

OPERATIONAL CRITERIA FOR DELIMITING SPECIES

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■ **Abstract** Species are routinely used as fundamental units of analysis in biogeography, ecology, macroevolution, and conservation biology. A large literature focuses on defining species conceptually, but until recently little attention has been given to the issue of empirically delimiting species. Researchers confronted with the task of delimiting species in nature are often unsure which method(s) is (are) most appropriate for their system and data type collected. Here, we review twelve of these methods organized into two general categories of tree- and nontree-based approaches. We also summarize the relevant biological properties of species amenable to empirical evaluation, the classes of data required, and some of the strengths and limitations of each method. We conclude that all methods will sometimes fail to delimit species boundaries properly or will give conflicting results, and that virtually all methods require researchers to make qualitative judgments. These facts, coupled with the fuzzy nature of species boundaries, require an eclectic approach to delimiting species and caution against the reliance on any single data set or method when delimiting species.

No one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species.

Darwin (1859/1964)

INTRODUCTION

Indigenous folk taxonomies spanning widely divergent cultures suggest shared and possibly innate cognitive mechanisms for recognizing biological categories (Atran et al. 1999, Hey 2001). The challenge for biologists is to go beyond subjective judgments and develop operational methods of delimiting species. Species are usually considered fundamental to studies of ecology, evolution, systematics, and conservation biology, yet the literature on the empirical methods of delimiting species has until recently been meager relative to the extensive literature on species concepts and on theory and methods of phylogenetic analysis (Wiens 1999). This is an odd state of affairs given that two frequently stated empirical goals of systematic biology are: (a) to discover monophyletic groups at higher levels, and

(*b*) to discover lineages (i.e., species) at lower levels (Wheeler & Meier 2000). Cracraft (2002) claims that the question, "What is a species?" remains the most crucial of the "seven great questions of systematic biology." Today, the empirical issue of species delimitation is receiving increased attention, and several novel methods have recently been proposed for delimiting species in a statistically rigorous framework (Puorto et al. 2001, Templeton 2001, Wiens & Servedio 2000, Wiens & Penkrot 2002). Nontree-based methods delimit species on the basis of gene flow assessments (Sites & Marshall 2003), whereas tree-based methods delimit species as historical lineages (Baum & Donoghue 1995, Goldstein & DeSalle 2000, Wheeler & Meier 2000).

Defining species conceptually has been contentious, as is evident by the plethora of species concepts (summaries in Zink 1997; Mayden 1997; Harrison 1998, table 2.1; Sluys & Hazevoet 1999), and some researchers have argued that there is no clear conceptual distinction between species and higher categories (Ereshefsky 2002, Mishler 2003). We share the view that species are spatio-temporally bounded entities (rather than classes defined by some common property; Baum 1998) that differ from higher categories in that they can originate by any number of mechanisms (Turelli et al. 2001) as a consequence of descent-with-modification (the unitary process of Frost & Kluge 1994). We anticipate that different data sets and different methods of delimiting species may give ambiguous or conflicting results (Hey et al. 2003, Sites & Marshall 2003) as a consequence of multiple evolutionary processes operating within and between populations across varying spatio-temporal scales (Harrison 1998, Lee 2003). Thus, the failure of any given discovery method in a particular case does not negate the reality of the species in nature (de Queiroz 1998, Wiley & Mayden 2000).

IMPORTANCE OF EMPIRICAL DELIMITATION OF SPECIES

Species delimitation is essential because species are used as basic units of analysis in several areas of biogeography, ecology, and macroevolution (Brown et al. 1996, Blackburn & Gaston 1998, Brooks & McLennan 1999, Barraclough & Nee 2001). Species are also the currency for global biodiversity assessments (Caldecott et al. 1996) and are therefore important to conservation biology (Agapow et al. 2004). At a more fine-grained level, species are often extensively subdivided (Hughes et al. 1997, Bohonak 1999), and their boundaries and population structures usually define the rates of, and limits within which, many evolutionary processes operate (Coyne et al. 1997). Empirical inference of species boundaries may be difficult when: (*a*) one or more characters acquires an independent geographic distribution within a species (which is likely given sufficient subdivision; Wade & Goodnight 1998) and therefore is/are not good whole-genome markers for the boundaries of the species (Porter 1990); or (*b*) a hybrid zone acts as a partial sieve (Martinsen et al. 2001) between two independent genealogical lineages that undergo some

intercrossing at points of parapatric contact (which may have several possible outcomes; Burke & Arnold 2001). Although these phenomena occasionally mislead delimitation of species, an understanding of evolutionary processes and mechanisms requires that we attempt to define objectively the arena within or across which these processes can act (see also Cracraft 2000) with rigorous empirical methods.

SPECIES CONCEPTS AND OPERATIONAL CRITERIA

Frost & Kluge (1994) and later Mayden (1997) distinguished between primary and secondary species concepts. The primary concept defines the entities believed to be species [the evolutionary species concept (ESC; Wiley 1978) was favored by these authors as the conceptual, or ontological definition], whereas secondary concepts are the operational methods for discovery of entities in accord with the primary concept. De Queiroz (1998) extended this idea further and noted that all modern species definitions either explicitly or implicitly equated species with “segments of population level evolutionary lineages,” which he termed the general lineage concept (GLC) of species. These authors all made the same distinction between the ESC ontological definition and criteria used to delimit species in nature (empirical discovery methods).

This distinction is useful. These authors’ perspectives capture the meaning of lineages (see Pigliucci 2003 for a similar perspective), and although lineage splitting (speciation) may result in species that can be empirically discovered (via fixation of a character, loss of a polymorphism, attainment of reproductive isolation, etc.), the processes causing the new species eventually to become distinct are usually unpredictable. A GLC (i.e., one defining species as ancestor-descendent populations) allows investigators to test species boundaries from different philosophical perspectives. Population biologists, for example, are likely to favor biological species criteria (giving primacy to gene flow), whereas systematists usually favor phylogenetic criteria (distinctness of lineages). Regardless, the delimitation of species requires that one have clearly defined operational criteria by which individuals can be tested for species membership, and the criteria must be understood within the context of what kind of entity (interbreeding versus historical lineage) each method is designed to test. In this review, we discuss 12 methods of species delimitation and consider the kinds of suitable data for, and the strengths and limitations of, each method (Table 1).

Nontree-Based Methods

Boundaries of sexually reproducing species (*sensu* Mayr 1942) are conceptually defined on the basis of reproductive compatibility within, or reproductive isolation between, species, and these boundaries can occasionally be tested by direct studies of crossability (Dettman et al. 2003b). This testing is not possible for many sexual species, however, and a number of methods are available for indirectly estimating gene flow within and between hypothesized species.

TABLE 1 Empirical methods for delimiting species in the context of properties/criteria, classes of data, generality (asexual versus sexual), and some important assumptions and/or limitations of each

Method ^a	Relevant biological properties/criteria ^b	Classes of data suitable to method	Assumptions/limitations
HZB ¹	Limited or no gene flow across hybrid zone	Nuclear genes with codominance	F_{ST} -based Nm estimator; assumes drift-gene flow equilibrium, with isolation-by-distance model
GenD _{GW} ¹	Gene flow within but not between species	Multilocus allele frequency data	Assumes drift-gene flow equilibrium
GenD _H ¹	Time-dependent emergence of reproductive isolation	Multilocus allele frequency data	Assumes a molecular clock correlated with a genomic basis for reproductive isolation
FFR ¹	Recombination within nuclear loci limited by extent of gene flow	Nuclear genes with codominance	Requires identification of all alleles segregating at a locus, and no gene flow between species
PAA ^{1,2}	Lineage isolation sufficient for fixation of character states	Allozymes, chromosomes, morphology, binary (presence/absence) of data	Assumes conspecificity of individuals from same locality; character fixation difficult to show at conventional levels ($\alpha = 0.05$) of confidence
Corr-D ¹	Correlated divergence in morphology and gene sequence mark discontinuity	Morphology, molecular markers (both converted to pairwise distances)	DNA and morphology must be available for same specimens; test may be circular if putative species are in sympatry
M/GC ¹	Morphological discontinuities or reduction/absence or gene flow mark boundaries species limits	Morphological or genetic characters	Between species variability is greater than within species variability; introgression limited or absent
PCT ^{1,2}	Lineage isolation sufficient for character divergence	Allozymes, chromosomes, morphology, DNA sequences	Some versions reduce speciation to single character substitutions, others require strong a priori rejection of some modes of speciation or anagenesis
CHA ^{1,2}	Lineage isolation sufficient for coalescence to monophyly of haplotypes at one locus	DNA haplotypes for one locus	Equates nonrecombinant haplotype clades to species
EXCL ¹	Lineage isolation sufficient for allelic coalescence to exclusivity at unlinked loci	DNA haplotypes for multiple loci	Requires unspecified number of unlinked genes with divergence profiles matched to timing of speciation events

(Continued)

TABLE 1 (Continued)

Method ^a	Relevant biological properties/criteria ^b	Classes of data suitable to method	Assumptions/limitations
WP ¹	Lineage isolation sufficient for geographical character divergence	DNA haplotypes, morphology, etc.	Assumes no gene flow between species; no interspecific recombination between haplotypes
TTC ^{1,2}	Lineage isolation sufficient for attainment of ecological or allopatric character divergence	Genetic, ecological, morphological, or physiological data; with DNA haplotypes	Inference key can be misled by insufficient sampling density; H ₂ can never be completely falsified; choice of candidate traits may be subjective

^aAbbreviations: cladistic haplotype aggregation (CHA), exclusivity criterion (EXCL), field for recombination (FFR), genetic distance Good & Wake (GenD_{GW}), genetic distance Highton (GenD_H), hybrid zone barrier (HZB), population aggregation analysis (PAA), Templeton's tests for cohesion (TTC), Wiens & Penkrot methods (WP), correlated distance matrixes (Corr-D), morphological-genotypic cluster (M/GC), phylogenetic/composite tree-based methods (PCT); for each of these methods, the superscripts 1 and 2 denote suitability of method to sexual and asexual taxa, respectively.

^bThese are deliberately general because multiple properties (criteria) are manifested during the speciation process, but both the order of their appearance and their relevance to testing species boundaries depend on many idiosyncratic conditions and mechanisms associated with a particular speciation event, and at what point along an evolutionary trajectory extant populations are sampled (de Queiroz 1998).

HYBRID ZONE BARRIER Porter (1990) used gene flow statistics with allozyme loci to test species boundaries in two hybridizing North American butterflies (*Limenitis lorquini*, *L. weidemeyerii*). The method derives from Wright's (1931) equilibrium relationship between gene flow and genetic differentiation and uses the following expression to estimate gene flow (number of migrants):

$$Nm_{ST} \approx (1/F_{ST} - 1)/4,$$

where the subscript *S* represents subpopulations, *T* represents the total set of populations, *N* is the effective population size, and *m* is the effective proportion of migrants between populations, under an island model of population structure. Porter used the estimator θ (Weir & Cockerham 1984) in place of F_{ST} and an isolation-by-distance model in place of Wright's (1931) island model, as justified by simulation studies (Slatkin & Barton 1989).

The method proceeds in two steps. First, Nm estimates are calculated between sympatric sibling species pairs to assess the strength of biases in the Nm estimator. Because this comparison is between sets of populations not currently connected by gene flow, a result showing $Nm > 0$ must be attributable to other factors that contribute to genetic similarity between populations. As a control, Nm estimates are also calculated among subpopulations within species and are expected to reflect gene flow at a level that presumably prevents fixation of alleles in the absence of selection ($Nm \geq 1.0$). The factors most likely to bias Nm in the direction >0 (giving the appearance of gene flow when there is none) include balancing selection, sister

taxa characterized by large N coupled with recent divergence, or the presence of undetected cryptic alleles (hidden heterogeneity; Coyne 1982). Porter (1990, pp. 134–35) gives details on calculating confidence intervals on Nm , as well as on what other kinds of information can be used to judge which of these biases is most likely in a given case.

The second step is to estimate Nm across a parapatric hybrid zone between nominal species under a null hypothesis (H_0) that the hybridizing populations are genetically isolated. Estimates of gene flow that are not significantly greater than those typical of sympatric sibling species would support the null hypothesis; i.e., these populations maintain separate gene pools despite some hybridization. The empirical examples presented in Porter (1990, table 1) suggest that the Nm estimator provides a reasonable estimate of gene flow except when it falls below ~ 0.5 .

If $Nm > \sim 1.0$, then ongoing gene flow is likely important in promoting genetic similarity between hybridizing groups, and they should be considered conspecific. If $\sim 0.5 < Nm < \sim 1.0$, then gene flow is weak but probably sufficient to permit rapid exchange of selectively favored alleles; this pattern would also reject H_0 in favor of conspecificity. In these cases, investigators should examine any relevant secondary evidence that could inform interpretation of the Nm result to determine what proportion of genetic similarity can be attributed to factors other than gene flow. If $0.0 < Nm < 0.5$, then the hybridizing populations are almost or fully isolated genetically; gene flow is unimportant relative to other processes, and the hybridizing populations would be considered separate species.

GENETIC DISTANCE: GOOD & WAKE Good & Wake (1992) described a method for qualitatively assessing gene flow as a function of geographic isolation over a range of spatial scales. The method involves plotting genetic distances (in the particular case in Nei 1978, distances summed over allozyme loci) against geographic distances for all pairwise comparisons made in a particular study. A regression line is then fitted to a set of points representing samples from a priori defined subsets of the total (i.e., all samples from a single river basin or some other geographically or taxonomically defined entity). If the regression line for such a sample passes through the origin of the graph, such a pattern is most readily explained by gene flow with isolation-by-distance, and populations are interpreted to be conspecific (or at least to represent part of a single genetically cohesive unit). Conversely, the regression for a sample of populations that includes genetically isolated groups will deviate significantly from a 0 origin because genetic divergence among samples is expected to be independent of their degree of geographical separation. The method is illustrated in its original application in the salamander genus *Rhyacotriton* (Good & Wake 1992) and has been used in studies of several other genera of salamanders, including *Desmognathus* (Tilley & Mahoney 1996), *Ensatina* (Jackman & Wake 1994), and *Plethodon* (Highton & Peabody 2000, Highton 2000).

GENETIC DISTANCE: HIGHTON Following a different line of reasoning, Highton (1989, 1990) suggested that, for some groups characterized by extremely slow

rates of morphological evolution (e.g., salamanders of the family Plethodontidae), species boundaries are most easily identified by multilocus allozyme data from which genetic distances (D of Nei 1972, 1978) are calculated and used to infer the distance that correlates with the origin of intrinsic reproductive isolation (refined arguments presented in Highton 1995, 1998, 2000). Specifically, Highton suggested that groups of samples differing by a Nei $D \geq 0.15$ should be considered distinct species; he recognized that this value was arbitrary, but he cited a review of the allozyme literature (Thorpe 1982) in which most (97%) genetic identities (I) between well-defined species of vertebrates are < 0.85 , whereas most ($> 98\%$) I values within a species are > 0.85 (a Nei I of ≈ 0.85 is the equivalent of a Nei D of ≈ 0.16). These patterns are general enough across nonavian vertebrates to suggest that, as a rule of thumb, the divergence usually needed to complete speciation is correlated with a D of ≈ 0.15 – 0.16 .

The method is implemented by plotting a histogram of D value frequencies for pairwise comparisons between populations (Highton 1998), and under a null model of conspecificity among all populations, the distribution should be approximately unimodal (presumably owing to isolation-by-distance within a species) with most values clumping below $D \approx 0.15$ (Figure 1). If the samples comprise different species, then the distribution of D values is expected to be bimodal, with a second peak well above $D \approx 0.15$ (Figure 1). The protocol includes two criteria: (a) that a $D > 0.15$ approximates that point at which reproductive isolation is completed, and (b) that pairwise D values will be bimodally distributed if two or more species are included in the sample (Figure 1). Only the second of these criteria is independent of the taxon (salamanders of the family Plethodontidae) upon which the method was based. Highton recommended that genetic data be corroborated with morphological and/or distributional data before making strong inferences about species boundaries (see Highton & Peabody 2000).

CORRELATED DISTANCE MATRICES A method described by Puerto et al. (2001) uses molecular and morphological data in combination to delimit species, specifically a combination of mtDNA haplotypes, a multivariate statistical summary of morphological variation, and numerical hypothesis-testing techniques, based on an example with taxonomically problematic pitvipers of the *Bothrops atrox* complex in Brazil. First, morphological variation was summarized as a matrix of Euclidean distances between specimens; this matrix is the set of dependent variables, and Mantel tests are then used to evaluate sequentially the potential alternative causal factors for morphological similarities or differences. The alternative factors tested included sex [matrix values were either 0 (between individuals of the same sex) or 1 (opposite sex)], geographic distances (straight-line distances between all paired combinations of individuals), and patristic distances of mtDNA haplotypes collected for the same individuals used to obtain Euclidian distances. Puerto et al. (2001) then tested whether morphology was associated with mtDNA distances, with the effects of the other factors regressed out; morphology was used as the only observed (dependent) variable. The expectation was that if two species

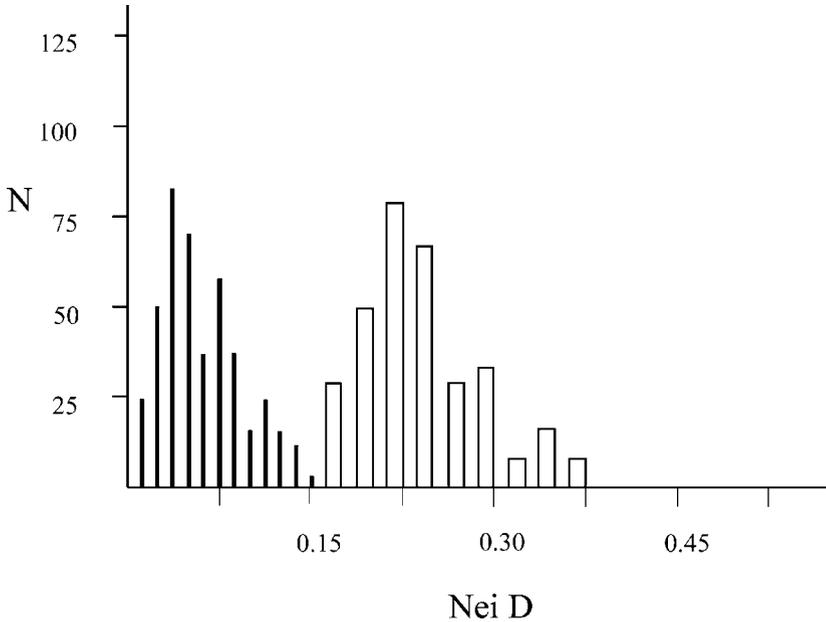


Figure 1 Histogram showing distributions of pairwise genetic distance values among multiple samples whose species status is of interest (Highton 1989, 1990, 2000). A unimodal distribution in which all D coefficients are <0.15 (Nei 1978) reflects divergence among populations within a single species (*solid bars*), and a bimodal distribution in which a substantial subset of pairwise D values are >0.15 (*both sets of bars*) represents between-species comparisons (see text for details).

are present in a sample, they should be characterized by possession of different mtDNA clusters, and specimens belonging to either species should show patterns of morphological variation congruent with species identity as revealed by mtDNA sequence variation (after regressing out the effects of sex and geographic distance). The alternative is that two (or more) mtDNA clusters may exist within a single species, in which case researchers would not expect patterns of morphological and mtDNA variation to correlate with each other.

The empirical results of this study revealed two overlapping mtDNA haplotype clusters of *Bothrops atrox* (Puerto et al. 2001, figure 3), a result compatible with a hypothesis of sympatry of two species, or conspecificity and the co-mingling of different genotypes in a single species. Mantel tests did not reveal any significant association between morphological variation and mtDNA affinities of the specimens, and in this case the authors failed to reject the single-species hypothesis.

MORPHOLOGICAL METHODS Until the 1940s, most biologists recognized species solely on the basis of morphological differences (Coyne 1994), following a methodology approximately as described by Cronquist (1978): “Species are the smallest

groups that are consistently and persistently distinct, and distinguishable by ordinary means." The implication is that the existence of "gaps in the pattern of visually observable phenetic diversity" is taken as evidence for reproductive isolation, and species are then delimited along the boundaries of morphological discontinuities. Cronquist recognized that opinions might differ on how to define the criteria for "consistent," "persistent," and "ordinary" means, but he insisted that any group of organisms that did not meet all three of these criteria to some reasonable degree should not be recognized as a species. Statistical rigor has been applied to quantify the criterion of phenetic discontinuity, most notably by numerical clustering approaches (Sokal & Sneath 1963); multivariate statistical methods identify samples divisible by phenetic gaps resulting from concordant differences in character states (i.e., minimally distinguishable in multivariate space), and these samples are often interpreted as species (see also Sokal 1973, Sneath 1976).

Mallet (1995) extended this reasoning to a genotypic cluster approach to delimit species as "clusters of monotypic or polytypic biological entities, identified using morphology or genetics, forming groups of individuals that have few or no intermediates when in contact." The morphological and genotypic clustering approaches are conceptually similar (statistical grouping algorithms, visual inspection to determine if clustering is significant), but neither is a discrete method in the sense of the others described in this review. However, both morphological and molecular characters can be coded in ways that permit implementation of some of the methods described here, particularly the population aggregation analysis (PAA).

POPULATION AGGREGATION ANALYSIS The PAA method was formulated by Davis & Nixon (1992) on the basis of two principles: (a) all individuals sampled from a local population are assumed to be conspecific, and (b) identical character attributes shared among individuals drawn from two or more populations provide evidence for conspecificity. The PAA is a formalized protocol for traditional approaches to identifying one or more diagnostic morphological characters for species delimitation (see also Nixon & Wheeler 1990); it codifies a way to identify the morphological discontinuities considered essential by Cronquist (1978), although Davis & Nixon (1992) recognized its applicability to many other kinds of data. The PAA requires a summary of character states for all individuals in a sample to estimate a population profile for those states, and then it combines all samples with identical profiles for all character states. This process is continued iteratively until the only remaining sample aggregates are those separated from each other by fixed character state differences; these samples are the smallest diagnosable units (Cracraft 1983, Nixon & Wheeler 1990) and are taken to be species. The method was illustrated in the original paper (Davis & Nixon 1992) and was recently used by Benavides et al. (2002).

FIELD FOR RECOMBINATION A method described by Doyle (1995) delimits species by using Mendelian loci (originally allozymes) to define a field for genetic

recombination (FFR, Carson 1957), which should be coincident with the boundaries of sexually reproducing species in which constituent populations are interconnected by gene flow. Because sexual reproduction defines the FFR for Mendelian loci, Doyle (1995) argued that the distribution of alleles would more faithfully define species boundaries than the phylogenies of those same alleles; the latter may not be equivalent to species boundaries because of idiosyncrasies of lineage sorting, between-locus mutation rate heterogeneity, and intralocus recombination (Avice & Wollenberg 1997, Hare 2001). Doyle's method seeks to identify natural discontinuities between different allelic states for a given locus on the basis of overlapping sets of heterozygous individuals. The presence of two alleles in a single individual is taken as evidence that these two alleles belong to the same allele pool, and so for a single locus, sampling of multiple individuals from multiple localities will permit grouping individuals together, or separating them into distinct groups, on the basis of overlapping or nonoverlapping sets of heterozygous genotypes. Better resolution of a gene pool boundary will likely be afforded by extending this procedure to additional loci. At the organismal level, the multilocus genotype provides evidence of gene flow among populations, and individuals possessing alleles belonging to a multilocus gene pool are part of the same FFR. Individuals not sharing alleles at any loci are considered to be members of a different FFR, and species are delimited on the basis of discontinuities in multilocus FFRs. This method was illustrated in the original description (Doyle 1995) and has been implemented in at least one system (J.C. Marshall, E. Arévalo, E. Benavides, J.L. Sites & J.W. Sites, Jr., submitted manuscript).

Tree-Based Methods

Systematists typically favor phylogenetic methods to delimit species, including several versions of the phylogenetic species concept (PSC; Rosen 1979, Mishler & Brandon 1987, Baum & Donoghue 1995), as well as more recent extensions and related discovery methods. Some of these methods were reviewed by Sites & Marshall (2003) and are again covered here but in the context of several versions of the PSC not covered by that review.

PHYLOGENETIC/COMPOSITE TREE-BASED METHODS The terminology of Brooks & McLennan (1999) is useful in recognizing tree-based methods originating from various versions of the PSC. These authors denoted an apomorphy-based method (Rosen 1979) as PSC-1, a lineage-splitting method as PSC-2, and a node-based composite species concept (CSC; see Kornet 1993). These methods differ from each other primarily with respect to how species are delimited on a phylogenetic tree and how ancestral species are handled. All rely on the reconstruction of a phylogenetic hypothesis from a data set for which discrete characters can be coded and polarized by standard cladistic methods followed by mapping character transitions onto the topology (see Brooks & McLennan 1999). These methods can be distinguished most clearly by comparing the number of species recognized and the

criteria for their recognition under PSC-1, PSC-2, and CSC for the same number of terminal units (i.e., potential species).

Figure 2 compares three hypothetical trees with identical topologies characterized by the same number of terminal taxa (seven species, A–G) but differing in the number or distribution of apomorphic transitions. Figure 2A presents a tree in which all terminal and interior branches are supported by one apomorphy each; here all three methods would recognize twelve species: all seven terminal (A–G) and all five internal (= ancestral) branches. The number of species delimited would be different if a data set produced the same topology but did not recover apomorphies for some terminal branches. In Figure 2B, for example, terminals B, C, and G lack apomorphies, and a strict application of the apomorphy-based

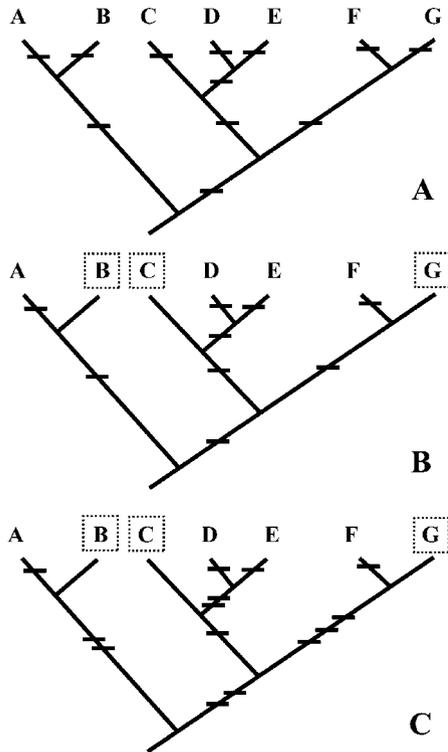


Figure 2 Hypothetical phylogenies with identical topologies for seven terminals (A–G), in which horizontal bars represent single apomorphic character transitions. (A) Tree in which each terminal and each interior branch are defined by single apomorphies; (B) tree in which each interior and all but three terminal branches are defined by single apomorphies; and (C) tree identical to B except that some internal branches are supported by two or three apomorphies. Terminals enclosed in squares in trees B and C are those not supported by apomorphies [modified from Brooks & McLennan's (1999) figure 1 with permission].

phylogenetic method (PSC-1) and the node-based CSC would recognize only nine species (terminals B, C, and G are considered species but are not distinguished from their common ancestors). The lineage-splitting PSC-2 would still recognize terminals B, C, and G as distinct species because it is based only on the branching structure of the tree and is not concerned with character evolution. Finally, in Figure 2C four internal nodes are characterized by more than one (two or three) apomorphic character transitions. All three methods recognize the seven terminals A–G as distinct species, but again the apomorphy method (PSC-1) collapses terminals B, C, and G with their common ancestors, whereas the CSC recognizes fourteen species—one for each apomorphic character on the tree. It thus permits recognition of multiple species on a single branch on the premise that the origin and fixation of each apomorphic trait required permanent lineage splitting; each apomorphy delimits the present or prior existence of a distinct species (Brooks & McLennan 1999).

CLADISTIC HAPLOTYPE AGGREGATION Brower (1999) extended the population aggregation analysis to encompass sequence data and cladistic analysis in an effort to take full advantage of the information content of the evidence. The use of DNA data requires one to decide how attributes are defined; one can either (*a*) use a well-defined region of DNA as a single attribute (Doyle 1995), in which haplotypes are scored as nonadditive alternative allelic states; or (*b*) atomize the sequence to the level of resolving single bases as attributes. Brower proposed to call these alternatives the PAA1 (sequence-as-a-single attribute) and PAA2 (string-of-attributes) methods and used a number of contrived and empirical data sets to compare the results of each with cladistic haplotype aggregation (CHA) (discussed below).

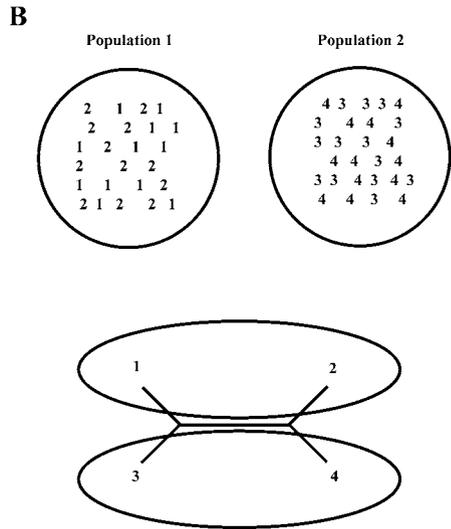
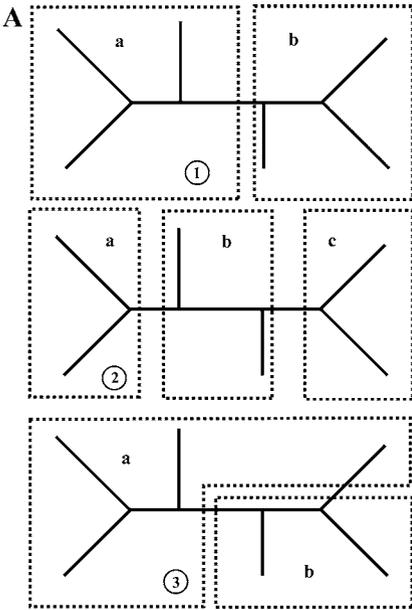
CHA is implemented by first identifying individual organisms as representatives of local populations, collecting DNA sequences (haplotypes) of exemplars, tabulating haplotypes to determine sample profiles, and aggregating sample profiles (by either PAA1 or PAA2) that do not have fixed character differences into a single population profile (as in the PAA of Davis & Nixon 1992). One then estimates a phylogeny of the original unaggregated haplotypes (all distinct population profiles) and uses this phylogeny to corroborate or refute the tentative species delimited by either PAA1 or PAA2. Brower argued that all members of a species will form a contiguous section of an unrooted tree and will be separated from all other groups of samples by a single branch along which a character transition leading to a fixed character difference can be inferred (Figure 3A). Therefore, groups identified by PAA will be corroborated by CHA if they form a contiguous section on the tree. The results of CHA “do not imply phylogenies, but rather represent parsimonious patterns of empirical grouping that corroborate or reject specific *a priori* hypotheses of species boundaries” (Brower 1999, p. 202).

Brower compared the performance of PAA1 and PAA2 with haplotype aggregation using contrived and real data. Figure 3B shows a hypothetical case in which two populations fixed for alternative attribute states would be successfully identified by PAA1 but not by a cladistic analysis of the gene genealogy (modified from Davis 1996). For data sets consisting of independently segregating attributes

(allozymes, chromosomes, microsatellites, morphology, etc.), alternative fixation of individual character states provides plausible evidence for separation of lineages (absence of gene flow), and PAA1 is appropriate. With sequence data, however, this distinction is not so obvious. Consider the hypothetical case illustrated in Figure 3C (for visual clarity different symbols were substituted for nucleotides). Here a homologous 13-bp sequence has been collected for six exemplars representing an outgroup and two population samples whose species status is of interest. If we apply PAA1, four distinct haplotypes are identified (in the two ingroup samples) because each sequence is considered a single character. The PAA1 approach delimits the two populations as two species because of fixed character differences [mutually exclusive allelic (haplotype) polymorphisms segregating at a single locus] without considering the phylogenetic relationship between haplotypes. Brower (1999) saw this delimitation of species as arbitrary. Alternatively, when each nucleotide is treated as an independent character, PAA2 identifies six characters (attributes 1–6) and seven traits (attributes 7–13) that are by definition not useful in delimiting species by the original PAA method of Davis & Nixon (1992). PAA2 collapses haplotypes 1 and 2 into a single population profile for sample 1, and haplotypes 3 and 4 for sample 2, and also delimits two species (Figure 3C). Brower's method attempts to make use of all 13 attributes present to test the validity of the species delimited by PAA. In this example, he recovers the seven most parsimonious cladograms, a strict consensus of which reveals no phylogenetic structure (Figure 3C) between the two populations, and thus rejects the hypothesis of distinct species. Brower argued that, by selecting from the observed data only those attributes that support the groups already assumed a priori to exist (the populations from which samples 1 and 2 were drawn), the PAA methods (in either form) are circular. Brower (1999) extended this argument by providing another hypothetical example in which the PAA failed to recover population differentiation where it actually existed, and he suggested that the reason for both kinds of inconsistencies is that the PAA does not use character congruence in a phylogenetic context to distinguish homology from homoplasy, whereas CHA is based on this premise.

GENEALOGICAL EXCLUSIVITY Baum & Shaw (1995) presented a different tree-based method that formalized a genealogical species concept, originally suggested by Avise & Ball (1990), in which relatedness is viewed in the context of genealogical descent of the genome. Operationally, the delimitation of genealogically exclusive species requires that: (a) species must be basal taxa, i.e., they must not themselves contain taxa; and (b) genealogical species reside at the boundary between reticulate and divergent genealogy (the tokogeny-phylogeny interface, *sensu* Hennig 1966). Species are therefore defined as exclusive groups; those in which all members are more closely related to each other than to any organism outside of the group. As in the above phylogenetic methods, such species can only be delimited when relationships are hierarchical, but the conceptual distinction here is that the idea of genealogical exclusivity derives from coalescent theory (Hudson 1990, Baum & Shaw 1995). Coalescent theory is concerned with tracing the genealogical

histories of extant samples of genetic elements (alleles or haplotypes) and is normally applied to multiple unlinked loci that characterize sexual species (Maddison 1997). Thus, one can infer that when organismal genealogy is divergent (phylogenetic) for a sufficient amount of time (Neigel & Avise 1986), then selectively neutral, unlinked genes should attain concordant genealogical histories, whereas these histories will become discordant when the organismal genealogy is reticulate (tokogenetic; see also Taylor et al. 2000).



attributes:	1	2	3	4	5	6	7	8	9	10	11	12	13	PAA 1	PAA 2	CHA	
outgroup:	a	a	a	a	a	a	a	a	a	a	a	a	a				
Pop. 1a:	+	+	+	+	+	+	q	q	q	q	q	q	q	1			
1 1b:	+	+	+	+	+	+	+	+	+	+	+	+	+	2			
profile 1:	+	+	+	+	+	+	±	±	±	±	±	±	±	Sp. 1	Sp. 1		
Pop. 2a:	q	q	q	q	q	q	+	+	+	+	+	+	+	3			
2 2b:	q	q	q	q	q	q	q	q	q	q	q	q	q	4			
2c:	q	q	q	q	q	q	q	q	q	q	q	q	q	4			
profile 2:	q	q	q	q	q	q	±	±	±	±	±	±	±	Sp. 2	Sp. 2		
PAA2	⏟						⏟										
identities:	6 characters						+	7 traits									

Operationally, the method requires reconstruction of genealogies for unlinked loci collected from the same exemplars and then the construction of a strict consensus of the separate gene trees to define points of concordance (resolved nodes). Species are delimited by exclusive nodes in the consensus tree. Figure 4 illustrates a contrived example in which eleven individuals are screened for four unlinked genes, which are then used to reconstruct independent gene genealogies for the eleven terminals. These four gene trees are taken to represent the coalescent histories of these loci among the organisms sampled. A strict consensus of the four genealogies defines one point of concordance among the independent gene genealogies, which shows that terminals f–k are exclusive. That is, the alleles of these terminals for all loci coalesce more recently within the group than between any member of this group and any of the other exemplars sampled. This same group is also basal—there are no other exclusive groups nested within it, and it therefore meets the criteria for recognition as a genealogical species. In contrast, exemplars a–e are not exclusive because the smallest group containing it (a–k) is not basal (it includes the exclusive group f–k; the entity a–e would be labeled a

Figure 3 (A) Hypothetical outcomes of CHA analysis (Brower 1999) in unrooted haplotype networks. Dotted lines identify distinct groups of haplotypes; two phylogenetic species are delimited in network 1 and three in network 2 because in both cases groups of haplotypes are identified by apomorphic character transitions along single branches in the network. In contrast, species-level distinctness is rejected in network 3 because the two clusters of haplotypes are connected to each other by two branches. (B) Hypothetical population samples characterized by fixed differences for alternate allelic states at a single locus (alleles 1 and 2, and alleles 3 and 4, in the two populations, respectively). In this example, the PAA1 of Brower (1999), as originally described by Davis & Nixon (1992), delimits the two species, whereas a gene genealogy with the topology depicted here would not delimit these populations as separate [modified from Brower's (1999) figure 2 with permission]. (C) Hypothetical sequence data matrix with thirteen bases collected for six exemplars (outgroup and two population samples of two and three individuals, respectively), and a comparison of outcomes of PAA1, PAA2, and CHA analyses for delimitation of species. The PAA1 (sequence region as the attribute) identifies four unique haplotypes in the five ingroup sequences given; two species are delimited, one for each sample because each segregates two mutually exclusive sets of alleles (as in panel B). The PAA2 (atomistic) also delimits two species (among the ingroup exemplars) on the basis of aggregated population profiles that show fixed differences at six characters (attributes 1–6), and seven polymorphic traits [attributes 7–13, following the Davis & Nixon (1992) protocol]. The CHA [a strict consensus of seven most parsimonious (MP) trees] resolves only one unambiguous clade (1b + 2a) because attributes 7–13 are polarized as synapomorphies; note that this result conflicts with a priori assumptions made about population membership [modified from Brower's (1999) figure 3 with permission].

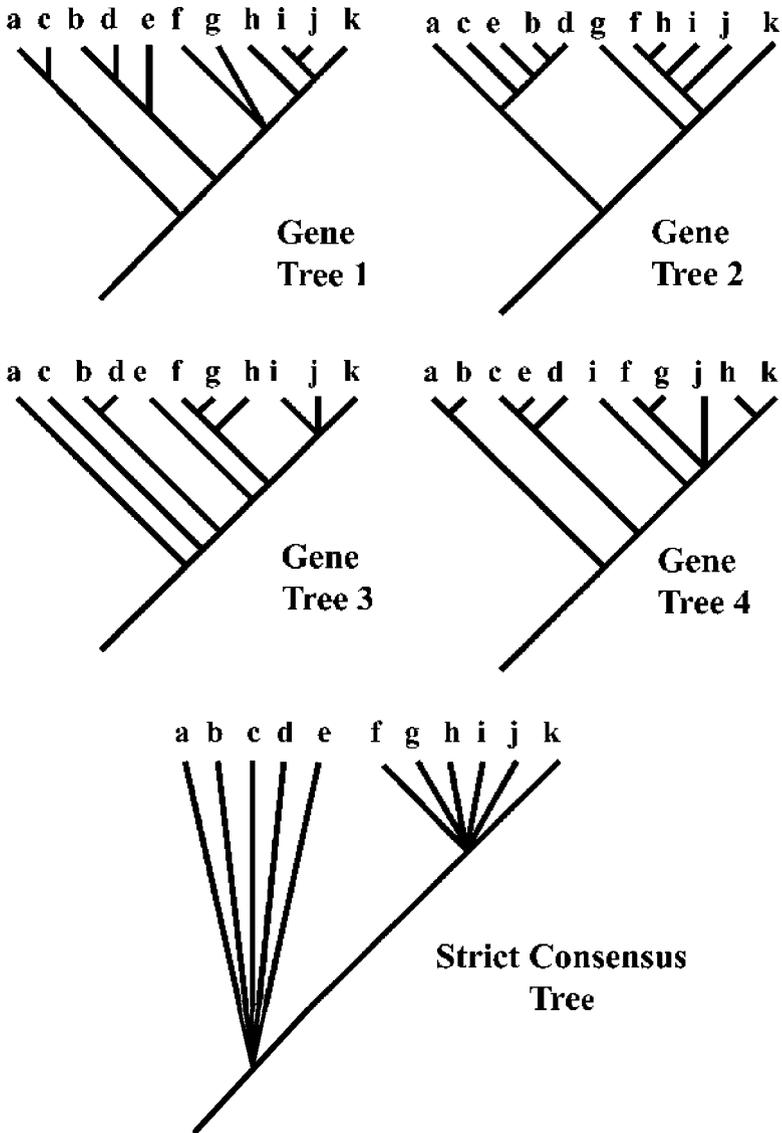


Figure 4 Hypothetical example in which gene genealogies are reconstructed from four unlinked loci for eleven individuals; the strict consensus tree recovers a single genealogical species that meets the two requirements specified by Baum & Shaw (1995): (a) genealogical species are basal taxa (i.e., they do not themselves contain taxa); and (b) these species are exclusive (i.e., all members are more closely related to each other than to any individuals outside of the group; see text for details). In this example, terminals f–k comprise a genealogical species, whereas the unresolved polytomy that includes terminals a–e does not.

ferespecies or metasppecies by some; Graybeal 1995). This approach was recently implemented by Dettman et al. (2003a), who used four nuclear gene regions to delimit species of the fungus *Neurospora* on the basis of concordance (with strong nodal support) for three of the four single gene genealogies. They added a novel criterion of genealogical nondiscordance to recognize species when a clade was strongly supported by a single-gene genealogy and not contradicted by any other single-locus genealogy at the same level of support.

WIENS-PENKROT PHYLOGENETIC METHODS Wiens & Penkrot (2002) recently described formal protocols for implementing molecular and morphological phylogenetic methods for delimiting species. They applied both to empirical data for *Sceloporus* lizards, along with results from a character-based morphological approach. The molecular method is intended to be used in combination with a nested clade analysis (Templeton et al. 1995) and is based on a sampling design that should ideally include: (a) a number of closely related reference species to test for exclusivity for the focal species (the species of interest in the study), and (b) sampling of at least two individuals from as many localities as possible within the focal species to increase the strength of between-population gene flow inferences. The method requires a phylogeny of nonrecombining haplotypes of known locality, and taxonomic designation (focal taxa are the testable hypotheses) and a topology that fails to recover haplotypes from a given locality as a clade is taken as evidence for potential gene flow with other populations.

The morphological method is similar but based on population, rather than individual, sampling to avoid the default treatment of all shared polymorphisms as homoplasies (rather than synapomorphies) if single individuals are used (see Wiens 2000). This method considers sets of populations that are strongly supported, exclusive, and concordant with geography as species. As with the molecular protocol, the morphological method assumes that gene flow among populations and recombination among characters will break up hierarchical patterns within species, and the absence of historical signal below the species level will permit recovery of only weakly supported trees that are discordant with geography. Both the methods are implemented using dichotomous flow charts that lead to several alternatives for making species-level decisions. Figure 5 diagrams the pathways for the morphological method.

TEMPLETON'S TESTS OF COHESION Extensions of Templeton's (1989) original, general hypotheses about genotypic and phenotypic cohesion have rendered both operational (Templeton 1994, 1998, 1999; Templeton et al. 1995) and include tests of species boundaries (Templeton 2001). Hypothesized species boundaries are statistically tested through a set of nested null hypotheses structured to evaluate the correlation of genotypes and/or phenotypes with geographic location [nested clade analysis (NCA); Templeton et al. 1995]. An inference key (Templeton 1998, 2004) is then used to derive the most likely biological cause(s) for observed patterns of variation. The method tests two hypotheses:

H₁ all organisms are sampled from a single evolutionary lineage

H₂ populations of separate lineages identified by rejection of H₁ are genetically exchangeable and/or ecologically interchangeable among themselves.

Species are recognized only after rejection of H₁ and H₂ at the same levels of divergence.

An NCA inference of historical fragmentation at some nested clade level is the only inference taken as evidence for the possible existence of separate evolutionary lineages (Templeton 2001) and requires rejection of H₁. When H₁ is rejected with statistical support for historical fragmentation, the two or more lineages inferred from the first NCA may constitute different cohesion species, and the second null hypothesis (H₂) must be tested. The question here (H₂) is whether populations comprising the two (or more) lineages defined by the historical fragmentation event(s) are genetically exchangeable and/or ecologically interchangeable among themselves. H₂ can be tested by direct statistical contrasts of the lineages previously identified or through the NCA to test for a statistical concordance of candidate traits for genetic exchangeability (i.e., those associated with prevention or promotion of gene flow) or for ecological interchangeability (life-history traits, ecological requirements, physiological tolerances, etc.). H₂ is rejected only when (a) a significant association is detected between geography and the genetic or ecological trait associated with genetic exchangeability or ecological interchangeability, and (b) the phylogenetic position of this association is concordant with the previously identified historical isolation event(s) (i.e., both are identified at the same clade level). Recent attempts to test H₂ include Shaw's (1999) use of song patterns (mate recognition) in the Hawaiian cricket genus *Laupala* and Templeton's (1999) use of chromosome number in races of mole rats (*Spalax*) as candidate traits for genetic exchangeability. Templeton (1999) also used nonshivering thermogenesis, hematocrit and hemoglobin concentrations, breathing and heartbeat frequencies, and oxygen and carbon dioxide pressures in subcutaneous gas pockets as candidate traits to test for ecological interchangeability in *Spalax*. More recently, Gomez-Zurita et al. (2000) used trophic selection and altitude of habitat in leaf beetles of the *Timarcha goettingensis* complex as candidate ecological traits.

DISCUSSION

General Observations

The methods surveyed in this review vary relative to biological properties and criteria, the kinds of data suitable, and some assumptions or limitations (Table 1). Methods appear to be biased in favor of using molecular markers, but six methods also include morphological data (PAA, correlated distances, morphological/genotypic cluster, phylogenetic/composite trees, Wiens-Penkrot, and Templeton's tests for cohesion), and tree-based methods can now accommodate allele frequency data and frequency and continuous morphological characters (Wiens & Penkrot 2002).

Most methods (hybrid zone barrier, Good-Wake distance, correlated distances, PAA, FFR, morphology/genotypic cluster, exclusivity, Wiens-Penkrot, and Templeton's tests for cohesion) refer explicitly or implicitly to gene flow and are applicable to sexually reproducing species, but some nontree-based methods (PAA and CHA) are operationally general enough to apply to asexual complexes, and the various tree-based approaches (phylogenetic/composite and Templeton's) are explicit in their incorporation of asexual taxa. The hybrid zone and Good-Wake distance methods implicitly assume that populations are in approximate mutation/drift equilibrium, a condition likely rarely met in nature (Pannell & Charlesworth 1999, Whitlock & McCauley 1999).

Several methods are likely to have technological limitations for most organisms. For example, the FFR, CHA, and exclusivity methods all implicitly assume perfect sampling for any locus; that is, no heterozygotes or distantly related alleles are missed for any given population. In all these methods, homoplasy will falsely identify different alleles as identical and either underestimate topological structure (CHA and exclusivity methods) or lead to inferences of gene exchange (FFR) when gene flow is absent. Both allozyme (Murphy et al. 1996) and microsatellite loci (Li et al. 2002, Estoup et al. 2002) can be characterized by allelic homoplasy between closely related species, which will introduce an unknown error into the use of either kind of data in FFR methods. Perhaps amplified fragment length polymorphisms (Sullivan et al. 2004) or single-nucleotide polymorphisms (Brumfield et al. 2003, Morin et al. 2004) might be viable alternatives. The methods described by Porter (1990), Good & Wake (1992), and Highton (1989, 1990) may also be sensitive to resolution of alleles, but the possible biases resulting from this phenomenon have not been carefully studied. The exclusivity criterion test requires sequences of unlinked gene regions that may not be available for many taxa, although this is changing (Hare 2001, Zhang & Hewitt 2003).

Operational Assumptions and Limitations

The hybrid zone method (Porter 1990) is the most restrictive relative to conditions that must be met in nature because it requires hybrid zones and regions of sympatry between closely related species, but many such complexes are now known that could be subjected to this test (Arnold 1997, Burke & Arnold 2001). The genetic distance method of Highton (1989, 1990) assumes that reproductive isolation is due to divergence across many loci scattered over the entire genome (Coyne & Orr 1989, Orr 1996, Hollocher & Wu 1996). This assumption is the same for the presumed clock-like accumulation of genetic distances by point mutations, and although there are broad correlations between D values and reproductive isolation in some groups (Coyne & Orr 1989, Sasa et al. 1998), there are enough exceptions to inflate error terms (on D estimates) sufficiently to compromise the diagnostic value of such correlations (Ferguson 2002).

The graphical genetic distance method (Good & Wake 1992) qualitatively delimits species on the basis of a correlation of pairwise D values with geography and thus depends more on patterns of gene flow than on whether D correlates tightly

with the emergence of reproductive isolation. The more quantitative correlated distance matrix method of Puerto et al. (2001) provides an explicit test of associations between independent data sets, and it discriminates between alternative hypotheses by partial correlation test to regress out factors that do not contribute to significant associations. It requires that both morphological and molecular divergence be concordant with species limits, and the tests are most powerful when both data sets are collected from the same individuals. One strength of this method is that in scenarios in which putative species are allo- or parapatric and there is an a priori basis for distinguishing these, then researchers can assign each specimen to a putative species and use such an assignment as another independent matrix in a Mantel test. A limitation is that such a test is not possible with putative species in partial sympatry because individual specimens cannot be assigned to putative species in the region of sympatry without causing the test to be circular.

The morphological methods summarized above have well-characterized limitations that need little elaboration here; they may be difficult to apply to organisms characterized by cryptic species (Mayden 1997), and when used to summarize patterns of geographic variation, phenetic clustering methods assume a causal connection between overall similarity and degree of genetic continuity (de Queiroz & Good 1997, but see Highton 2000).

Virtually all methods are sensitive to sampling design, with different implications for individual, geographic, and character sampling, and these points have been explicitly addressed in some methods. PAA (Davis & Nixon 1992) specifies the clearest relationship between fixed character differences and species boundaries; characters must be present at 100% frequency to be diagnostic of species limits. However, this criterion cannot be established with statistical confidence given finite sample sizes (Wiens & Servedio 2000). Wiens & Servedio (2000) replaced the 100% fixation criterion with one that is less restrictive (e.g., 95%) but that can be achieved with statistical confidence and may still indicate an absence of gene flow. This method enables one to evaluate the statistical confidence that a character is diagnostic (present at a frequency established by the investigator) in the context of finite sampling. The FFR method (Doyle 1995) is likely susceptible to false positives in favor of gene flow owing to retained ancestral polymorphisms (J.C. Marshall, E. Arévalo, E. Benavides, J.L. Sites & J.W. Sites, Jr., submitted manuscript).

Tree-based methods provide various criteria for species delimitation as described above, but several of these methods (the various phylogenetic/composite, CHA, and exclusivity) do not explicitly emphasize any geographic component, which will likely be relevant for inferences about gene flow in sexually reproducing species and are emphasized by other approaches (Wiens-Penkrot and Templeton methods). All methods that rely on gene genealogies can be misled by discordance if these genealogies coalesce above or below the divergence/reticulation interface because there is no basis for equating gene trees to species lineages if divergence has been recent (Hudson 1990, Avise 2000; but this may become less of a problem as time since divergence increases, see Neigel & Avise 1986). The

Wiens-Penkrot approach does emphasize the rapid sorting of the mtDNA locus in these cases, presumably because mtDNA is more likely to track recent species splits relative to nuclear loci (Moore 1995, 1997). However, stochasticity associated with population structure, recurrent mutation, selection, and other processes generate sufficiently large confidence intervals for mtDNA coalescent times that they may overlap extensively with nuclear gene coalescents (Hudson & Turelli 2003). Thus caution is required when interpreting single gene genealogies for any method.

Until recently, most DNA-based studies of species boundaries have been based on single loci, and the over-reliance on single gene regions to delimit species has been problematic because phylogenetic structure can extend below the level of species (as for asexual taxa or haploid genomes), and/or tokogenetic relationships may extend above the level of species (interspecific hybridization or reticulation; de Queiroz & Donoghue 1990, Davis 1996). If hybrids must be categorized and narrow hybrid zones are common in a particular group, then notions of diagnosability or monophyly are not useful, and other approaches must be sought. Detailed population sampling of a diversity of taxa often reveals widespread intraspecific paraphyly and polyphyly resulting from hybridization and other processes (Funk & Omland 2003) as well as mismatches between nuclear and mitochondrial genomes in cases where both have been sampled (Shaw 2002). In this case, an advantage of Templeton's tests for cohesion is that they do not require absolute properties (character fixation, exclusivity, complete absence of gene flow) if both individual and geographic sampling are adequate (Templeton 2001), but an operational limitation is the extensive sampling required for all taxa included in a study. A conceptual limitation of Templeton's methods is that negative evidence for H_2 (failure to reject) cannot be taken as evidence that different lineages belong to the same cohesion species because this outcome may simply result from failure to select the appropriate ecological or reproductive trait(s) to delimit lineages in nature that really do represent distinct species. Other concerns about the accuracy of the nested clade inference key (Knowles & Maddison 2002) can at least partly be addressed by cross-validation of inferences with other analyses based on different assumptions (Masta et al. 2003, Morando et al. 2004).

Finally, all methods that require monophyly or exclusivity (CHA, genealogical exclusivity) or that insist on the primacy of branching structure over character evidence (PSC-2 in Figure 2B), do not permit recognition of ancestral species that retain plesiomorphic characters relative to their descendents. This pattern is likely to occur when peripheral populations speciate from a widespread ancestor (Talbot & Shields 1996, Hedin 1997) and will result in paraphyly of the ancestral species. Adoption of these methods thus requires a priori rejection of some modes of speciation that may be important processes in nature (see also Brooks & McLennan 1999, Barraclough & Nee 2001). At another extreme, the node-based composite species method does not allow for within-species anagenesis, an assumption that also severely constrains the kinds of evolutionary processes that biologists might study in phylogenies (Brooks & McLennan 1999).

Where Do We Go from Here?

The emerging debates over empirical methods of species delimitation suggest that evolutionary biologists are giving the issue serious consideration (Brower 1999 versus Davis & Nixon 1992, Wake & Schneider 1998 versus Highton 1998). Researchers agree that speciation processes create fuzzy boundaries under which all methods will occasionally fail or be discordant with each other and that this is an unavoidable consequence of the many combinations of deterministic and stochastic processes associated with any speciation event (de Queiroz 1998, Harrison 1998, Frost 2000). Empirical examples bear out this expectation: In salamanders of the *Ensatina eschscholtzii* complex, one (Wake & Schneider 1998), two (Frost & Hillis 1990), seven (Graybeal 1995), or eleven (Highton 1998) species can be recognized on the basis of different methods. This result reflects both the multiple properties that change at different rates and in different order during speciation and the fact that different methods were often designed to detect different kinds of entities (distinct lineages versus interbreeding populations).

When viewed in this context, all methods will sometimes fail to delimit species boundaries properly, and virtually all will require researchers to make qualitative judgments. For example, there is no objective criterion for how much morphological divergence is enough to delimit a species, what threshold frequency of intermediates is needed to delimit species by genotypic clusters (Mallet 1995), what proportion of unlinked loci are needed to delimit coalescent species (Hudson & Coyne 2002), or what frequency cutoff most appropriately indicates that no significant gene flow is occurring between populations (Wiens & Servedio 2000). Proponents of the apomorphy-based criterion for historical delimitation of species have described auxiliary ranking criteria as a means of providing independent justification to assign species rank to some monophyletic groups and not others (Mishler & Brandon 1987). These criteria may include causal processes, such as interbreeding, selective constraints, and developmental canalization, as well as practical criteria (Mishler & Theriot 2000), such as number and quality of synapomorphies, bootstrap values, etc.

The impasse in the debate surrounding species concepts and species delimitation has precipitated recent proposals to dump the species category altogether because some insist that species be defined in a manner consistent with all other Linnaean ranks (Pleijel & Rouse 2000, but see Lee 2003). Others suggest that groups of organisms be delimited by the amount that they differ from other groups, independent of the concept of species (Hendry et al. 2000, but see Avise & Walker 2000). What has been missing in most empirical studies are honest assessments of uncertainties of methods (Adams 2001, Hey et al. 2003) and cross-validation of inferences from a single method or data set. The particular features of the biology of some groups make them well suited to particular methods and data sets (see Adams 1998 and Taylor et al. 2000 for reviews of species concepts and recognition in nematodes and fungi, respectively), but even when this is not the case, the variety of methods now available (Table 1) allows investigators to select the combination

of data and analyses that best matches the reality (i.e., individual or character sampling limitations) of a particular study. In most cases, researchers should be able to analyze the same data set with several methods and/or to collect multiple data sets for independent corroboration; both provide opportunity for cross-validation of hypotheses based initially on one analysis of a single data set (Agapow et al. 2004, Templeton 2004).

We think evolutionary biology would now be well served by detailed comparative studies applying multiple methods and data sets to species delimitation in natural populations. Some studies (Wiens & Penkrot 2002; Dettman et al. 2003a,b; Fukami et al. 2004) show that when different methods and data give conflicting results for the same organisms, researchers can often sort out the most likely cause(s) for the conflict. We can make few generalities about which methods are best for a variety of taxa and biological properties, but additional studies should reveal how alternative methods designed to recover the same entities (i.e., interbreeding populations) compare with each other and under what conditions the boundaries of different entities (interbreeding populations versus historically distinct lineages) will coincide with each other.

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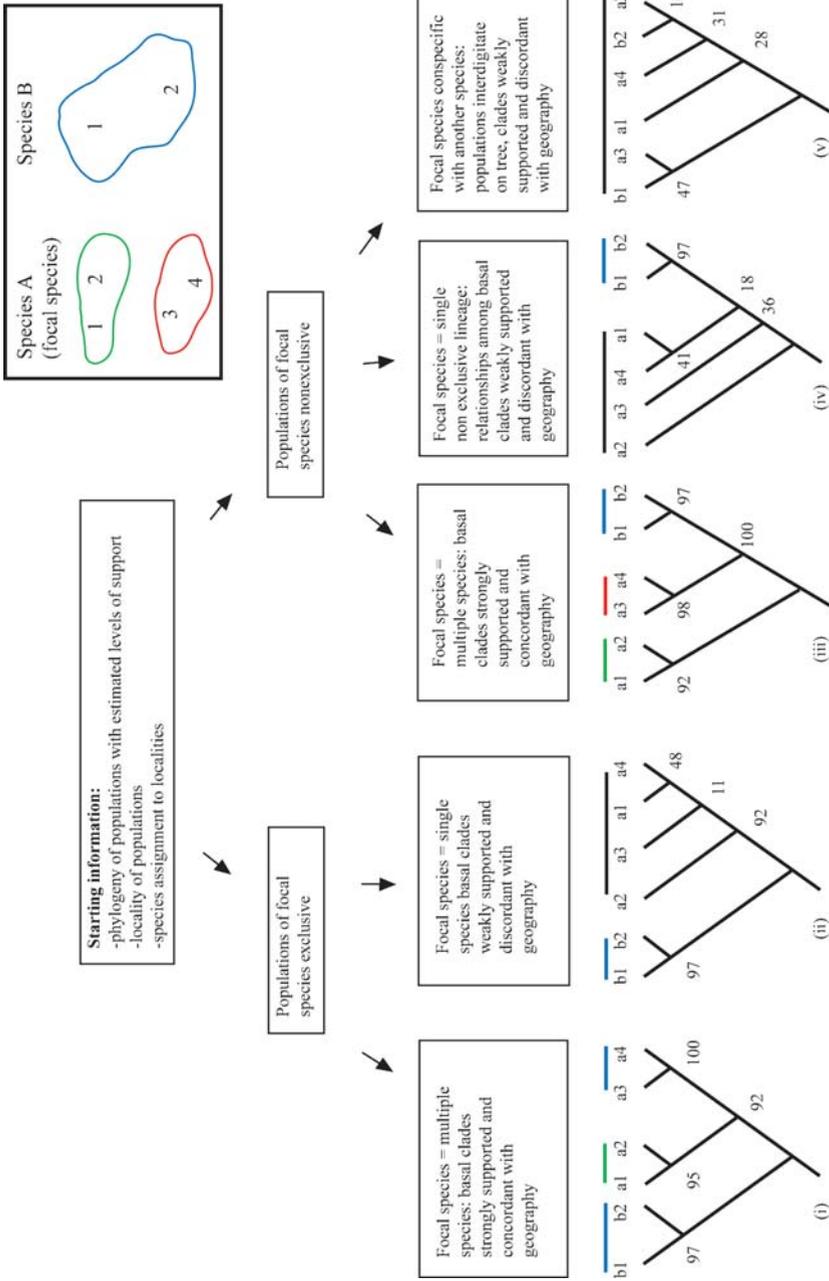
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Figure 5 Morphological tree-based method of species delimitation described by Wiens & Penkrot (2002). Species *a* represents the focal species in which population samples are drawn from two geographically distinct regions (indicated by *green* and *red lines*), and species *b* represents a closely related nonfocal species (delimited by *blue lines*). Phylogenetic relationships are inferred from population samples, and species are delimited on the basis of the positions of the terminal branches in the tree (the five possibilities for completely resolved trees are the topologies i–v; numbers at nodes represent bootstrap values), relative to their geographic points of origin, by following the pathways depicted here. The DNA tree-based method (not shown) is similar but uses individual haplotypes rather than population samples (see Wiens & Penkrot 2002 for more detail).