergence the mid-point root (m) and the out-group root (o) on any one cladistic level of a Wagner tree will tend to coincide. Consequently, in an ancestral to descendant lineage the patristic distance (D) between the outroot and the midroot ($D_{o,m}$) will tend to zero at any cladistic level and will tend not to be highly correlated with the patristic distance ($D_{r,o}$) between the putative root (r) of the total lineage and the outroot at the various levels. This is illustrated as line C in figure 1 where the hypothesized direction of evolution ($r \rightarrow r'$) is correct.

If however the putative root r is erroneous (and if in fact r′ is the correct ancestral root) then $D_{o,m}$ should be highly inversely correlated with $D_{r,o}$ and $D_{o,m}$ should only tend to zero at r′. In other words when the direction of evolution is incorrect the further one is from the real root of the lineage (in toto) then the greater the distance between the outroot and midroot at a given cladistic level. The outroot and midroot should only coincide at r′ where $D_{o,m}$ should obviously tend to zero irrespective of whether the direction of evolution is correct. This is line I in figure 1 where the hypothesized direction of evolution ($r \rightarrow r'$) is incorrect.

If the extent of divergence is not well correlated to time of divergence $D_{o,m}$ may not tend to zero and there may be a significant regression of $D_{o,m}$ against $D_{r,o}$. Nevertheless the slope of the regression (b in $y=a+bx$) should be less for the correct direction of evolution than the incorrect direction of evolution. The opposite will only be true if the most recently evolved taxa are more divergent than older taxa. This is likely only for small island populations which diverge rapidly due to the founder effect. Consequently, small island populations should be excluded from consideration.

One can use these expectations to statistically test which is the most likely direction of evolution and whether reticulate evolution has occurred. The taxa used as a test case is the grass snake *Natrix natrix*. The eastern and western semi-species are thought to have split into separate southern refugia due to the ice caps and after diverging to have undergone post-Pleistocene expansion northwards to occupy their present range[2-4].

Whilst a cladistic analysis of the grass snake population affinities shows the eastern clade to have a southern root with progressive evolution of more northerly populations both the midroot and outroot of the western clade are northern. The mid-point between the midroot and outroot ($\bar{r}$ in fig. 2) is a node giving rise to Holland in the far north.

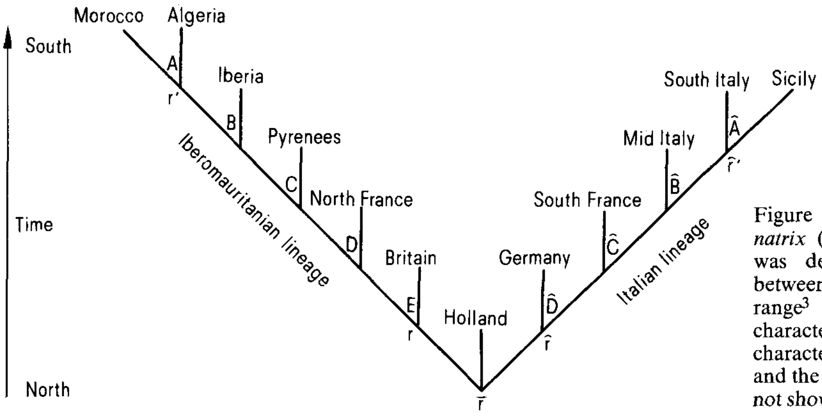

Figure 2. Cladogram of western population of *Natrix natrix* (small islands excluded). The Wagner network was derived from a Manhattan distance matrix[1] between *N. natrix* population[3] from the entire species range[3] and is based on 53 normalized morphometric characters. These characters are listed as Clc[3] (plus character 22). $\bar{r}$ is the midpoint between the outgroup root and the midpoint root of the western lineage. Anagenesis not shown.

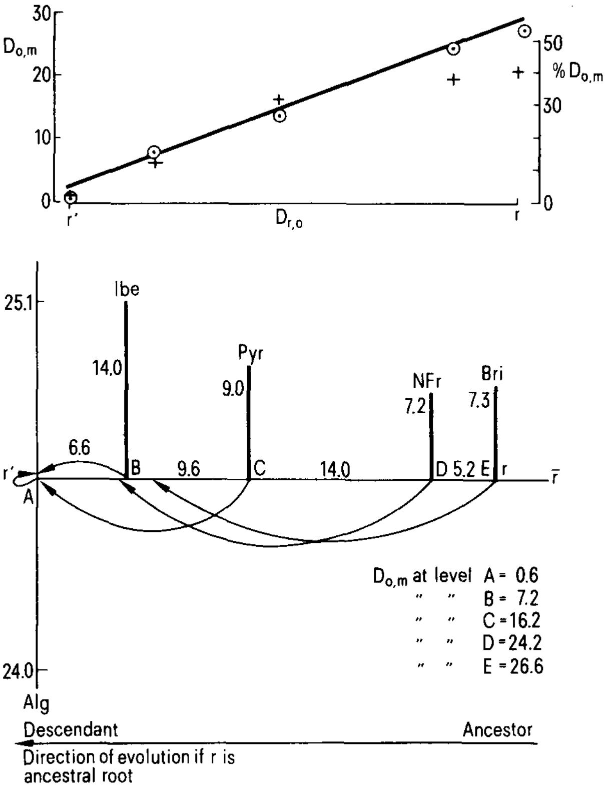

Figure 3. Relationship between outroots and midroots of the Iberomauritarian lineage at any given level when r is taken as the ancestral root and the direction of evolution is $r \rightarrow r'$ (as assumed in fig. 2). The midpoint root for any given cladistic level (E to A) is indicated by an arrow. $D_{o,m}$ is indicated by a circle and % $D_{o,m}$ by a cross. The least squares fit of $D_{o,m}$ against $D_{r,o}$ is given as a line. The patristic distances are indicated on the network. This conforms to the expections of r being the incorrect root (line I in fig. 1).

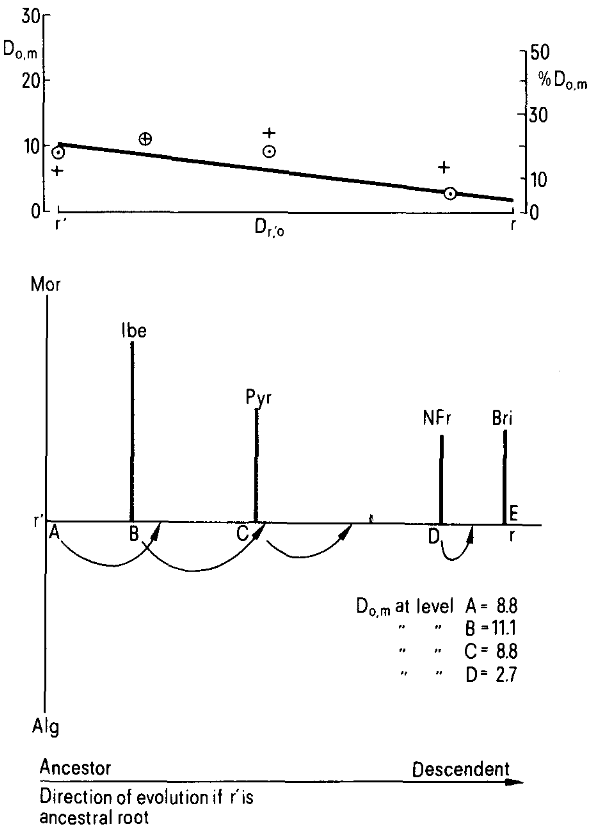

Figure 4. Relationships between outroots and midroots of the Iberomauritarian lineage when r′ is taken as the ancestral root. Key as for figure 3. This conforms to expectations of r′ being the correct root (line C in fig. 1).

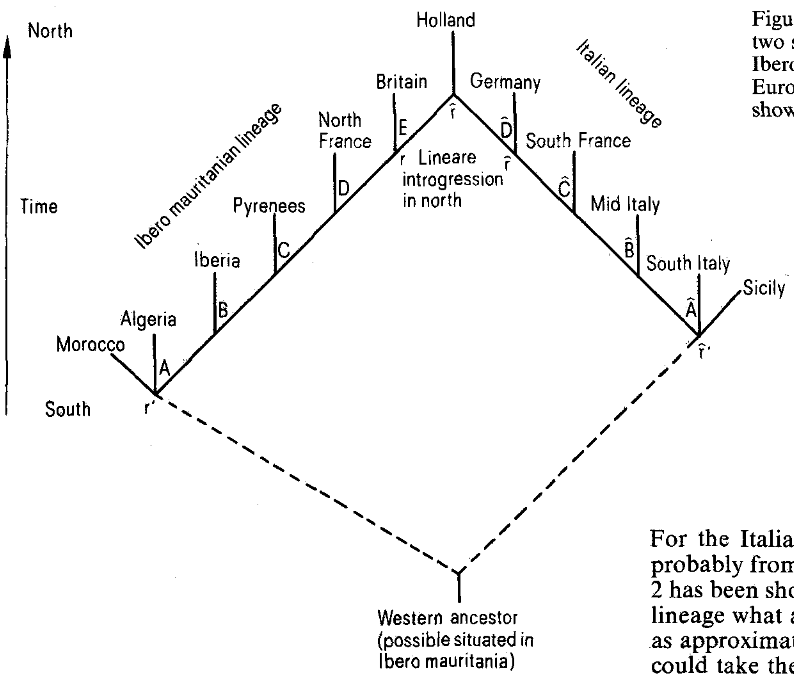

Figure 5. Cladogram of the western populations showing two southern roots and reticulate evolution between the Iberomauritarian and Italian lineages in northern Europe. r′ and r̂′ are taken as the roots. Anagenesis not shown.

Since this area was covered in ice until geologically recent times[5] it could not have supported a Pleistocene refuge population. This cladogram (fig. 2) can be tested for reticulate evolution using the above expectations.

If we assume that r in the north (figs 2 and 3) is the ancestral root of the Iberomauritarian lineage (and divergence is well correlated to time) then we would expect $D_{o,m}$ (through cladistic levels E to A) to be uncorrelated and conform to expectation C in figure 1. This hypothesis can be tested an rejected since when r is assumed to be the ancestral root (and the direction of evolution from r→r′) the percent $D_{o,m}$ (where $D_{o,m}$ at a given cladistic level is expressed as percent of the greatest patristic distance between taxa at that cladistic level) is significantly correlated to $D_{r,o}$ (correlation = 0.94, $P < 0.01$). Moreover, the slope of $D_{o,m}$ against $D_{r,o}$ is steep ($b = -0.73$) and passes close to the distal origin ($y = 2.12$ at r′).

This conforms to the expectations (I in fig. 1) or r not being the correct root and evolution progressing in the opposite direction (from r′ to r). If we assume that r′ (in the south) is the ancestral root (fig. 4) and the direction is from r′ to r then $D_{o,m}$ against $D_{r',o}$ (through levels A to D) gives the expectations associated with the correct direction of evolution (i.e. C in fig. 1). That is, the percent $D_{o,m}$ is not significantly correlated with $D_{r',o}$ (correlation = 0.06, $p > 0.05$) and the slope of $D_{o,m}$ against $D_{r',o}$ is slight ($b = 0.23$).

Consequently the above information is sufficient to test whether r or r′ is the correct ancestral root for the Iberomauritarian lineage with the conclusion that r′ is correct and the direction of evolution is not as indicated in figure 2.

If we drop the assumption that the extent of divergence is well correlated to the time of divergence then we can decide whether the direction of evolution is from r to r′ or r′ to r by comparing the regression coefficients of $D_{o,m}$ against $D_{r,o}$ and $D_{o,m}$ against $D_{r',o}$ to see which has the shallowest (inverse) slope. $D_{o,m}$ against $D_{r',o}$ has a significantly shallower slope than $D_{o,m}$ against $D_{r,o}$ ($F = 19.70$, df 1,5, $p < 0.01$) so r′ to r is confirmed as the most likely correction direction of evolution.

For the Italian lineage the direction of evolution is most probably from r̂′ to r̂ and not as in figure 2. Since r̂ in figure 2 has been shown not to be the ancestral root of the western lineage what are the correct roots? One could take r′ and r̂′ as approximations to the ancestral roots. Alternatively, one could take the average position of the mid points for A, B, C, D and E in the Iberomauritarian lineage as in figure 3 (and for Â, B̂, Ĉ and D̂ in the Italian lineages). These show little variation and are extremely close to r′ and r̂′ as expected. (There may be several other ways to detect reticulate evolution, for example a significantly lower distance between the midpoint roots along a lineage is another indication that the hypothesized direction of evolution is wrong or one could test the pattern of anagenesis by monotonic regression to see if the populations nearest the root were progressively more divergent.

When a tree or cladogram is constructed using r′ and r̂′ as roots then reticulate evolution between the northern populations is hypothesized (fig. 5). This is entirely consistent with the geographic position of the populations, with the occurrence of a southern root in the eastern form and with Pleistocene events.

When the Pleistocene ice caps began to retreat 20 thousand years ago[5] grass snake populations could expand northwards out of the Iberian peninsula and colonize the Italian peninsula. Ten thousand years ago as the climate suddenly deteriorated[5] the Italian and Iberomauritarian populations would have been temporarily isolated into separate refugia. Northward range expansion as the climate ameliorated would result in expansion across the Pyrenees into northern France and Britain (from Iberomauritaria) and expansion into mid Italy, south France and Germany (from southern Italy) with reticulate evolution between the northern populations.

Numerical cladistics can therefore be used in situations where reticulate evolution occurs and are one of a range of techniques[6] that are useful for investigating population affinities.

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