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Using phylogeographic analyses of gene trees to test species status and processes

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Abstract

A gene tree is an evolutionary reconstruction of the genealogical history of the genetic variation found in a sample of homologous genes or DNA regions that have experienced little or no recombination. Gene trees have the potential of straddling the interface between intra- and interspecific evolution. It is precisely at this interface that the process of speciation occurs, and gene trees can therefore be used as a powerful tool to probe this interface. One application is to infer species status. The cohesion species is defined as an evolutionary lineage or set of lineages with genetic exchangeability and/or ecological interchangeability. This species concept can be phrased in terms of null hypotheses that can be tested rigorously and objectively by using gene trees. First, an overlay of geography upon the gene tree is used to test the null hypothesis that the sample is from a single evolutionary lineage. This phase of testing can indicate that the sampled organisms are indeed from a single lineage and therefore a single cohesion species. In other cases, this null hypothesis is not rejected due to a lack of power or inadequate sampling. Alternatively, this null hypothesis can be rejected because two or more lineages are in the sample. The test can identify lineages even when hybridization and lineage sorting occur. Only when this null hypothesis is rejected is there the potential for more than one cohesion species. Although all cohesion species are evolutionary lineages, not all evolutionary lineages are cohesion species. Therefore, if the first null hypothesis is rejected, a second null hypothesis is tested that all lineages are genetically exchangeable and/or ecologically interchangeable. This second test is accomplished by direct contrasts of previously identified lineages or by overlaying reproductive and/or ecological data upon the gene tree and testing for significant transitions that are concordant with the previously identified lineages. Only when this second null hypothesis is rejected is a lineage elevated to the status of cohesion species. By using gene trees in this manner, species can be identified with objective, a priori criteria with an inference procedure that automatically yields much insight into the process of speciation. When one or more of the null hypotheses cannot be rejected, this procedure also provides specific guidance for future work that will be needed to judge species status.

Keywords: fragmentation, haplotype tree, hybridization, nested clade analysis, phylogeography, species

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Introduction

Hull (1997, 1999) pointed out that scientists ideally would like their concepts to be as general, applicable and theoretically significant as possible. With respect to species concepts, these goals are often in conflict with each other, leading Hull (1997, 1999) to conclude that there is no ideal species concept and that a plurality of species concepts may be needed. One of the species concepts evaluated by Hull is the cohesion species concept (Templeton 1989). A cohesion species is an evolutionary lineage whose boundaries arise from the genetic and ecological forces that create cohesive reproductive communities (Templeton 1998a, 1999). An evolutionary lineage is a reproducing population with sufficient historical continuity (ancestral-descendant

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relationships) to have its own evolutionary trajectories and tendencies (an operational definition will be given later).

The cohesion concept scored well by Hull's first criteria of generality. All life evolves and forms lineages, so defining a species as an evolutionary lineage connects the cohesion concept to a true biological universal. In particular, Hull points out that the major impediments to universality of most species concepts are the opposite challenges of asexual reproduction and hybridization. Both sexual and asexual taxa can define evolutionary lineages, so the cohesion concept applies to all life on this planet and not just the small subset that reproduces in a manner similar to humans. Moreover, a novel evolutionary lineage can start from a stabilized or recombinant hybridization event between species (Templeton 1981), a fact overwhelmingly documented in the botanical literature (Rieseberg 1997) and found in the animal literature as well (e.g. Vyas *et al*. 1990; DeMarais *et al*. 1992; Bullini 1994; Dufresne & Hebert 1994; Schartl *et al*. 1995; Carmona *et al*. 1997; Kenyon 1997; Grant & Grant 1998; Parris 1999; Vila & Wayne 1999). The cohesion species concept can embrace these lineages founded by hybridization events (Templeton 1989). Moreover, cohesion lineages can show limited hybridization without losing their lineage status by using explicit, objective and quantifiable criteria (Templeton 1994a). Hence, the cohesion species concept acknowledges hybridization as an important and potentially creative force in speciation.

The cohesion concept did not score well with the criterion of applicability under Hull's reckoning. This is not surprising, as Hull (1997, 1999) only cites Templeton (1989) when discussing the cohesion concept. That paper was designed to introduce the cohesion concept into the evolutionary literature, but did not nor was intended to address the issue of making the concept operational. The issue of applicability was addressed in subsequent papers (Templeton 1994a, 1998a, 1999), none of which were considered by Hull (1997, 1999). These subsequent papers show with worked examples that the cohesion concept can be rephrased as a set of testable null hypotheses. The operational implementation of the cohesion concept requires data gathering techniques (e.g. DNA sequencing, restriction site mapping, etc.) that are now readily available. Given that virtually all data sets represent a sample and that measurement errors are possible, null hypotheses are formulated that must be rejected with statistical significance before inferring a cohesion species. Such use of statistical tests is a widespread operational procedure in virtually all science. Finally, the criteria for the biological interpretation of statistically significant results are made in an explicit a priori fashion to insure replicable and objective inference.

This practical inference procedure automatically yields many insights into fragmentation, hybridization, range expansions, colonization events, gene flow patterns, novel adaptations, etc.; forces and historical factors that can contribute directly to the process of speciation (Templeton 1994a, 1998a, 1999). Accordingly, the cohesion concept has great theoretical significance, as was also recognized by Hull (1997). Because the perceived weakness of the cohesion concept concerns its applicability, this paper will focus upon the practical applicability of the cohesion concept when rephrased as a set of testable null hypotheses.

Using gene trees to detect multiple lineages

The cohesion species is first and foremost an evolutionary lineage. Therefore, the first null hypothesis to be tested is that *the organisms sampled are derived from a single evolutionary lineage*. This hypothesis can be tested through the use of gene trees. A gene tree is an evolutionary reconstruction of the genealogical history of the genetic variation found in a sample of homologous genes or DNA regions that have experienced little or no recombination. In practice, one can usually only discern this genealogical structure to the level of haplotypes; that is, a set of genes that share an identical nucleotide sequence (or restriction site state if restriction sites are being used to assay variation in the DNA). The evolutionary history captured in a haplotype tree can span genetic variation found both within and among species; hence, haplotype trees have the potential of straddling the interface between intra- and interspecific evolution. It is precisely at this interface that the problem of discerning species occurs, and haplotype trees can therefore be used as a powerful tool to probe this interface to define species (Templeton 1994a). However, this tool must be used with caution because there is no theoretical basis for equating haplotype trees to population lineages (Hudson 1990; Wu 1991, 1992; Avise 1994, 2000; Hey 1994). This does not mean that haplotype trees contain no information about population level events, but it does mean that this information must be extracted with rigorous analytical safeguards and criteria to prevent a naive equation of haplotype trees with population trees.

A nested clade analysis (NCA) of a haplotype tree is one such analytical procedure (Templeton *et al*. 1995; Templeton 1998b). NCA converts a haplotype tree into a hierarchical set of nested branches or clades using the nesting rules given in Templeton *et al*. (1987) and Templeton & Sing (1993). Basically, these nesting rules start at the tips of the haplotype network and move one mutational step into the interior, uniting all haplotypes that are connecting by this procedure into a '1-step clade.' After pruning off the initial 1-step clades from the tips, this procedure is then repeated on the more interior portions of the haplotype network as needed until all haplotypes have been placed into 1-step clades. The next level of nesting uses the 1-step clades as its units, rather than individual haplotypes. The nesting rules are the same, but result in '2-step clades' this time. This

nesting procedure is repeated until a nesting level is reached such that the next higher nesting level would result in only a single category spanning the entire original haplotype network. The resulting nested clades are designated by 'C-N' where 'C' is the nesting level of the clade and 'N' is the number of a particular clade at a given nesting level. Some special nesting rules are needed to deal with symmetries and ambiguities in the estimated haplotype network (Templeton & Sing 1993).

An NCA can be used to test the first null hypothesis both in the case in which a priori lineage categories have been proposed and in the case in which no a priori categories have been stipulated. As an example of the first case, Matos (1992) studied restriction site variation in chloroplast DNA (cpDNA) among pine trees in the *Pinus montezumae* complex of Mexico. Three prior species categories (*P. hartwegii*, *P. montezumi*, and *P. michuocana*) had been proposed on the basis of morphological, ecological and habitat distinctiveness. To test the null hypothesis that these prior categories do not correspond to phylogenetic lineages, the cpDNA haplotype tree obtained by maximum parsimony was converted into a hierarchical set of nested clades. The null hypothesis of no association between the phylogenetic structure of the haplotype tree and the prior taxonomic categories was tested by applying exact random permutation tests to the nested design (Matos 1992; Templeton 1994a), and the null hypothesis was strongly rejected. The NCA identified those parts of the cpDNA tree that corresponded to lineages and demonstrated that each of the three prior categories were behaving as statistically distinct evolutionary lineages.

A more detailed phylogeographic analysis is required when no a priori categories exist. The NCA phylogeographic analysis and its inference criteria are discussed at length along with a detailed worked example in Templeton *et al*. (1995). A validation of the inference criteria are given in Templeton (1998b), and a program and documentation are available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm. Hence, only a brief summary will be given here. In the procedure of Templeton *et al*. (1995), the geographical data are quantified by the clade distance (D_c), which measures the geographical range of a particular clade, and the nested clade distance (D_n), which measures how a particular clade is geographically distributed relative to its closest evolutionary sister clades (i.e. clades in the same higher-level nesting category). When using geographical distances, the D_c measures the average great circle geographical distance (the shortest distance between two points on the Earth's globe) that an individual bearing a haplotype from the clade lies from the geographical centre of all individuals bearing haplotypes from the same clade. Hence, the D_c measures the geographical spread of a clade. The D_n measures the average geographical distance that an individual bearing a haplotype from the clade lies from the geographical centre of all individuals bearing haplotypes from the next higher level nesting clade that contains the clade of interest. Therefore, the D_n measures how far a clade lies from the geographical centre of all of its closest evolutionarily neighbouring clades (including itself). For some organisms (e.g. a riparian organism), geographical distances may not be the most relevant, so user-defined distances between sampling locations can also be entered into the programme. In this case, the D_c is the average pairwise user-defined distance between individuals bearing haplotypes from the same clade. The D_n in this case is the average pairwise distance of individuals bearing a haplotype from the nested clade of interest to all individuals bearing haplotypes from the nesting clade containing the nested clade of interest. In either case, contrasts in these distance measures between tip clades (clades that are not interior nodes in the haplotype tree) and the clades immediately interior to them in the cladogram are important in discriminating the potential causes of geographical structuring of the genetic variation (Templeton *et al*. 1995). The statistical significance of the different distance measures and the interior-tip contrasts are determined by random permutation testing that simulates the statistical null hypothesis of a random geographical distribution for all clades within a nesting category given the marginal clade frequencies and sample sizes per locality.

If statistically significant patterns are detected by the above analysis, they next need to be interpreted biologically. The strength of the phylogeographic NCA is that it can separate the effects of recurrent gene flow from those of historical events affecting whole populations as causes of geographical associations within the haplotype tree (Templeton *et al*. 1995, 1998b). Explicit criteria are given in an inference key (Templeton *et al*. 1995) for discriminating between gene flow, population range expansions, long-distance colonization events, and fragmentation events. NCA does not treat these patterns as mutually exclusive but rather searches for multiple, overlaying patterns within the same data set. The key given in Templeton *et al*. (1995) incorporates not only the expected patterns under gene flow, fragmentation, and range expansion, but also incorporates the types of pattern artefacts that can arise from inadequate sampling. As a consequence, even though statistical significance may have been detected, the inference key can sometimes result in no definitive biological inference. The ability of the key to yield an inconclusive outcome is a strength, not a weakness, of this procedure because the application of the key identifies the deficiencies of the current sample that must be corrected for strong biological inference.

For the problem of inferring species, the relevant biological inference from a phylogeographic NCA is the inference of fragmentation. The null hypothesis that *the organisms sampled are derived from a single evolutionary lineage* is

rejected only when one or more fragmentation events are inferred. There are two attributes of this inference procedure that are particularly noteworthy for the problem of inferring species. First, in most tests of a null hypothesis, the failure to reject the null hypothesis does not necessarily constitute evidence that the null hypothesis is true. The failure to reject could be due either to the null hypothesis being true or a lack of statistical power. For example, sample sizes may simply be inadequate to detect geographical associations, in which case the NCA would not yield statistically significant distances relative to the statistical null hypothesis of no geographical association. Alternatively, the geographical sampling scheme may have been inadequate to infer fragmentation even though significant geographical associations were discovered. For example, Templeton (1999) used a phylogeographic NCA on the mitochondrial DNA (mtDNA) haplotype tree estimated by Nevo *et al*. (1993) in the *Spalax ehrenbergi* complex of mole rates in Israel. The NCA revealed two statistically significant fragmentation events, subdividing these mole rates into three lineages by the rigorous criteria given in the key. Nevo *et al*. (1993) had subdivided these mole rats into four groups based upon chromosome number, and the two of the significant NCA lineages corresponding to two of these chromosome 'races', whereas the third pooled two chromosome races together. As discussed in Templeton (1999), there was much evidence leaning in the direction that these two 'races' were indeed cohesion species, so that augmenting sample sizes and/or the number of sample locations of these two groups should be a high priority in future studies on this complex. In this case, the failure to reject the null hypothesis that *these two chromosomal 'races' are derived from a single evolutionary lineage* could well be due to inadequate sampling, so no firm conclusion about their species status is justified at present.

However, in some cases the sampling scheme is adequate, statistically significant associations are found and the inference key yields unambiguous conclusions of gene flow among all sample locations, but no fragmentation events are inferred. In such cases, the NCA test of the null hypothesis that *the organisms sampled are derived from a single evolutionary lineage* can yield support for the null hypothesis; that is, the test justifies the biological inference that the organisms sampled constitute a single cohesion species. For example, many recent papers have argued that humans are split into more than one lineage, minimally into African and non-African lineages that split approximately 100 000 years ago (Stoneking 1997; Tishkoff *et al*. 1998). This interpretation of recent human evolution was motivated by haplotype trees (Cann *et al*. 1987; Vigilant *et al*. 1991) and haplotype trees continue to be cited as powerful evidence for a recent split between Africans and non-Africans (Stoneking 1997). However, none of the papers using haplotype trees to support a split between Africans and non-Africans have performed any statistical phylogeographic analyses of any sort but instead equate haplotype trees to population trees or make subjective arguments based upon a visual geographical overlay upon the haplotype tree — trees that were estimated incorrectly by the criteria given in the original papers (Templeton 1992, 1993, 1994b, 1996, 1997a,b). However, phylogeographic NCA's of human mtDNA (Templeton 1993, 1997b, 1998c), Y-DNA (Hammer *et al*. 1998), and nuclear autosomal DNA (Templeton 1998c) reveal no fragmentation events between Africans and non-Africans. The only major class of DNA inheritance pattern not included in these previous human NCA studies is X-linked DNA. Recently, Kaessmann *et al*. (1999) published a haplotype tree on a noncoding X-linked region with little recombination, but without performing any phylogeographic analyses. Figure 1 shows the haplotype tree for this region as estimated through statistical parsimony (Templeton *et al*. 1992; Crandall 1994). This tree is identical to the one given in Kaessmann *et al*. (1999) except that the sole loop of phylogenetic ambiguity in their network is resolved by statistical parsimony, yielding the single haplotype network with no ambiguity. Figure 1 also depicts the nesting

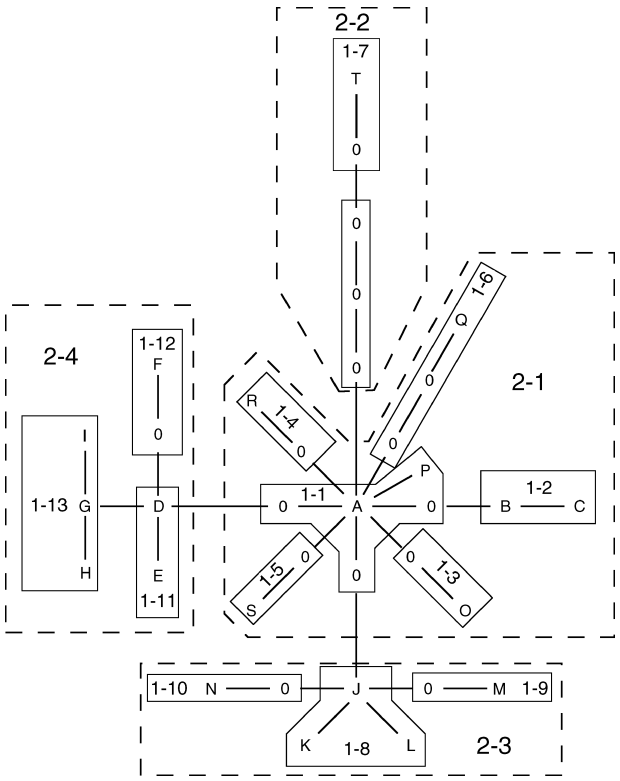

Fig. 1 Haplotype tree and nested clade design for a noncoding region of the human X chromosome showing low recombination, from Kaessmann *et al*. (1999). The distinct haplotypes are indicated by the letters A through T. One-step clades are indicated by solid boxes and polygons, dashed boxes and polygons indicate 2-step clades.

Table 1 Pooled sample sites used in the phylogeographic nested clade analysis of an X-linked region showing little recombination studied by Kaessmann *et al*. (1999)

Population/Site name	Pooled site numbers from Kaessmann *et al*. (1999)
Australian Aborigine	1 and 2
Nasioi	31, 52 and 59
Papua New Guinea	32, 33, 51 and 62
Phillipines	13
Thailand	47
China	39, 40, 41 and 42
Korea	43
Japan	44, 45 and 55
Chuckchi	3
Eskimo	54
Evenk	46
Buriat	38
India	17 and 36
Kyrgyz and Uzbek	15, 16 and 49
Georgia and Iran	4 and 14
Crimea	48 and 60
Western Europe	18, 19, 20 and 61
Great Britain	21 and 22
Scandanavia	28, 29, 30, 35 and 53
Mandenka	11 and 26
West Africa	6, 9, 10, 23, 34, 57 and 69
Sudan	7 and 12
Biaka	5, 67 and 68
East Africa	24 and 25
Mbenzele	27 and 58
Mbuti	63 and 64
San	8, 65 and 66
North Amerindian	50
South Amerindian	37 and 56

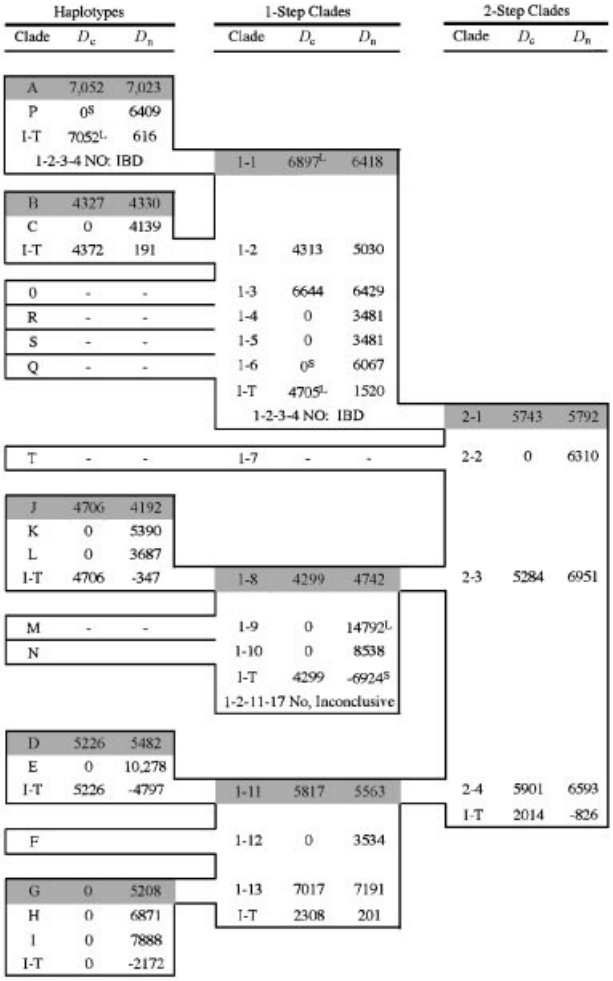

Fig. 2 Results of phylogeographic nested clade analysis of the human haplotype tree shown in Fig. 1. Clades at one level that are enclosed within a box are nested together, with an opening in the box leading to the nesting clade to the right. The average clade, D_c, and nested clade, D_n, distances were calculated and tested for significant deviations from the null hypothesis of no association between nested clades and geographical location within a nesting clade. A superscript 'L' by a distance measure indicates a significantly large distance (probability < 0.05 under the null hypothesis), and a superscript 'S' indicates a significantly small distance. In addition, clades were classified as either tips or interiors. Interior clades and their associated distances are indicated by stippled areas in the figure. 'I-T' refers to the average distances between interior minus tip clades within a nesting clade. If significant deviations from the null hypothesis of no geographical associations were detected among a set of nested clades, the inference key given in Templeton *et al*. (1995) was used to draw a biological conclusion. The numbers in the bottom of the nested clade boxes with one or more significant deviations from the null hypothesis refer to the sequence of question numbers obtained by use of this key along with the answer to the last question (Yes, No, or Inconclusive), followed by the biological inference. The only biological inference obtained was: IBD = recurrent gene flow restricted by isolation-by-distance.

design obtained with this network. Kaessmann *et al*. (1999) reported some 69 different sample locations, but small sample sizes precluded any meaningful analysis of all 69 sites. Hence, geographically close sites were pooled together and the geographical centre of the pooled sites was used as the location for the pooled sample. The populations used in the present analysis are given in Table 1. Figure 2 gives the results of the phylogeographic NCA. As can be seen, statistically significant signals are obtained, but in every case the inference key leads to the conclusion of gene flow restricted by isolation by distance. There is no fragmentation event between Africans and non-Africans, nor anywhere else among these human populations. However, note that we have not merely failed to reject the null hypothesis that *the organisms sampled are derived from a single evolutionary lineage*. In this case, the NCA has detected significant gene flow among these human populations (Fig. 2). This means that the NCA provides statistically significant evidence for all humans being a single cohesion species. The same is true for the mtDNA, Y-DNA, and

autosomal DNA analyses referred to above: all detected significant gene flow among human populations, and in particular between Africans and non-Africans (Hammer *et al*. 1998; Templeton 1993, 1997b, Templeton 1998b,c). Thus, all the major types of DNA in humans (maternal haploid, paternal haploid, autosomal diploid, and X-linked haplodiploid) are concordant: Africans and non-Africans have never been fragmented and Africans and non-Africans have experienced recurrent gene flow on the time scales defined by mutational accumulation in these various genetic systems — time scales that extend much farther back in time than the presumed 100 000 years ago 'split' between Africans and non-Africans. Note that the concordance among these haplotype trees is at the level of inference from the NCA; there is no expectation of concordance in haplotype tree topologies. This inference concordance indicates that humans are a single cohesion species. As this example illustrates, the NCA can provide evidence for both multiple lineages within the group of organisms being studied (significant fragmentation events) or evidence that these organisms are a single cohesion species (significant gene flow among all locations). This is a particularly powerful attribute of NCA with respect to the problem of inferring species.

The second powerful attribute of NCA is that it accommodates transspecific polymorphism, lineage sorting and hybridization. Transspecific polymorphism and lineage sorting can cause even populations that are absolutely fragmented to display haplotype trees that are not cleanly subdivided into monophyletic or even paraphyletic clades that correspond to the fragmented populations (Templeton 1999). This represents a serious problem to species concepts based on absolute phylogenetic patterns, such as the phylogenetic species concept (Cracraft 1989) or the genealogical species concept (Baum & Shaw 1995). The problem of hybridization presents an even greater challenge: how much hybridization can be tolerated and still regard two populations as separate lineages? A stringent application of the biological species concept would demand complete and absolute reproductive isolation under all circumstances in order to regard a population as a species, but most advocates of the biological species concept are willing to accept some level of hybridization. The reason for this tolerance is straightforward: as shown by many and particularly by Hewitt and coworkers (Hewitt 1990, 1993, 1996, 1999; Nichols & Hewitt 1994; Virdee & Hewitt 1994; Cooper *et al*. 1995; Ferris *et al*. 1995, van Oppen *et al*. 1997, 1998), hybridization is a common phenomenon among previously fragmented lineages that should not and cannot be ignored. Hybridization represents one of the primary challenges to the biological species concept (Templeton 1989; Hull 1997) because it undercuts the defining criterion of species under that concept; yet, it is such a widespread phenomenon that some degree of hybridization is allowed by even the strongest advocates of the biological species concept (O'Brien & Mayr 1991; Mayr 1992). However, this tolerance is subjective with no explicit, consistent criteria or quantification.

The cohesion species concept as implemented here addresses the problems of transspecific polymorphism, lineage sorting, and hybridization with objective and quantifiable criteria. With an NCA, evolutionary lineages are inferred by phylogenetic patterns having statistical significance; not an absolute pattern. As a consequence, lineages do not need to be clean, monophyletic groups in the haplotype tree, and the standard probability levels emerging from the NCA provide a straightforward, quantitative measure of the strength of the lineage inference. For example, none of the karyotypic races of mole rates recognized by Nevo *et al*. (1993) represents a strict monophyletic group in the mtDNA haplotype tree; yet, the NCA still detected three significant evolutionary lineages within this group that corresponded to the chromosomal races. Similarly, none of the pine tree lineages recognized by NCA correspond to monophyletic groups in the cpDNA tree due to rare hybridization (Templeton 1994a). The problems of transspecific polymorphism, lineage sorting, and rare hybridization become worse when dealing with recently speciated groups (Wu 1991, 1992; Avise 1994; Hilton & Hey 1997), as is certainly the case in the mole rats and pine trees. Hence, this pattern discrepancy between the haplotype tree topology and the fragmented lineages merely reflects a highly probable outcome expected under coalescent theory in recently derived groups. Therefore, any species concept that is going to be applicable to common situations encountered in nature must deal with these phenomenon. The cohesion concept does because the NCA automatically evaluates the strength of the signal for fragmentation vs. the confounding effects causes by transspecific polymorphism, lineage sorting, and hybridization in a manner that uses objective and quantifiable statistical criteria. No other species concept deals with hybridization in an objective, explicit and quantifiable fashion.

Although the NCA described in Templeton *et al*. (1995) accommodates hybridization in inferring lineages, it does not provide criteria for directly detecting hybridization among previously fragmented groups. Obviously, the NCA can detect haplotypes associated with different fragmented groups, so one criterion for hybridization would be the presence of such haplotypes in the same population. Note that this criterion is different from than suggested by Avise (2000) or Avise *et al*. (1987) of detecting admixture through the presence of highly divergent haplotypes in the same population. This pattern can arise from mechanisms other than secondary contact. For example, suppose a species is widespread geographically with no fragmentation but with extreme isolation by distance coupled with rare long distance dispersal. This situation could also result in the pattern suggested by (Avise *et al*. 1987; Avise 1994,

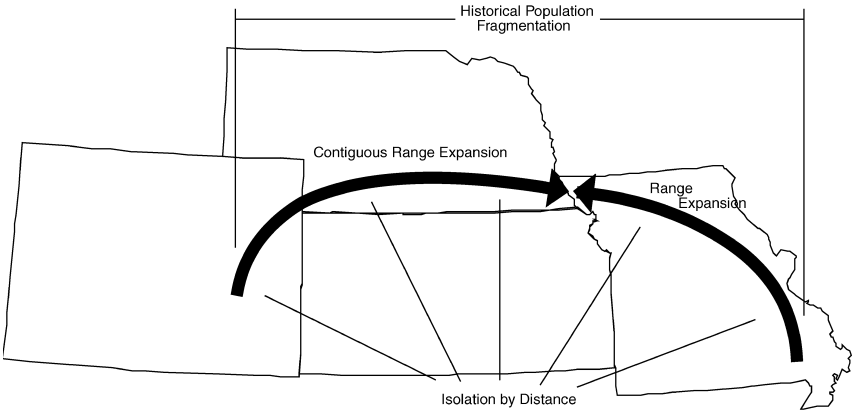

Fig. 3 A summary of the statistically significant inferences made with the *Ambystoma tigrinum* mtDNA haplotype tree with the phylogeographic nested clade analysis given in Templeton *et al*. (1995).

2000). In contrast, the criterion suggested here requires the prior statistically significant detection of a fragmentation event and detects hybridization through the common presence in a single population of haplotypes from clades marking different population fragments (not just sequence divergence).

This new criterion for hybridization can be quantified and made subject to statistical testing by a new addendum to NCA. In the original NCA, haplotypes and clades are the units of analysis and the NCA measures how geographically widespread they are, as shown in Fig. 2. Now consider looking at a single sample site and asking the question, how far apart are the geographical centres of the clades or haplotypes found at this site? The geographical centres of all haplotypes and clades are already calculated as part of the standard NCA, so the average pairwise distance between geographical centres of the haplotypes or clades found at a particular site can be calculated as well. Under panmixia, all haplotypes and clades have the same expected geographical centre, although fluctuations are expected due to drift and sampling error that can be readily tested through random permutation testing. Under isolation by distance, these average pairwise location distances are expected to be small relative to the total geographical range of the species, but when hybridization occurs between previously fragmented populations, haplotypes or clades with very divergent geographical centres can be placed at the same location. As one moves from haplotypes to higher level clades, the average pairwise location distances at a site should start approaching zero under isolation by distance, but it should stay large or even increase at those sites reflecting secondary contact. Indeed, when one reaches the clade level most strongly associated with the previously detected fragmented populations (through the standard NCA) that are now hybridizing, the discrepancy between the sites with and without secondary contact should be maximized.

An illustration of this new technique for detecting hybridization of previously fragmented lineages is provided by the salamander *Ambystom tigrinum* in central North America. Figure 3 shows a summary of the statistically significant inferences made from a phylogeographic NCA (Templeton *et al*. 1995). In particular, the oldest inferred event is fragmentation between the eastern and western populations (corresponding to two named subspecies, *A. t. mavortium* in the west, and *A. t. tigrinum* in the east), followed by subsequent range expansion of both fragmented populations into the great plains and the establishment of gene flow restricted by isolation by distance within each of the two lineages. However, the expansions of the two lineages apparently have brought them into secondary contact in north-western Missouri (Templeton *et al*. 1995). Because the distribution is basically east to west, Fig. 4 presents the average pairwise location distances between the geographical centres of the haplotypes found at a single sampling site plotted by the sites arranged by longitude. If all haplotypes at a site are of the same type, this pairwise location distance must be zero; otherwise, it can take on non-zero values. There is one sampling site that has a much larger pairwise location distance among the haplotypes found at that site (Fig. 4A), and this site is indeed in north-western Missouri and represents an area in which haplotypes from the two clades marking the two inferred fragmented populations are co-occurring. As one goes from haplotypes to 1-step clades as the genetic units of analysis, the average pairwise location distances tend to drop in most sites, many going to zero (Fig. 4B). This reflects the fact that under an isolation by distance model, which the original NCA (Templeton *et al*. 1995) indicated was an important factor in structuring the variation within (but not between) the two recognized subspecies, that most haplotypes found in a single geographical site tend to be closely related evolutionarily. This trend continues at most sites as one goes to 2-step clades (Fig. 4C), 3-step clades (Fig. 4D) and finally to 4-step clades (Fig. 4E). The original NCA (Templeton *et al*. 1995) identified a statistically significant fragmentation event defined by the contrast of the two 4-step clades found in the *A. tigrinum* mtDNA tree. As predicted above,

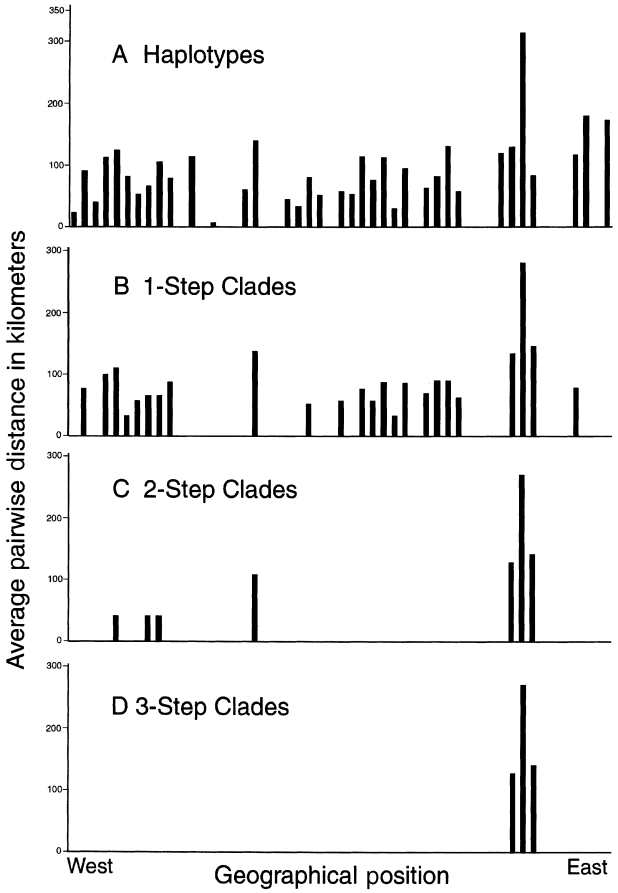

Fig. 4 Average pairwise location distances for haplotypes and clades for the *Ambystoma tigrinum* mtDNA haplotype tree given in Templeton *et al*. (1995). The 53 sample locations given in Templeton *et al*. (1995) are ordered by longitude, going from west (left) to east (right). Panel A shows the average pairwise distance between the geographical centres of haplotypes found at each of the 53 sampling sites, panel B shows the comparable distances for 1-step clades found at each site, panel C shows 2-step clade distances, and panel D shows 3-step clade distances.

the contrast between sites with and without secondary contact becomes most extreme at the 4-step clade level under this new metric. As Fig. 4(E) shows, most geographical locations have a pairwise location distance of zero, indicating that all haplotypes at these locations come from a single 4-step clade. However, there are three sampling sites that have non-zero pairwise location distances. They include the sampling site with the largest distance at the haplotype level, but in addition two other nearby sampling sites that did not have unusually large distances at the haplotype level (Fig. 4A). These three sites, all located in north-western Missouri, are the only three sites affected by admixture; that is, the joint presence of haplotypes from the two clades, 4-1 and 4-2, that mark a statistically significant fragmentation event. Hence, the clade level that marks a significant fragmentation event also is the clade level that most clearly (and completely cleanly in this example) marks the geographical sites at which secondary contact has occurred.

The above results indicate that this new test is a promising addendum to the original phylogeographic NCA that allows the objective detection and localization of yet another type of historic event: admixture following secondary contact of previously fragmented lineages. This promise has great implications, because one of the major controversies in population genetics and in the speciation literature is how to discriminate between primary differentiation (associated with isolation by distance) vs. secondary contact due to hybridization and introgression of previously fragmented lineages (Endler 1977). As shown by Fig. 4(D), the NCA with this new addendum cleanly discriminates between those populations affected by isolation by distance (zero pairwise location distances) vs. those affected by secondary contact of previously fragmented lineages (large pairwise location distances).

There are many other desirable attributes of this approach to testing the first null hypothesis that the organisms being sampled all come from a single evolutionary lineage, but these are discussed elsewhere (Templeton 1994a, 1998a, 1999). The ability of this approach to deal with hybridization in a simple, objective and quantifiable fashion clearly distinguishes the cohesion concept from many other species concepts. Hybridization and its attendant complications represent a major stumbling block for many species concepts, both in theory and in practice (Hull 1997, 1999). This stumbling block is simply a building block in the cohesion species inference chain as implemented here.

At the end of this first phase in testing for a cohesion species, an investigator will arrive at one of three possibilities. First, the NCA may indicate that the sampled organisms are all from one evolutionary lineage and therefore one cohesion species. Under this situation (illustrated by humans), the inference chain is now terminated because species identification has been achieved. Second, the NCA may have failed to reject the null hypothesis of one lineage, but not have yielded evidence for one lineage. This situation is illustrated by the 2n = 52 and 54 chromosomal races of mole rats (Templeton 1999). Under this situation, the current data set is inadequate for inference, so the inference procedure is suspended until additional samples or genetic data can be obtained. The third and final situation occurs when the null hypothesis of one lineage is rejected; that is, the organisms sampled are found in two or more evolutionary lineages, as shown by the pine trees, total mole rat complex, and the *A. tigrinum* complex.

Some species concepts (e.g. the diagnostic species concept, Hull 1997) equate species to evolutionary lineages, but the cohesion species concept does not. Cohesion species must be at least a single evolutionary lineage, but they can also contain more than one evolutionary lineage

if those lineages have not significantly diverged with respect to the attributes that define cohesion mechanisms (Templeton 1989). Hence, when the first null hypothesis is rejected with the result of having identified two or more lineages in the sample, the inference chain must proceed to a second phase.

Using haplotype trees to detect divergence in cohesion mechanisms

Given that the null hypothesis of a single evolutionary lineage has been rejected, a second null hypothesis must then be tested that *the previously identified lineages are genetically exchangeable and/or ecologically interchangeable.* The two major classes of cohesion mechanisms are genetic exchangeability (applicable primarily to sexual outcrossers) and ecological interchangeability (originally called demographic exchangeability, Templeton 1989). For sexual organisms, hypotheses about candidate traits for both genetic exchangeability and ecological interchangeability should ideally be tested; for asexual taxa only hypotheses about ecological interchangeability are relevant. As many worked examples of testing this second null hypothesis have already been published relating to both genetic and/or ecological ex/interchangeability (Templeton 1994a, 1998a, 1999), only some general comments will be made in this paper.

This second null hypothesis can be tested through direct statistical contrasts of the lineages previously identified in testing the first null hypothesis, or they can be tested through NCA. The NCA provides a robust procedure for testing phylogenetic associations with many types of data, including quantitative and categorical phenotypes (Templeton *et al*. 1987, 1988; Templeton & Sing 1993; Markham *et al*. 1996), categorical frequency data (Templeton 1995), and distance data, as illustrated in the previous section of this paper. This provides the needed flexibility for testing diverse types of data that could pertain to genetic exchangeability or ecological interchangeability. When NCA is used, the second null hypothesis is rejected only when a significant change related to genetic or ecological ex/interchangeability is detected, and the phylogenetic position of that change is concordant with the previously identified evolutionary lineage transitions.

Consider first genetic exchangeability. The cohesion concept recognizes two major classes of mechanisms related to genetic exchangeability; those preventing and those promoting gene flow. The mechanisms preventing gene flow are simply the reproductive isolating mechanisms that are used under the biological species concept (Templeton 1989). Many experiments on reproductive isolation, particularly but not exclusively those dealing with premating isolation, yield the analogues of a 'distance.' Hence, genetic exchangeability as assayed through reproductive isolation indices can be analysed in a fashion similar to that of geographical distance (Templeton 1998a). Some phenotypes can be related directly to reproductive isolation by appropriate experiments and observations, such as variation in chromosome structure and number. NCA can be applied to those phenotypes of reproductive isolation (Templeton 1999). The mechanisms that promote gene flow are more akin to the 'fertilization mechanisms' and 'mate recognition systems' used under the recognition species concept (Paterson 1993). Many phenotypes, both quantitative and categorical, can be related to mate recognition systems, and NCA of these phenotypes also represents a test of genetic exchangeability (Templeton 1998a, 1999).

Ecological interchangeability can be tested through NCA of many phenotypes, such as life history phenotypes (Templeton 1994a), habitat requirements or preferences (Templeton 1994a), and physiological data related to environmental tolerances and adaptation (Templeton 1999). Of course, many such phenotypes can be tested on the same group of organisms (Templeton 1999), and each case of concordance among transitions in ecological relevant phenotypes with lineages strengthens the inference of a cohesion species in a quantifiable and explicit fashion.

There are three causes for failure to reject the second null hypothesis. The first is that (i) the different evolutionary lineages detected previously are truly a single cohesion species; that is the lineages are genetically exchangeable (if sexual) and ecologically interchangeable. If the lineages are in fact distinct cohesion species, failure to reject the second null hypothesis could be due either to (ii) the fact that the appropriate reproductive or ecological traits that define the species in nature have not been included in the study and/or (iii) there is a lack of statistical power. Often it is not possible to discriminate among these possibilities. By increasing sample sizes, one can address the power issue, but the possibility of having overlooked a trait or suite of traits that are important in nature can never be fully discarded. Unlike the first null hypothesis, the failure to reject this second null hypothesis should never be interpreted as evidence for the different lineages being the same cohesion species. However, elevating one or more lineages to the status of cohesion species due to the rejection of this second null hypothesis is based on evidence for these lineages being distinct species. Thus, one needs evidence to recognize more than one cohesion species in the original sample, and this evidence is made explicit with objective and quantifiable inference criteria under this implementation of the cohesion species concept.

Discussion

The cohesion concept is made operational by testing two null hypotheses:

1 *the organisms sampled are derived from a single evolutionary lineage*, and
2 *the lineages identified by rejecting hypothesis 1 are genetically exchangeable and/or ecologically interchangeable*.

Only when both null hypotheses are rejected can a sample of organisms be split into two or more cohesion species. Otherwise, the organisms are regarded as members of a single cohesion species until future data or analyses allow the rejection of the null hypotheses.

With the development of this testable definition of cohesion species (Templeton 1994a, 1998a, 1999), the cohesion species concept emerges as the only species concept ranking high in all three criteria discussed by Hull (1997, 1999): it is general, applicable and theoretically significant. The generality and theoretical significance of the cohesion concept had previously been recognized (Hull 1997, 1999), so this paper focused on its applicability. What was shown here and in previous worked examples (Templeton 1994a, 1998a, 1999) is that when the cohesion concept is rephrased in terms of testable null hypotheses, it becomes an operational species concept that can be implemented by applying explicit statistical and biological inference criteria to types of data commonly gathered in current studies.

The cohesion species concept is also applicable at the individual level, that is, when a taxonomist wants to classify an individual specimen into a single species category. The procedure leading to an inference of a cohesion species requires a sample of many individuals. However, once the inference is made, the cohesion species concept can be used to classify individuals by using the same genetic data used to infer lineages and/or the lineage-associated morphological, life history or behavioural data used to reject the null hypotheses of genetic exchangeability and/or ecological interchangeability. For example, a sample of individuals drawn from the current taxonomic category *Drosophila silvestris* was split into two cohesion species that were cleanly separated by both genetic and morphological criteria related to genetic exchangeability (Templeton 1998a). In that case, all individual *Drosophila* specimens could be unambiguously classified as to cohesion species status by either their mitochondrial haplotype or by the number of bristle rows on their front tibia. In the analysis of a sample of mole rats, the genetic, morphological and behavioural data would sometimes not be individually diagnostic of an individual's cohesion species status (Templeton 1999), but using all these traits together in a multivariate sense would allow an unambiguous classification of the individuals. In the case of the tiger salamanders, a variety of genetic, life history or morphological characters would allow the unambiguous classification of almost all individuals sampled into the two inferred cohesion species, with the exception of some of the individuals from the three locations in north-western Missouri that had been subject to hybridization (Fig. 4). The fact that a few hybrid individuals would not be classifiable is true under other species concepts as well. Hence, the cohesion species is applicable at the individual classification level even though it demands population level sampling in its original inference.

This method of classifying individuals under the cohesion concept may at first seem similar to Mallet's 'genotypic cluster' definition of species (Mallet 1995). However, Mallet (1995) only considers current patterns of variation in defining genotypic clusters. In contrast, the variation used under the cohesion concept is restricted to that with statistically significant lineage associations. Evolutionary history is missing in 'genotypic clusters' but is critical to applying the cohesion concept.

Applicability does not necessarily mean easy. The operational cohesion concept requires much data to make inference, and some of these data may be difficult or expensive to gather for certain groups. Thus, the practical dominance of morphological distinctiveness as the criterion for species status will undoubtedly continue, but the increasing ease of gathering the molecular data needed to implement the cohesion concept as outlined above implies that the practicality of the cohesion concept, in terms of labour and expense, should rise in the near future. Moreover, science has frequently been driven and even redirected by a few well-worked examples, so even an occasional implementation of the data rich inference procedure outlined here could have a major impact on thinking about species or on the taxonomic status of groups deemed important for some reason.

Although this paper focused on applicability, the operational nature of the testable cohesion concept enhances both its generality and theoretical significance. By using explicit a priori criteria, the cohesion concept allows the use of a broader range of candidate traits than those under alternative species concepts while still providing focus to species inference. Data on reproductive isolation, mate recognition, and ecology can all be used. This enhances the generality of the cohesion concept. In particular, generality is enhanced by allowing the use of ecological data to test the second null hypothesis. This allows the cohesion concept to be implemented with asexual taxa in a straightforward manner. For example, much current microbial taxonomy is based upon a series of tests of growth under varying food media, antibiotics, etc., that is, these tests define at least a subset of the ecological niche that allows successful reproduction. The data from these growth tests could easily be incorporated into the testing scheme outlined here by the addition of molecular phylogenetic data. Such molecular data is becoming increasingly abundant for microbes, so all that is needed is a rigorous integration of the molecular data with the growth test data already in common use in order to implement the cohesion concept. This ecological dimension of a reproductive community is generally ignored by advocates of the biological species concept

when dealing with sexually reproducing organisms, but the ecological dimension of reproduction is relevant to the sexual world as well. For example, multiple lineages were found in *Ambystoma tigrinum* (Templeton 1994a), the *Pinus montezumae* complex (Templeton 1994a), and the *Spalax ehrenbergi* complex (Templeton 1999), and many of these lineages were elevated to cohesion species status on the basis of rejecting ecological interchangeability (although the mole rat lineages were elevated on the basis of both reproductive and ecological criteria). Often it is more practical to gather ecological data on natural populations than measuring such elusive traits such as the potential for reproductive isolation. Thus, the incorporation of ecological criteria in a focused manner enhances both the operationality and the generality of the cohesion concept.

Because the criteria for inference are statistical and quantitative, absolute categorical properties are not required for cohesion species status. This also greatly enhances the generality of the cohesion concept by providing the needed flexibility in dealing with a variety of biological processes, such as lineage sorting and hybridization. For example, none of the species inferred in *A. tigrinum* (Templeton 1994a), the *P. montezumae* complex (Templeton 1994a), or the *S. ehrenbergi* complex (Templeton 1999) were 'clean' in a phylogenetic sense; every group showed evidence of either hybridization, lineage sorting or both. These are real and common biologically phenomena, and the cohesion concept displays unique strengths in dealing with such phenomena.

The fact that cohesion species inference does not depend upon absolute categorical properties also enhances its theoretical significance. Lineage sorting and hybridization are most common in actively speciating groups, and these are precisely the groups most informative about the process of speciation. Hence, the cohesion concept is applicable in exactly those circumstances that have the most potential for yielding insights into the mechanisms of speciation. Moreover, the data rich inference procedure of the cohesion concept automatically insures much insight into likely contributors to the speciation process. The investigator will have information about biogeography; historical events such as range expansions, long distance colonization events, fragmentation events, and secondary contact; and evolutionary transitions in reproductive and/or ecological attributes as they relate to inferred historical events. Such information delimits or even defines the types of processes that were involved during speciation. Hence, the inference chain outlined above is both a procedure for inferring species and a procedure for studying the process of speciation. Pattern and process are intimately intertwined under the cohesion concept.

Advocates of the use of absolute categorical pattern properties in defining species often make the claim that such definitions are theory-free or neutral in their implications for processes (Hull 1997; Goldstein *et al*. 2000). However, all pattern definitions of species have implications for processes or theory, although these implications are unintended and hidden. For example, some pattern definitions allow no paraphyly. Because the cohesion species concept does not elevate all lineages to species status (e.g. the *P. montezumae* complex, Templeton 1994a), it can result in a paraphyletic relationship among inferred cohesion species (Templeton 1998a). However, the pattern criterion of no paraphyly has some ludicrous implications for speciation theory. For example, Templeton (1998a) gave an example showing how this pattern criterion can 'cause' speciation to occur between two populations showing no adaptive or reproductive divergence of any sort by evolutionary changes in a third population. This 'speciation by remote control' is patently absurd, but it is one of the unintended theoretical implications of this pattern definition of species. Hull (1997) also discusses the impossibility of these definitions of being truly theory-free.

In summary, the cohesion concept is general, applicable and theoretically significant. It integrates pattern and process through explicit statistical testing to infer species and to gain much insight into speciation. Hybridization, a common and important phenomenon as shown by the work of Hewitt (1993, 1996, 1999) poses a serious stumbling block for many species concepts, but not the cohesion concept. As molecular genetic data sets become increasingly common and less expensive, this approach to inferring species by testing null hypotheses also becomes increasingly easy to implement. This coming century should be an exciting and productive one for students of species and speciation.

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