Testing Species Boundaries in Biodiversity Studies

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Abstract: *A paper recently published by Phillips et al. (1996) reported a molecular genetic study of all recognized subspecies of the common snapping turtle (*Chelydra serpentina*) and concluded from patterns of geographic variation in isozyme and mitochondrial DNA restriction fragment pattern data that three evolutionary species should be recognized in this group. We suggest that this paper is fundamentally flawed because it fails to present any species concept as a testable hypothesis. Data are collected in the absence of any conceptual framework for diagnosing species boundaries, so no criteria for acceptance or rejection of a preferred hypothesis are formulated, and species boundaries are determined in a nonrigorous, post hoc manner. Further, the absence of specific criteria for species diagnosis in this case leads to flaws in sampling design, data collection, and data analysis. Because these design flaws are typical of many studies, we briefly outline three different lineage-based operational species concepts (phylogenetic, concordance, and cohesion) and present an alternative interpretation of the* Chelydra *data, insofar as this is possible given the design limitations. We conclude the following: (1) species status may not be warranted for the Central and South American taxa, (2) more-detailed analysis is warranted in the U.S. population because distinct lineage may be obscured by poor lab technique or introgression of mtDNA, and (3) the Ecuadorean population may deserve species status based on fixed nuclear isozyme loci. We recommend the following procedures for implementation of lineage-based species concepts within a rigorous hypothesis testing framework. First, if an animal is to be sacrificed, proper care should be taken to utilize different tissues for multiple pass electrophoresis. This will unmask hidden heterogeneity and maximize the number of resolved loci. Second, when phylogenetic relationships are reconstructed with restriction fragment length polymorphism data, fragment data should be converted to site data to avoid uncertainties in homology. Finally, a proper sampling scheme should be designed to address the question of species status in terms of numbers of individuals, genetic loci, populations, and geographic regions.*

Comprobar Demarcaciones de Especies en Investigaciones de Biodiversidad

Resumen: *Un artículo publicado recientemente por Phillips et al. (1996), reportó un estudio acerca de la genética molecular en todos las subspecies reconocidos de la tortuga común (*Chelydra serpentina*). Se concluyó, con base en los patrones de variación geográfica en isoenzimas y en fragmentos de restricción de ADN mitocondrial, que tres especies evolutivas deberían ser reconocidas en este grupo. Nosotros sugerimos que ese artículo está mal fundamentado porque no presenta un concepto de especie como hipótesis a probar. Por consiguiente, los datos son obtenidos en ausencia de un marco conceptual para diagnosticar límites de especies. Esto es, que no se formula un criterio para la aceptación o rechazo de una hipótesis adoptada y los límites de especie son determinados de una manera post hoc y no rigurosa. Además, la ausencia de criterios específicos para la diagnosis de especies, en este caso, lleva a un inadecuado diseño de muestreo, obtención de datos y análisis de los mismos. Debido a que estos diseños inadecuados son típicos en muchos estudios, nosotros damos brevemente los lineamientos de tres diferentes conceptos operacionales de especie basados en linajes (filogenético, de concordancia y de cohesión) y presentamos una interpretación alternativa para los datos de* Cheyldra, *hasta donde es posible, dadas las limitaciones del diseño. Concluimos lo siguiente: (1) el estatus de especie puede no ser garantizado para los taxa de Centro y Suramérica, (2) un análisis más detallado es requerido en las poblaciones de U.S. ya que técnicas deficientes de laboratorio o la introgresión de mtADN pueden dificultar la distinción de linajes, y (3) las poblaciones ecuatorianas podrían recibir el estatus de es-*

pecie con base en loci fijados de isoemzimas nucleares. Recomendamos lo siguiente para la implementación de conceptos de especie basados en linajes dentro de un riguroso marco de hipótesis a probar. Primero, si un animal va a ser sacrificado, se debe asegurar el uso de diferentes tejidos para múltiples corridas de electroforesis. Esto descubrirá heterogeneidad escondida y maximizará el número de loci resueltos. Segundo, al construir las relaciones filogenéticas con datos de polimorfismos en longitud de fragmentos de restricción, se debería de convertir los datos de fragmentos en datos de sitios para evitar dudas en la homología. Finalmente, se debería diseñar un apropiado esquema de muestreo para abordar la pregunta del estatus de especie en términos de número de individuos, loci genéticos, poblaciones y regiones geográficas.

Introduction

This journal recently published a paper by Phillips et al. (1996) centered on the conservation genetics of the common snapping turtle (*Chelydra serpentina*). The data collected for this study included two classes of molecular markers, one consisting of mitochondrial DNA (mtDNA) fragments obtained by digestion with restriction endonucleases and the second from a multilocus isozyme data set resolved by starch-gel electrophoresis. Samples of all four recognized subspecies of *C. serpentina* were included in the analyses, but the two subspecies indigenous to Central and South America, *C. s. rossignonii* and *C. s. acutirostris* (hereafter referred to as the CSA taxa), respectively, were each sampled from only a single locality. The authors emphasized two major findings of this study. First, mtDNA divergence was sufficiently large between the Central and South American subspecies and between these two as a monophyletic group relative to a monophyletic group consisting of the two U.S. subspecies (*C. s. osceola* and *C. s. serpentina*) that species status was warranted for each of the two CSA subspecies. Second, allozyme divergence was minimal among the four subspecies and was deemed not to be useful for diagnosis of species boundaries in this group. The authors then stressed the point that the recognition of three distinct groups of *Chelydra*— *C. acutirostris*, *C. rossignonii*, and *C. serpentina* (with subspecies *C. s. osceola* and *C. s. serpentina*)—rather than one widespread polytypic species, will have important conservation implications because it will draw attention to the poorly known CSA taxa (species in their view).

The paper by Phillips et al. (1996) is typical of many that have been published recently in that (1) it advocates the use of a lineage-based species concept, in this case the evolutionary species concept (ESC) of Wiley (1978), as the best descriptor of biodiversity and then (2) it fails to specify any a priori operational criteria by which the species concept may be empirically accepted or rejected, relative to the data being presented. In other words, the issue of ontological status of species (i.e., their real nature) is not separated from the issue of their

practical recognition (for a detailed consideration of these issues see Frost & Kluge 1995). It is our view that, like all other propositions in science, species concepts need to be treated as hypotheses testable by rigorous criteria, which should be described in detail in the introduction and methods sections of papers dealing with the practical issue of species recognition. More typically, papers refer to the ESC or some other species concept in general terms, present data and analyses thereof, and then offer an ad hoc interpretation as to whether or not the populations in question are distinct evolutionary lineages. Such noncritical assessments of taxonomic diversity will hinder rather than aid the cause of biodiversity inventory and conservation; hence, our intent is to clarify the points we think are most relevant to the practical issue of determination of species boundaries. We note that this journal has previously published papers stressing the importance of better communication and interaction between systematists and conservation biologists (Rojas 1992, for example); so we offer a constructive critique of the paper by Phillips et al. (1996) in this context and mention other studies where relevant.

Although our paper focuses on species concepts and the diagnosability of species, we do not endorse the view of the species as the central focus for conservation efforts. The present reality, however, is that little has changed since Rojas (1992) pointed out that, although conservation biologists are one of the primary users of species-level taxa, they have participated little in the debate over the meaning of different species concepts and what conservation implications these might have. As a general category, species are used to define areas of endemism and "hotspots" of diversity (identified by comparisons of species lists) and in the selection, design, and management of reserves (Rojas 1992:172–173). At the level of individual taxa, conservation efforts have historically aimed at conserving species as "types"; the names are literally taken from a checklist or field guide, and for the most part species are delimited operationally without regard to any specific concept. Good (1994) refers to these taxa as being operationally defined by an "inertial species concept" in which species limits are set solely by historical precedence. Populations defined in

this way (probably the majority of species even in the best-known groups in the best-studied temperate regions) are considered conspecific or not because biologists are used to thinking of them as such, not because evidence for or against the original proposition has been rigorously examined (Good 1994:194).

We stress two points here. First, it is worth emphasizing again the issue raised by Rojas (1992:173) with regard to treating species as static "types" in conservation programs: this disregards the importance of intraspecific variation. Intraspecific variation and genetic heterozygosity was high-lighted as a major concern of conservation biology very early in the development of the discipline (Frankel & Soulé 1981). Most conservation biologists in principle therefore consider species as evolutionary units and therefore seek to understand how genetic variability (broadly defined) is apportioned within and among populations so that evolutionary potential may be conserved (many examples are reviewed in Avise & Hamrick 1996). This leads to a focus on individual populations as the primary concerns of conservation because evolutionary mechanisms operate at the level of local populations and their conservation maximizes adaptive potential and the possibility for speciation (Soulé 1989; Lesica & Allendorf 1995).

Second, a more effective approach to conservation biology is an ecosystem approach whereby large numbers of endemic species and unique habitats can be protected as a functional ecosystem (for a recent report on this approach see Schmidt 1996). Unfortunately, the constraints of the U.S. Endangered Species Act require protection efforts through the federal government to be focused at the species level. Ideally, an endangered species eventually listed by the federal government will serve as an umbrella species for an entire ecosystem. Because conservation activities at supraspecific (ecosystem) and intraspecific (local population) levels will depend heavily on the concept of species employed, diagnosing species with the ability to rigorously defend such diagnoses in court is essential to protection efforts in the United States.

The Ontological Status of Species

Philosophical definitions of species attempt to find some biological universal that unites a group of entities to form a "species." Two basic forms of these biological universals have been proposed: (1) reproductive communities (e.g., biological species concept, recognition concept) and (2) evolutionary lineages (e.g., phylogenetic species concept, evolutionary species concept) (Templeton 1994). From a philosophical standpoint, operationality need not enter into a discussion of species definition. But if a species diagnosis is based on criteria that cannot be evaluated empirically, the concept is of

little value to the conservation biologist. Therefore, many species concepts have explicit operational criteria for determining species boundaries within a hypothesis testing framework. We submit that these are the concepts of greatest value for conservation biology. Furthermore, researchers need to design studies that collect data relevant to the operational criteria of the species concept being employed.

Operational Considerations of Lineage-Based Species Concepts

The original description of the evolutionary species concept by Wiley (1978:18) reads as follows: "A species is a single lineage of ancestral descendant populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate." This concept neither precludes nor requires morphological differences between species, and reasons for its popular use in descriptions of taxonomic diversity have to do both with correctly identifying the fundamental units of evolution (the independently evolving lineages; see McKitrick & Zink 1988; Frost & Hillis 1990) and in avoiding dilution of the species concept by excessive reliance on polytypic species (those with allopatric and morphologically distinct populations; Collins 1991). Wiley's original formulation of the ESC, however, did not include sufficient operational criteria for actual identification of populations that were on their own "separate evolutionary trajectories."

Subsequently, Cracraft (1983) offered a more detailed lineage-based concept, the phylogenetic species concept (PSC), in which species were defined as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (see also Donoghue 1985; Cracraft 1987; de Queiroz & Donoghue 1988; McKitrick & Zink 1988). The PSC has been widely adopted, amplified, and put into operation (Nixon & Wheeler 1990; Frost & Kluge 1995); explicit rules have been published for its use (Davis & Nixon 1992); and a distinction has recently been made between character-based and tree-based applications of the PSC (Baum & Donoghue 1995; Doyle 1995; Davis 1996).

Application of the PSC has been criticized because of problems that may arise in distinguishing the histories of characters from the histories of species (Avise & Ball 1990). Fixation of diagnostic characters is not an emergent property of newly evolved species, and species and character genealogies may be discordant under many plausible scenarios (Neigel & Avise 1986; Pamilo & Nei 1988; Doyle 1992; Smith 1992; Moore 1995). Thus use of different characters may result in recovery of different diagnosable units, and, because many species may be extensively subdivided over time, fixation of any rapidly

evolving character in an ephemerally isolated deme will produce a diagnosable unit, which may reticulate with other conspecific lineages at some point in the future (Fig. 6 in O'Hara 1993). The heavy reliance on mtDNA haplotypes in conservation genetics studies must be tempered with extreme caution with respect to both of these issues (Moritz 1994). Discordance between a character tree and a species tree, for example, may result from hybridization and subsequent transfer of mtDNA lineages between species that are otherwise distinct in their morphologies and/or nuclear genomes (Degnan 1993). The fixation of diagnosable mtDNA haplotypes in small demes is expected to be common within species with strongly structured populations because the small inbreeding effective population size (N_e) for nonrecombining haploid loci means that they will sort to fixation about four times faster than a single-copy Mendelian locus (Birky et al. 1983, 1989). The use of mtDNA markers alone, therefore, will identify population units that are demographically independent over ecological time (Moritz 1994, 1995; Avise 1995), but diagnosis of such groups as species in the absence of support from independent characters would trivialize the concept of species as evolutionarily independent lineages (Kluge 1990).

Practical Identification of Species Lineages

Operational difficulties exist for the identification of distinct historical entities in nature regardless of the species concept applied (Frost & Kluge 1995), but we wish to summarize briefly some empirical methods recently proposed to test hypotheses of species recognition. The species concept that has dominated much of the literature in the past 40 years has been the biological species concept (BSC). Mayr (1963:19) defined species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups." One reason for the dominance of this concept has been its testability in sexual species. The BSC defined species in terms of population genetic parameters that can be measured in natural populations, thus providing a consistent framework within which one could test hypotheses of species status. The BSC has been criticized for a number of reasons, however, including the fact that it does not apply to asexual species (Templeton 1989), it has difficulty with species that hybridize (Whittemore 1993), and it is nonhistorical in perspective (Donoghue 1985). Furthermore, in many instances the operational criteria of the BSC is that of morphological differences, not the diagnostic criterion of reproductive isolation (Vogler & DeSalle 1994). For example, a reanalysis of an application of the BSC to native plant species of Concord, Massachusetts (Mayr 1992) demonstrated that the criteria used to diagnose species (morphological criteria) were inconsistent with the stated theoretical operational criteria of re-

productive isolation (Whittemore 1993). We have therefore limited our discussion to lineage-based species concepts with operational criteria for species diagnoses that are consistent in theory and practice.

Phylogenetic Species Concept

Davis and Nixon (1992) suggested implementation of what they call a population aggregation analysis (PAA) to delimit species boundaries on the basis of within- and between-population character polymorphisms. This method summarizes profiles of character states in all individuals of a sample and then compares this "population profile" sequentially with profiles for all other samples. A profile of characters summarized in this manner will quickly reveal the absence, fixed presence, or polymorphic presence of character states across all samples. When two samples are distinct on the basis of at least one character state that is fixed in one and absent in the other, they become candidates for separate species. If fixed differences do not exist among samples, they are interpreted as conspecific, and an aggregate profile of multipopulational variation is then produced by subsuming all conspecific samples sequentially under the same heading in the matrix. Additional samples and/or characters can be added to the matrix, and the process proceeds iteratively. The analysis terminates when further aggregation is unwarranted, at which point all samples have either been aggregated into a single species (an indivisible aggregate of polymorphic populations) or apportioned among two or more species characterized by at least one fixed difference. By this method, "the empirical delimitation of phylogenetic species amounts to the partitioning of attribute variation into characters (fixed differences among species) and traits (variation among individuals within species), and the local population is the arena within which the relevant observations can be made" (Davis & Nixon 1992:430).

Davis and Nixon (1992:432–433) recognized several potential limitations to this approach. First, undersampling of attributes will reduce the discriminating power of the method and consistently bias the results toward the recognition of fewer species than actually exist. Second, if sample sizes of individuals are very small within a population, some attributes might appear either fixed or absent from a population, when in fact they are polymorphic. Undersampling of individuals thus biases the analysis toward the recognition of more species than might actually exist. Third, undersampling of populations can have either effect. If a previously unsampled species is added to the analysis and properly diagnosed, it will increase the total number. Alternatively, the addition of populations polymorphic for characters previously diagnosed as "fixed" between samples will cause populations originally considered specifically distinct to be aggregated into one.

Note that this procedure will sometimes require that samples be aggregated as conspecific even though they may differ from each other, as when a character state is constant in one sample or set of samples, absent in a second, and polymorphic in a third. Mink and Sites (1996) criticized the requirement of "fixation" of all diagnostic character states and argued that unique character states might also be diagnostic by virtue of being "trapped" in a species after reproductive closure had been attained, even though they had not reached fixation. Such a pattern can still be accomodated within a PAA if the unique polymorphic trait is hypothesized to be the exclusive transformed state of the original fixed state of an attribute (Davis & Nixon 1992). But defining the state of an attribute as "trapped" in a single population or set of populations is subject to the same character, individual, and population sampling errors considered above with respect to determination of the "fixation" of characters (Davis & Nixon 1992).

Concordance Principles Concept

A more conservative operational methodology, introduced by Avise and Ball (1990), stresses concordant support from unlinked and non-epistatic markers for diagnosis of species boundaries (see also Mallet 1995). The rationale for this approach is that such concordances are likely to arise only when populations have been separated from one another for long periods of time (i.e., they are on separate evolutionary "trajectories"), such that the major phylogenetic subdivisions in the gene genealogies become coincident with the major population-level subdivisions (Avise & Ball 1990). Given the small N_es for haploid genomes mentioned above and the rapidity with which they are expected to attain monophyly in recently diverged lineages (Moore 1995), we suggest that the strongest evidence for evolutionary independence of lineages would be concordance among unlinked Mendelian nuclear gene loci, either with each other or with other classes of single-locus or polygenic characters (morphological, behavioral, physiological, etc.). Although Avise and Ball (1990) emphasized molecular genetic characters in their paper, we do not consider these kinds of characters superior to nonmolecular characters; many valid species may be morphologically distinct in the absence of divergence of any molecular marker (pupfishes of the genus *Cyprinodon*; Echelle & Dowling 1992), whereas other species are cryptic morphologically but are characterized by very large genetic discontinuities (as occurs in many genera of plethodontid salamanders; reviewed by Larson 1989).

Genealogical concordance is a conservative approach because it requires that the phylogenetic histories of independent markers separate samples into the same arrays, and it interprets such a pattern as consistent with long-term isolation of those arrays. As a consequence of this requirement, it will overlook species that may have evolved intrinsic barriers to reproduction so recently that lineage separation and concordance are not yet evident for any markers (molecular or morphological) other than the loci directly controlling the isolating mechanisms themselves. Further, subjective judgments may be required for intermediate levels of congruence and divergence (i.e., is concordance between two characters enough to diagnose a species?). A final limitation of the genealogical concordance approach is that well-supported phylogenies of independent genes (or other characters) may be difficult to collect on a microevolutionary scale, but the distributions of phylogenetically unordered markers (multilocus isozyme markers, for example) will continue to be used as a first-order approximation of long-term population isolation (Avise & Ball 1990).

We note that many of these same issues plague the application of other species concepts (Frost & Kluge 1995), and the use of concordance principles does allow for the recognition of distinct populations within species, such as those that might be diagnosed on the basis of single characters or deep mtDNA splits between groups otherwise connected by nuclear gene flow (Moritz 1994; Avise 1995). This phylogeographic diversity can then be recognized for its own conservation value (Allendorf & Leary 1988; Dizon et al. 1992; Bowen et al. 1994) and is clearly covered under the Endangered Species Act, at least for vertebrates (O'Connell 1992).

Cohesion Species Concept

A final example of an operational, lineage-based species concept is the cohesion concept. A cohesion species is the most inclusive population of individuals with the potential for phenotypic cohesion through intrinsic cohesion mechanisms that are classified into two major categories: (1) genetic exchangeability, factors that define the limits of spread of new genetic variants through gene flow and (2) demographic exchangeability, factors that define the limits of spread of new genetic variants through genetic drift and natural selection (Templeton 1989). Like the approach of Avise and Ball (1990) described above, this approach looks for concordance. Unlike the approach of Avise and Ball (1990), the cohesion concept does not limit concordance analysis to multiple genes and/or geographic distribution but also examines morphological and ecological information within a phylogenetic framework to determine the extent to which there may be genetic and demographic exchangeability.

The cohesion concept can be applied as a set of testable null hypotheses. The first is that all organisms under study represent a single evolutionary lineage (Templeton 1994). Failure to reject this null hypothesis implies that there is no evidence for more than a single

cohesion species. If the null hypothesis is rejected, the data represent two or more diagnosable evolutionary lineages. But, as discussed, this alone does not indicate species status because of problems of discordance between gene trees and species trees (Pamilo & Nei 1988). Thus, being an independent evolutionary lineage is a necessary but not sufficient condition to be considered a species under the cohesion concept (Templeton 1994). To be considered a cohesion species, one or both of two additional null hypotheses must be tested: the evolutionary lineages are (1) genetically exchangeable and (2) demographically exchangeable (Templeton 1994). Lineages are genetically exchangeable if there is evidence of recent gene flow among lineages. Genetic exchangeability not only determines the boundaries of genetic exchange but also determines the extent to which natural selection and genetic drift influence lineages. If the null hypothesis of genetic exchangeability is rejected, then the independent lineages are considered different cohesion species. Demographic exchangeability, on the other hand, refers to ecological or demographic limitations to reproduction. For example, if two distinct lineages demonstrated distinct mating behaviors as well, then the hypothesis of demographic exchangeability would be rejected and the two independent lineages would be considered distinct species. Exchangeability is tested statistically within the phylogenetic framework by the methods of Templeton and Sing (1993). One of the advantages of this approach, besides placing species delineation within a hypothesis testing framework, is that the cohesion approach does not require gene trees to form monophyletic groups of species (Templeton 1994). Therefore, it is robust to the problem of gene trees and species trees discussed above. For worked empirical examples demonstrating this approach to determining species boundaries, see Templeton (1994).

These are but three of several empirical approaches that can be used to delimit real species in nature, and all are characterized by certain strengths and limitations (details in Avise & Ball 1990; Davis & Nixon 1992; Avise 1994:252–257; Baum & Donoghue 1995; Doyle 1995; Frost & Kluge 1995). The important point here is that in each case authors can be very precise about the relevance of the data they collect to address the question and the criteria they will use to accept or reject the hypothesis of species rank. Some aspects of this issue have been debated previously in the pages of this journal, most extensively with regard to the taxonomic status of the red wolf (*Canis rufus*; reviewed by Brownlow 1996), and this case typifies the confusion surrounding excessive reliance on the mtDNA locus alone for species diagnosis. We are convinced that much of the confusion surrounding the discovery of species boundaries in nature would disappear (not to be confused with discussions on the ontological status of species), or at least be clarified by, the formulation of an explicit a priori opera-

tional hypothesis for species diagnosis and the design of a study tailored to test this proposition rigorously.

Alternative Interpretations of the *Chelydra* Data

In documenting the deep mtDNA split between taxa of Central and South America and of the United States, Phillips et al. (1996) pointed out an important phylogeographic discontinuity within *Chelydra serpentina*, but, for the reasons given above, this does not by itself indicate species status for either of the CSA taxa. These authors argued that on the basis of estimated sequence divergences among taxa, and the isolation times into which these translated (based on mtDNA sequence divergence rates of 0.2–0.4% per million years [Avise et al. 1992]), the U.S. and CSA groups would have a divergence time of $11.125–22.25 \times 10^6$ years. But these estimates were based on mtDNA restriction fragment profiles (versus mapped restriction sites, which are expected to provide a more accurate estimate of sequence divergence; Dowling et al. 1996) and assumed neutrality of the mtDNA haplotypes and the operation of an approximate molecular clock. The authors recognized the large error terms on their divergence estimates but did not acknowledge the possible influence of nonneutral mtDNA evolution on their divergence estimates (Ballard & Kreitman 1995; Rand 1996), or the severe limitations of molecular clock calibrations, even if the neutrality assumption is met (Hillis et al. 1996:531–540). We suggest that, given these uncertainties and the previously discussed mtDNA lineage sorting issues, the use of mtDNA or similar markers to define species by estimated divergence times or percent sequence divergence (e.g., Densmore et al. 1992) be avoided.

From the opposite perspective, Phillips et al. (1996) considered the two U.S. taxa conspecific because of a lack of divergence in mtDNA, and this absence of diversity in the North American taxa led them to conclude that the Central and South American taxa are specifically distinct. Given that similarity between the Florida and central U.S. taxa in isozymes could be an artifact of "single-pass" electrophoresis and the demonstrated potential for mtDNA to introgress readily across species boundaries (Barton & Hewitt 1989; Cronin, 1993), these forms may actually be different, with the divergence masked by mtDNA introgression. A more detailed study of the U.S. populations is justified.

An interesting aspect of the data presented by Phillips et al. (1996) but overlooked by them is the apparent fixation of a unique allele at each of two nuclear isozyme loci (M-Icdh and S-Icdh) in the sample of *C. s. acutirostris* from Ecuador (their Table 4). These characters were scored from only two individuals from a single locality, but, if this pattern holds with more extensive sampling, this taxon would merit species recognition by the popu-

lation aggregation approach, the genealogical concordance approach (substituting the cooccurence of fixed but phylogenetically unordered alleles at two loci as an approximation of concordant gene genealogies), and the cohesion approach (two distinct evolutionary lineages with a rejection of the genetic exchangeability null hypothesis). The fixation of distinct alleles at two nuclear gene loci in this taxon almost certainly required more time than did the mtDNA divergence that separates the CSA from the U.S. taxa (for reasons given in Avise and Ball [1990] and Moore [1995]) and can therefore be taken as stronger evidence for a distinct evolutionary lineage (e.g., Daugherty et al. 1990). Contrary to the authors' virtual dismissal of the isozyme data set, we suggest that the deep mtDNA split between U.S. and CSA taxa serve as a stimulus for more-detailed sampling of both populations and isozyme characters. Failure of enzyme loci to reveal more-distinctive differences between these two groups of *Chelydra* could result from (1) insufficient sampling of loci and/or (2) screening of markers on single electrophoresis buffer ("single-pass" electrophoresis). Both of these limitations are easily corrected.

Recommendations

If animals are to be sacrificed for any reason, then investigators must take the time to remove several types of tissues. In vertebrates, for example, protocols have been developed for screening a potentially large number of enzyme loci (Murphy et al. 1996); and tissues such as blood, heart, liver, skeletal muscle, kidney, and stomach/duodenum should be assayed for loci with restricted expression (Murphy & Crabtree 1985). Second, the well-documented phenomenon of "hidden heterogeneity" (Coyne 1982; Barbadilla et al. 1996) reveals that single-pass gel electrophoresis will frequently underestimate electromorphic variability, both within and between populations, and that loci should be routinely screened sequentially with multiple buffers to maximize the number of alternative electromorphs identified (Aquadro & Avise 1982; Hedges & Burnell 1990; Highton & Hedges 1995).

When analyzing data in terms of phylogenetically based species concepts, one should incorporate data most useful for phylogenetic estimation. Phillips et al. (1996) used restriction fragment data to estimate phylogenetic relationships instead of converting the fragments into restriction sites. A major concern with fragment data in phylogenetic analyses is that these data, unlike site data, are not independent: for example, the gain of a restriction site between two existing sites can result in two small fragments that are homologous to one large fragment, leaving two samples that share two of three restriction sites but no restriction fragments (Swofford et al. 1996). Hillis (1994) details two additional sources of error associated with the violation of the homology assumption with restriction fragment data. The first is in the convergence of size classes of fragments relative to the resolving power of the gel. The second is that insertion-deletion events can change the size of fragments and obscure homologies. These three sources of error in the assessment of homology argue strongly for the use of restriction site data over restriction fragment data. Also, information about the relative probabilities of alternative tree topologies (Crandall & Templeton 1993), root probabilities (Castelloe & Templeton 1994), and the neutrality or nonneutrality of loci (Rand 1996) can be inferred from analyses with restriction site data but not with restriction fragment data. Thus, restriction site data are much more powerful in generating reliable phylogenetic estimates upon which hypotheses of species status can then be tested.

A discussion of recommendations would be incomplete without mention of sample sizes, both in terms of the number of independent gene regions surveyed and the number of individuals surveyed. Because of the problems with surveys looking at only mtDNA, it is important to include data from other independent loci. The question then becomes how many loci must be surveyed to avoid the gene tree–species tree problems. One study that examined this question in relation to the segregation of ancient polymorphisms among three species suggested that in general more than five loci are needed to resolve the species phylogeny accurately (Wu 1991), and this number may be considerably higher if the internal nodes are relatively short (Moore 1995). Likewise, how many individuals must be sampled to obtain accurate estimates of genetic diversity within and among populations? This question was addressed in part by Crandall and Templeton (1993), who suggested that, for biologically realistic levels of genetic diversity, sample sizes of 50 individuals will provide accurate estimates of both genetic diversity and relative frequencies of haplotypes in a population. We realize that these are large and perhaps unrealistic goals, especially for conservation biology; they nonetheless represent our current theoretical understanding of appropriate sample sizes for such studies.

In addition to a sample of gene regions from individuals, a thorough geographic sampling with information on numbers of populations sampled and geographic locations is imperative for determining species boundaries. Only with such sampling can one diagnose lineages accurately and partition current from historical population structures at work within the species (Templeton et al. 1995). Even large numbers of individuals and genes sampled cannot compensate for poor geographic sampling (Templeton 1993).

We have used the study of Phillips et al. (1996) as only one example among many, and we reiterate the point that authors must be as complete in data collection and

as critical in analysis as current technologies permit in geographic population surveys of taxonomically poorly known groups. Given the enormity of the biodiversity crisis (Soulé 1990; Raven & Wilson 1992; Systematics Agenda 2000 1994), accurately describing species diversity is arguably the most fundamental chore at hand, and published work must be legally and scientifically as credible as we can make it. Nothing less will get the job done.

Acknowledgments

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