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SPECIES-LEVEL PARAPHYLY AND POLYPHYLY: Frequency, Causes, and Consequences, with Insights from Animal Mitochondrial DNA

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■ **Abstract** Many uses of gene trees implicitly assume that nominal species are monophyletic in their alleles at the study locus. However, in well-sampled gene trees, certain alleles in one species may appear more closely related to alleles from different species than to other conspecific alleles. Such deviations from species-level monophyly have a variety of causes and may lead to erroneous evolutionary interpretations if undetected. The present paper describes the causes and consequences of these paraphyletic and polyphyletic patterns. It also provides a detailed literature survey of mitochondrial DNA studies on low-level animal phylogeny and phylogeography, results from which reveal the frequency of nonmonophyly and patterns of interpretation and sampling. This survey detected species-level paraphyly or polyphyly in 23% of 2319 assayed species, demonstrating this phenomenon to be statistically supported, taxonomically widespread, and far more common than generally recognized. Our findings call for increased attention to sampling and the interpretation of paraphyletic and polyphyletic gene trees in studies of closely related taxa by systematists and population geneticists alike and thus for a new tradition of "congeneric phylogeography."

INTRODUCTION

Intraspecific variation is at the core of modern evolutionary biology, its prevalence and importance having been increasingly documented at the phenotypic and genotypic levels over the course of the twentieth century. Whereas many biological disciplines implicitly adopted a more typological approach—studying the physiology or molecular biology of individuals, then extrapolating to entire species and beyond—evolutionary biology has long emphasized the importance of appropriately sampling any trait or process so as to identify, and thus have the opportunity to interpret, important elements of variation.

Interestingly, however, while an early source of molecular data (allozymes) greatly motivated interest in variation, the more recent introduction of DNA sequences initially reduced the emphasis on certain aspects of variation in studies of evolutionary history. This de-emphasis presumably occurred for the same reason that variation is largely ignored in other fields, namely, constraints on money and effort that restricted the number of individuals that could practically be studied. The sampling traditions of two groups of biologists who embraced these new data reflect different responses to these constraints (Barraclough & Nee 2001, Funk 1999). To caricature these two traditions: Systematists began to use DNA sequences to study the phylogenetic relationships among taxa by sampling a single individual per species, whereas population biologists began to evaluate phylogeographic patterns in DNA sequence variation among many individuals within a single species (Avise 2000, Avise et al. 1987).

In such cases of extremely restricted intraspecific or interspecific sampling, the accuracy of various evolutionary inferences depends on the assumption that individual study species are monophyletic with respect to the alleles at the study locus. That is, they assume that all the DNA sequence alleles that might be collected from individuals of a given species are more closely related to each other than to any alleles that exist in any other species. In turn, this assumption requires that nominal study species represent genetically and reproductively independent lineages whose boundaries have been accurately identified by taxonomists and whose reconstructed gene trees are accurate approximations of organismal history, i.e., species trees. However, only by sampling multiple individuals from each of multiple species can both intraspecific and interspecific variation be assessed, allowing the hypothesis of species-level monophyly to be tested.

The alternatives to species-level monophyly are species-level paraphyly or polyphyly (Figure 1*a*) in which gene trees reveal an allele from one species to be more closely related to particular alleles in a different species than any conspecific allele (but see Wheeler & Nixon 1990). In this review, we use the term polyphyly in referring to both paraphyly—in which all the haplotypes of one or more species are phylogenetically nested within the haplotypes of a second, paraphyletic, species—and narrow-sense polyphyly—in which various haplotypes from the polyphyletic species are phylogenetically interspersed with those of other species such that they are not phylogenetically contiguous with each other on the gene tree. We use polyphyly as our more general term rather than nonmonophyly to avoid awkward prose; we use it rather than paraphyly because polyphyly is the older term and we hope that temporarily expanding its meaning to include paraphyly will be less discordant with past systematics literature than the reverse. We commonly use the term polyphyletic species as convenient shorthand in referring to currently recognized species taxa whose alleles exhibit a polyphyletic pattern in the broad sense outlined above. This pattern is significant both because of what it may reveal about the biology of the polyphyletic species and because of the consequences it may have for evolutionary inference if undetected.

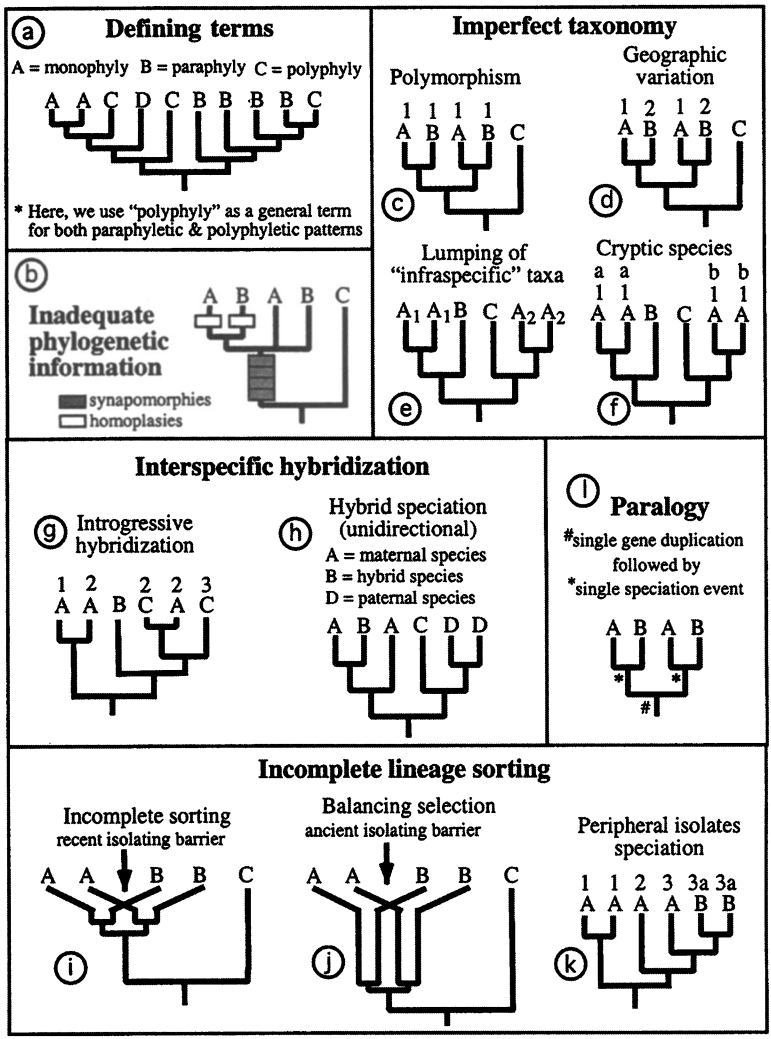

Figure 1 Species-level polyphyly and its causes. This figure illustrates patterns of gene-tree topology that are consistent with various causes of species-level polyphyly. Capital letters represent nominal species; numbers represent geographic regions; the subscripts in (*e*) identify recognized subspecies; the lowercase letters in (*f*) represent subtle phenotypic differences subsequently found to distinguish cryptic species first identified from a gene tree. See text for details.

In this review, we provide the first analysis of the observed frequency and taxonomic distribution of species-level polyphyly. We describe and contrast the various mechanisms that yield polyphyletic patterns and report on the frequency with which particular causes are invoked in the literature. We discuss the implications of polyphyly, describe patterns of sampling from the literature, and recommend sampling strategies for future research. Our survey and discussion emphasize studies of mitochondrial DNA sequence variation in animals, reflecting the authors' expertise, the widespread use of these data, and practical limitations on the scope of this study. Mitochondrial DNA offers a particularly valuable source of markers for the study of closely related taxa and the causes of polyphyly owing to its lack of recombination (but see Maynard Smith & Smith 2002), maternal mode of inheritance (but see below), simple genetic structure, rapid rate of mutation, and reduced N_e (Avise et al. 1987, Harrison 1989, Moore 1995, Moritz et al. 1987). The general principles discussed here, however, apply to the study of gene trees across diverse loci and taxa.

The broadest goals of this review are to provide investigators with a framework for thinking through the unexpected patterns revealed by their gene trees and to encourage sampling practices that maximize the detection of important elements of intra- and interspecific variation. Some workers dismiss all polyphyly as reflecting bad taxonomy. And indeed, imperfect taxonomy and inadequate phylogenetic information are two of the causes we will discuss below. However, we also emphasize introgression and incomplete lineage sorting following recent speciation as major causes of species-level polyphyly that reflect fundamental aspects of organismal biology with important evolutionary implications. This review does not address the observation of polyphyly at higher taxonomic levels or its practical implications for nomenclatural issues.

THE PREVALENCE OF POLYPHYLY

The Literature Survey

To evaluate the importance of species-level polyphyly as an empirical observation, we conducted an intensive survey of studies that evaluate mitochondrial DNA variation in animals in a phylogenetic context. This survey included only those studies with a theoretical possibility of observing polyphyly. Since many of the causes of polyphyly are most likely to affect closely related taxa, we further and arbitrarily limited our data collection to studies of congeners. In order to avoid inflating our estimates of polyphyly, we excluded explicit studies of hybrid zones, where polyphyly would be expected. Included studies were all others that treated at least two congeneric species, at least two individuals from one of these species, and an outgroup. For each species represented by multiple individuals (and thus potentially polyphyletic) we recorded: number of individuals, localities, congeneric species, and congeneric individuals sampled; whether or not polyphyly was observed; and (where presented by the authors, when polyphyly was observed) bootstrap support

and possible explanations for the observed polyphyletic pattern. Polyphyly was evaluated using the species-level taxonomy adopted by our studies' authors and their published mtDNA-only phylogenies. Where multiple trees were published, the phylogeny derived from the most data was used. Where unresolved haplotype relationships were consistent with either monophyly or polyphyly, the species was removed from the analysis. When multiple studies treated the same species, the species was recorded as polyphyletic if polyphyly was detected in any study. The large majority of included studies treated mtDNA sequence data, but appropriate mtDNA restriction analysis studies were also evaluated. Surveyed studies were those published between 1990 and 2002 in 14 leading journals: *Annals of the Entomological Society of America, Biological Journal of the Linnaean Society, Copeia, Evolution, Genetics, Heredity, Journal of Evolutionary Biology, Journal of Mammalogy, Journal of Molecular Evolution, Molecular Biology and Evolution, Molecular Ecology, Molecular Phylogenetics and Evolution, Systematic Biology*, and *The Auk*. Citations for all papers treated in our survey are available via the Supplementary Materials link in the online version of this chapter at http://www.annualreviews.org/.

The Distribution of Polyphyletic Species

Our 13-year survey treated 584 studies, 526 genera, and 2319 potentially polyphyletic species (Table 1). Overall, 535 species proved to be polyphyletic, representing 23% of those surveyed. Forty-four percent (44%) of genera included at least one polyphyletic species, with more than half of these study genera including at least two polyphyletic species. A number of studies showed rampant polyphyly involving many (up to 12) congeners, phylogenetically far-flung haplotypes,

TABLE 1 Results of the literature survey

Taxa	Number of:			Percent spp. polyphyletic[a]
	Studies	Genera	Spp.	
Mammals	139	102	469	17.0
Birds	74	87	331	16.7
Reptiles	56	45	147	22.4
Amphibians	35	26	137	21.3
Fishes	100	99	371	24.3
Arthropods	143	126	702	26.5
Other Invertebrates	37	41	162	38.6
TOTAL	584	526	2319	23.1

[a]Percentage of surveyed species observed to exhibit a paraphyletic or polyphyletic pattern of haplotype relationships.

extreme polyphyly, or multiple species emerging from widespread polyphyletic forms (e.g., Crandall & Fitzpatrick 1996, Demboski & Cook 2001, Funk 1999, Porter et al. 2002, Sota & Vogler 2001, van Oppen et al. 2001). The incidence of polyphyly was also taxonomically widespread, observed for at least 15% of species in each evaluated animal class and phylum (Table 1; this is also true of cnidarians, mollusks, insects, crustaceans, arachnids and echinoderms when these invertebrate taxa are considered individually). Interestingly, there seemed to be a negative correlation between intensity of study and proportion of polyphyletic species across taxa, with birds and mammals exhibiting less than half the incidence of polyphyly observed in nonarthropod invertebrates, a pattern than might partly reflect inadequate taxonomy (see below). In sum, these results clearly indicate that species-level monophyly cannot be assumed and that species-level polyphyly is a much more important phenomenon than is generally recognized. To the degree that any bias exists against publishing untidy results, this survey may yet underestimate polyphyly's actual prevalence. Such a bias might explain an apparent recent decline in the reported incidence of polyphyly (1990–1999 = 28.2%, 2000–2002 = 19.7%) that isn't readily explained by changes in sampling or phylogenetic information content. Alternatively, this pattern could reflect a tendency for early studies on a group to focus on problematic taxa.

CAUSES AND INTERPRETATIONS OF POLYPHYLETIC PATTERNS

An observation of polyphyly should prompt a consideration of its particular causes. When interpreting molecular variation, however, it is often tempting to offer ad hoc explanations for unusual patterns without fully considering alternatives. This tendency is exacerbated when certain explanations have achieved wide recognition only recently or by workers in certain fields or students of certain taxa. In this section we try to alert workers to the full range of phenomena that may produce species-level polyphyly and to explain how they do so (Avise 1994, Funk 1996, Slowinski & Page 1999). In some cases, observed polyphyly is an artifact of misidentified specimens, species limits, and study loci, or of inadequate information. In others, it reflects aspects of allelic history that provide important insights into species biology. Where possible, we recommend means of distinguishing among these alternative explanations. Unfortunately, however, clear one-to-one correspondence between specific causes and particular patterns often does not exist so that definitive conclusions may frequently remain elusive.

Inadequate Phylogenetic Information

One potentially quite general cause of observed polyphyly is weak phylogenetic signal, which may result in poor phylogenetic resolution or inaccurate gene trees as an artifact of phylogenetic reconstruction. Phylogenetic algorithms can create topologies regardless of the amount and quality of the data. Thus, if a gene is evolving too slowly relative to the rate of speciation in one's study taxa or if too small a

fragment of that gene is analyzed, obtained data may provide too few synapomorphies to robustly recover the underlying gene tree, a challenge that becomes greater if positively misleading homoplasies confound the few variable sites (Figure 1*b*). Although rapidly evolving mitochondrial sequences are less prone to inadequate information than most loci, even mtDNA may exhibit insufficient variation for the accurate reconstruction of very recent phylogenetic radiations. On the other hand, sequences from a gene that evolves very rapidly relative to speciation rates might be saturated and produce an inaccurate gene tree owing to high levels of homoplasy. Thus, even if all study species are in fact monophyletic, a reconstructed gene tree may erroneously exhibit polyphyletic groupings that do not accurately represent the history of the analyzed alleles or species.

The studies in our survey adopted various approaches to assess the likely historical accuracy of polyphyletic gene trees. Some studies tested whether a topology constrained to be monophyletic represented a significantly worse fit to the data than the observed polyphyletic topology using, for example, the method of Kishino & Hasegawa (1989). More commonly, Bremer support (Bremer 1988) and especially bootstrap support (Felsenstein 1985) were offered as estimates of the degree to which the data supported haplotype groupings. Here, we use reported bootstrap values to assess the generality of statistical support for observed polyphyletic patterns. Specifically, for each polyphyletic species (A), we recorded the largest bootstrap value that grouped any haplotypes of A with one or more haplotypes from any other species to the phylogenetic exclusion of some other A haplotypes. This provided a conservative estimate of the support for polyphyly because only one of potentially multiple supporting nodes was considered.

We found that 85% of polyphyletic species were from studies that employed bootstrap proportions, providing a large sample for this analysis. Among these studies, the percentages of polyphyletic species supported by various bootstrap proportions were as follows: <50 = 17% of species, 50–69 = 15%, 70–94 = 22%, and ≥ 95 = 46%. Thus, in two-thirds of observed cases polyphyly was supported by $\geq 70\%$ of bootstrap replicates. These results provide compelling evidence that the prevalence of polyphyly documented by our survey reflects a common aspect of true mitochondrial gene trees and is not simply a common artifact caused by inadequate data.

The remaining causes of polyphyly result not from imperfect phylogenetic reconstructions, but despite well-supported gene trees with topologies that likely depict the true origins and relationships among sampled alleles. Such gene trees may nonetheless disagree with recognized species boundaries—and produce polyphyly—for a number of reasons. To simplify and separate our discussions of these reasons, we hereafter assume that the phylogenetic patterns invoked are strongly supported, unless stated otherwise.

Imperfect Taxonomy—Inaccurate Species Limits

One important reason for the observation of polyphyly is a failure of the taxonomic circumscription of a nominal species to correspond to patterns of gene flow.

That is, polyphyly sometimes results from "bad taxonomy" when named species fail to identify the genetic limits of separate evolutionary entities. This failure can occur either by underestimating or by overestimating the field of genetic exchange among individuals and populations. In both situations, polyphyly can be validly eliminated simply by changing current taxonomy. More trivially, polyphyly can result from the misidentification of samples, providing a strong argument for maintaining voucher specimens.

SPECIES OVERSPLIT—MISIDENTIFYING INTRASPECIFIC VARIATION AS SPECIES-LEVEL VARIATION Taxonomy underestimates the breadth of species limits when anatomical (or behavioral, ecological, etc.) variants of a single species have erroneously been described as separate nominal species. This may occur, for example, when distinctive variants coexist within individual populations of a single polymorphic species. Under this scenario, no phylogenetic substructure as a function of variant type is expected (e.g., Demastes et al. 2002, Nice & Shapiro 2001, Small & Gosling 2000) (Figure 1*c*). This is because local gene flow among variants should produce a gene tree in which sympatric haplotypes from each variant are cladistically intermingled with those of the other(s), rendering each polyphyletic. Furthermore, levels of genetic variation among these "oversplit" nominal species are expected to be typical of within-species variation in the taxa under study. Incomplete lineage sorting (see below), however, can produce the same patterns.

Species polyphyly may similarly be observed if two nominal species actually represent geographic variants (races, subspecies) of a single species that continue to exchange genes. In this case, the observed phenotypic divergence may be either environmentally induced or genetically based and maintained by strong selection despite gene flow. If haplotypes of these geographic variants do not phylogenetically segregate into separate clades, conspecificity is supported (Figure 1*d*). Unlike the polymorphism example above, however, some degree of phylogenetic substructuring by variant type might be observed if gene flow is geographically restricted, yielding isolation by distance. In such cases, distinguishing between intraspecific variation and interspecific introgression (see below) as a cause of these patterns may be difficult.

SPECIES OVERLUMPED—MISIDENTIFYING SPECIES-LEVEL VARIATION AS INTRASPECIFIC VARIATION Just as intraspecific variants may be mistaken for species, traits diagnostic of species are sometimes assumed to represent intraspecific variation or are simply difficult to detect at all. This may result in the taxonomic "lumping" of multiple species under a single name and the observation of polyphyly when these species are not sister taxa. In such cases, current taxonomy overestimates the breadth of species limits. This is sometimes observed, for example, with respect to subspecies, geographic forms, morphotypes, and other nominally infraspecific taxa that have been recognized on the basis of divergence in particular traits. When one or more infraspecific taxa within a nominal species prove to be mitochondrially monophyletic, a substantial history of genetic isolation of these taxa from other "conspecific" populations is indicated. In the case where distinct

clades of this kind separate fully sympatric taxa, reproductive isolation between them is further indicated, as is species status under most species concepts. In the case where distinct clades are also geographically separated (Fukatsu et al. 2001, Kotlik & Berrebi 2002, Riddle et al. 2000), evidence on reproductive compatibility is ambiguous, and the decision of whether or not to recognize these genetically differentiated entities as separate species depends on the species concept applied. In either case, the telltale polyphyletic pattern is caused by the nesting of one or more additional nominal species among the haplotypes of the overlumped species (A) (Figure 1*e*). When such nesting renders certain infraspecific taxa of A monophyletic, elevating these taxa to species rank is one strategy for taxonomically removing the species-level polyphyly (Omland et al. 1999, Voelker 1999).

Sometimes, clues to lumping may be scarce owing to the highly similar morphologies of unrecognized species. If other described species are more closely related to such "cryptic species" than the cryptic species are to each other, a mitochondrial gene tree might hint at cryptic taxa by revealing polyphyly in the form of two phylogenetically separated clades (Figure 1*f*) (Omland et al. 2000; Williams et al. 2001; D.J. Funk 1998, unpublished data). Such cryptic species might reflect the retention of ancestral morphology (Jarman & Elliott 2000). However, the same polyphyletic pattern would be expected if cryptic species resulted from the convergent evolution of similar morphologies (Kim et al. 2000, Rees et al. 2001, Richmond & Reeder 2002, Su et al. 1996). This might be expected if divergent lineages were responding to similar selection pressures, as in threespine stickleback fishes that have repeatedly evolved complex benthic- and pelagic-adapted morphologies (Bell 1987, Schluter & Nagel 1995). Such convergence creates special problems when traits under selection are also those used by taxonomists to define species.

In the scenarios just reviewed, polyphyly results when the described phenotypic boundaries of nominal species do not adequately or accurately reflect the history of population differentiation and speciation. That is, polyphyly results even if a species tree can be safely assumed to be identical to the gene tree used to infer it. By contrast, the remaining causes of polyphyly generally reflect situations where the history of alleles revealed by a gene tree is incongruent with the actual organismal history embodied by the species tree (but see Doyle 1997, Maddison 1997). This "gene tree/species tree problem" (Avise et al. 1983; Brower et al. 1996; Doyle 1992; Goodman et al. 1979; Maddison 1996, 1997; Nichols 2001; Pamilo & Nei 1988; Slowinski & Page 1999; Wu 1991) represents a major limitation on evolutionary inferences from single-locus (e.g., mitochondrial) gene trees that has not yet been fully incorporated into certain areas of systematic biology.

Interspecific Hybridization

One potential cause of gene tree/species tree discordance and accompanying polyphyly is the occasional mating between otherwise distinct species and resulting transfer of parental alleles to hybrid offspring. Two aspects are worth noting.

INTROGRESSION Alleles from one species may penetrate the gene pool of another through interspecific mating and the subsequent backcrossing of hybrids into parental populations, a process known as introgressive hybridization, introgression, or interspecific gene flow. Introgression yields polyphyly by introducing phylogenetically divergent allelic lineages across species boundaries (e.g., Boyce et al. 1994; Patton & Smith 1994; Shaw 1999, 2002). The phylogenetic effects of mitochondrial introgression are particularly great because a lack of recombination entails that all mitochondrial base positions introgress as a completely linked block (Smith 1992). Thus, any analyzed fragment of introgressed mtDNA will entirely reflect the heterospecific origin of its mitochondrial genome. Furthermore, mitochondrial alleles might be expected to introgress farther, on average, than nuclear loci if their persistence in a foreign gene pool is less constrained by linkage to selected loci than are the alleles of nuclear genes (Barton & Jones 1983, Harrison et al. 1987, Marchant 1988, Tegelström 1987; reviewed in Harrison 1990, Arnold 1993). For these reasons, mitochondrial gene trees could be particularly susceptible to the effects of introgression. An interesting exception is offered by female heterogametic taxa following Haldane's rule, such as birds (Tegelström & Gelter 1990) and butterflies (Sperling 1993). In such cases, female hybrids show reduced viability that might restrict the introgression of maternally inherited mtDNA between species, offering a potential explanation for low mtDNA introgression in several avian hybrid zones (e.g., Allen 2002, Brumfield et al. 2001, Sattler & Braun 2000). More generally, the exposure of haploid mtDNA loci to selection in all individuals may also impede its introgression (Brumfield et al. 2001). The differential introgression of mitochondrial versus nuclear alleles and its effects on polyphyly is an important topic that deserves further attention.

Recognizing mitochondrial introgression requires evaluating a mitochondrial gene tree against a nuclear background that identifies the participating taxa. This background can be provided by gene trees from nuclear loci or simply by consistent taxon-specific phenotypic differences that presumably have a nuclear basis (Smith 1992). The clearest signature of introgression is the sympatric sharing of geographically localized mtDNA sequence haplotypes between otherwise genetically and morphologically divergent species (Figure 1*g*). Such a pattern is hard to interpret as anything but ongoing (or very recent) and geographically localized interspecific gene flow. Importantly, introgression may not be detected in such situations unless populations are indeed sympatrically sampled because species that share haplotypes in regions of geographic overlap may otherwise exhibit reciprocal monophyly in gene trees based on allopatric samples (e.g., Masta et al. 2002, Redenbach & Taylor 2002).

Unfortunately, confidently attributing polyphyly to introgression becomes progressively more difficult the farther in the past that gene flow last occurred. Species that have rather recently ceased exchanging genes may no longer share haplotypes (because of post-introgression mutation) yet still possess very closely related haplotypes that are nested together within the gene tree. However, as the time since last gene flow increases, those introgressed allelic lineages that do persist are more likely to be phylogenetically basal (as a result of the sorting out of allelic

polymorphisms dating to the time of introgression) and less likely to show any geographic association with the population from which they introgressed (to the degree that populations change distributions over time).

Other factors can further complicate the recognition of introgression as a cause of polyphyly. If mitochondrial gene flow is bidirectional, very common, or occurs among multiple species, its affect on mitochondrial tree topology may be profound, making it difficult or impossible to confidently infer patterns of genetic exchange or even to determine which mitochondrial clade represents the "native" lineage of particular species. Mitochondrial gene trees may be especially misleading in cases where introgressed haplotype lineages become fixed, leaving no hint that they are of heterospecific origin. The smaller N_e of mtDNA compared with nuclear loci may facilitate this process, such that even low levels of introgression may be sufficient to establish a neutral mitochondrial genotype in a foreign population (Takahata & Slatkin 1984). Patton & Smith (1994), for example, attributed complicated polyphyletic patterns in pocket gophers to sporadic episodes of hybridization combined with small, patchy gopher populations that facilitated the fixation of introgressed alleles. Recurrent hybridization has been similarly invoked to explain rampant polyphyly in a variety of taxa (Freeland & Boag 1999, Funk 1999, Shaw 2002, Sota & Vogler 2001).

HYBRID SPECIATION Polyphyly may also result from the spontaneous formation of a new species through interspecific hybridization, a mechanism that has been demonstrated in various animal taxa (e.g., Moritz et al. 1992; reviewed by Dowling & Secor 1997). In such instances, the initial relationship among parental and a new hybrid species' mitochondrial alleles will depend on the number and symmetry of hybrid speciation events. Most hybrid species appear to originate via asymmetrical hybridization. A hybrid species formed by a single such event will itself be mitochondrially monophyletic, while specifically rendering the mitochondria-contributing maternal species paraphyletic (Figure 1*h*). A hybrid species formed through repeated asymmetric hybridizations (all involving, e.g., a female of species A and a male of species B) will be monophyletic if the participating females have identical mitochondrial haplotypes, polyphyletic otherwise (e.g., Mantovani et al. 2001). A hybrid species formed through symmetric hybridization events would be polyphyletic, as would both parental species. Because hybrid speciation is often associated with polyploidy or asexual reproduction, knowledge of such traits may bolster a suspicion that hybrid speciation is the cause of observed polyphyly (e.g., Johnson & Bragg 1999). However, in several cases of putative hybrid speciation (Hedrick et al. 2002, Wayne & Jenks, 1991; also see Salzburger et al. 2002) alternative explanations have proven difficult to rule out.

Incomplete Lineage Sorting

The incomplete sorting of ancestrally polymorphic allelic lineages represents a very general source of polyphyly, potentially afflicting any single-locus gene tree in any taxon. Within any species, the various alleles at a particular locus have

their own history, with some alleles sharing more recent, and others more ancient, coalescent events (Pamilo & Nei 1988). Thus, the random division of allele copies at speciation will generally result in each daughter species possessing certain alleles that are most closely related to those in the other daughter species. For this reason, new species are initially expected to exhibit polyphyletic gene trees (Figure 1*i*). Over time, allelic lineages in each daughter species will be randomly lost by drift, and new alleles will be formed by mutation until eventually only one of the (ancestrally polymorphic) allelic lineages present in the parent species survives in each daughter species and all intraspecific variation reflects post-speciation mutation. At this point, sorting has gone to completion, and alleles in the two daughter species are reciprocally monophyletic. This progression from polyphyly (narrow-sense) to paraphyly to monophyly is expected to take on the order of $4N_e$ generations for mitochondrial loci and ultimately results in a gene tree that accurately reflects the species tree (Avise 1989, Avise & Ball 1990, Harrison 1991, Neigel & Avise 1986, Pamilo & Nei 1988, Tajima 1983, Takahata & Nei 1985).

Because the mitochondrial genome is haploid and maternally inherited, the N_e of mitochondrial loci is generally one-quarter that of nuclear loci (but see Hoelzer 1997), and stochastic lineage sorting is expected to progress more rapidly for mitochondrial alleles. Thus, incomplete sorting is less of a concern for mitochondrial than for nuclear loci, other things being equal, providing one advantage to using mitochondrial gene trees as estimates of species trees for closely related taxa (Hudson & Turelli 2003). Indeed, theory predicts that if one can be 95% certain that an internode in a single mitochondrial gene tree has not been affected by incomplete sorting, 16 independent nuclear gene trees would be required to justify an equal level of confidence (Moore 1995). Nonetheless, incomplete sorting also affects mitochondrial gene trees and can have especially major effects in the case of rapidly radiating taxa, in which succeeding speciation events occur before sorting is completed. This scenario has been invoked to explain the sharing of alleles among multiple species in the rampant polyphyly exhibited by cichlid fishes and other taxa (Moran & Kornfield 1993, 1995; also see Crandall & Fitzpatrick 1996, Goodacre & Wade 2001, Klein & Payne 1998).

Unfortunately, it is impossible to demonstrate conclusively that incomplete sorting explains any particular case of polyphyly. One problem is that because species remain incompletely sorted for a narrow window of evolutionary time, a dearth of accumulated synapomorphies may often make it difficult to distinguish incomplete sorting from inadequate phylogenetic information as a cause of observed polyphyly (Slowinski & Page 1999). Another problem is the difficulty of distinguishing the effects of incomplete sorting and introgression, an issue of considerable interest. A phylogenetically basal position of polyphyly rendering haplotypes hints at retained ancestral polymorphism, while recently introgressed alleles may assume a highly derived position in the gene tree. Also, incomplete sorting is not predicted to promote the geographic proximity of interspecifically shared alleles that may be seen under local introgression (Hare & Avise 1998, Masta et al. 2002). However, these criteria are often inadequate to distinguish ancient mitochondrial introgression

from incomplete sorting (Schneider-Broussard et al. 1998). Comparisons with nuclear markers and geography can provide additional insights (e.g., Redenbach & Taylor 2002, Tegelström 1987, Weckstein et al. 2001), as may nested clade analysis (Templeton 1998, Templeton et al. 1995; but see Knowles & Maddison 2002). Moore (1995) suggested comparing maximum intraspecific sequence divergences between a polyphyletic species and related species in an empirical method for evaluating the likelihood of incomplete sorting (see Baker et al. 2003, Holder et al. 2001, Knowles 2000, Mason et al. 1995, Palumbi et al. 2001, Rees et al. 2001). Other, more statistical, methods have also been described (e.g., Nielsen & Wakeley 2001, Sang & Zhong 2000, Wakeley 1996). However, a generally diagnostic and widely agreed-upon approach for documenting incomplete sorting and distinguishing it from introgression has not yet emerged (Holder et al. 2001).

SPECIATION AND SORTING Although the progression of new species from initial polyphyly through paraphyly to monophyly follows quite generally on the heels of speciation, the particular pattern and time course of this progression may be rather distinctive in the case of peripatric, peripheral isolates, or "budding" speciation (Frey 1993; Harrison 1991, 1998; Rieseberg & Brouillet 1994), in which populations along the periphery of a species range become spatially isolated and speciate. To the degree that a "parental" species exhibits geographic substructure and a peripherally speciating population is small and local, this population may be predicted to initially possess a phylogenetically restricted subset of parental alleles and may lose alleles under drift at a faster rate than the larger parental population. For these reasons, peripheral isolates speciation may commonly yield a geographically restricted daughter species whose monophyletic set of haplotypes is embedded within a widely distributed and still paraphyletic parental species (termed a ferespecies by Graybeal 1995; also see Baum & Shaw 1995, Olmstead 1995) (e.g., Avise et al. 1990, Funk et al. 1995a, Hedin 1997, Marko 1998; but see Knowles et al. 1999) (Figure 1*k*). This deep phylogenetic nesting is not expected under large-scale vicariant or parapatric modes of speciation, although it might also be observed (in a different phylogeographic context) in the case of rapid, local sympatric speciation (Harrison 1998). This asymmetrically paraphyletic relationship will persist until sorting renders the parental species monophyletic.

In the case of budding speciation, forcing taxonomy to reflect gene tree monophyly by synonymizing the nested and parent species or by elevating lineages in the paraphyletic lineage to species status ignores the distinctive nature of the nested lineage (de Queiroz & Donoghue 1988; Harrison 1991, 1998; Olmstead 1995; Rieseberg & Brouillet 1994; Rodríguez-Robles & De Jesús-Escobar 2000; Sosef 1997; Wiens & Penkrot 2002). Under budding speciation, the cause of paraphyly is incomplete lineage sorting, yet the gene tree accurately reflects the history of population divergence. Thus, although gene trees from different loci are ordinarily expected, by chance, to be incongruent under incomplete sorting, budding speciation is predicted to produce parallel patterns of paraphyly across nuclear and mitochondrial loci (Hedin 1997, Marko 1998, Petren et al. 1999; but

see Ballard 2000, Tosi et al. 2000). Because it reflects population history, this nested pattern is evolutionarily informative, allowing the polarization of the speciation event and of transitions between traits (host plant associations, plumage patterns, geographic ranges, etc.) that accompany and may have promoted speciation (e.g., Brown et al. 1994, 1996; Funk et al. 1995b; Omland 1997).

SELECTION AND SORTING The expected time to complete sorting invoked above, $4N_e$ generations, applies to strictly neutral mitochondrial alleles. However, mtDNA variation may often be subject to selection (Ballard & Kreitman 1995, Hudson & Turelli 2003, Rand 2001), which will affect the rate at which reciprocal monophyly is attained. While positive selection will accelerate allele fixation and sorting, balancing selection may preserve ancestrally polymorphic alleles within a population indefinitely (Figure 1*j*). Polymorphic nuclear MHC alleles, for example, are shared between otherwise genetically divergent species in several animal taxa (Klein et al. 1993, 1998). However, although there is some evidence for balancing selection on mtDNA in animals (James & Ballard 2000) and plants (Städler & Delph 2002), it has not yet been documented as a cause of species-level mitochondrial polyphyly.

Unrecognized Paralogy

Orthologous alleles derive from the same locus whereas paralogous alleles derive from different loci that originated by a gene duplication event. A gene tree that includes paralogous alleles may depict polyphyletic species because its topology reflects gene duplication as well as speciation (Figure 1*l*). The cause of this polyphyly may be misinterpreted if the orthology of alleles is assumed. Because mitochondrial loci are single-copy genes rather than members of multigene families, it was long considered safe to assume the orthology of alleles sequenced with mitochondrial primers. Two phenomena illustrate exceptions to this rule that cause polyphyly.

NUCLEAR PSEUDOGENES It is now well understood that segments of mitochondrial DNA are sometimes transposed into the nucleus where they become functionless pseudogenes (Bensasson et al. 2001, Collura & Stewart 1995, Sorenson & Fleischer 1996, Sunnucks & Hales 1996, Zhang & Hewitt 1996). When such nuclear copies of mtDNA exist, using mitochondrial primers for PCR amplification from whole-genomic DNA extractions (a common approach) may yield sequences of nuclear as well as mitochondrial origin. Indirect evidence for nuclear copies may be provided by unusual patterns of molecular evolution that are consistent with the reduced functional constraint (e.g., elevated frequencies of nonsynonymous substitutions, indels, frameshifts, and stop codons) or nuclear location (slowed rates of substitution) of pseudogenes. Nuclear copies may be more directly detected through the isolation of mtDNA, cloning, and rtPCR (Collura et al. 1996). Nuclear copies of mtDNA and their effects on polyphyly have now been documented in a variety of taxa.

PATERNAL INHERITANCE In a few cases, paternally inherited mitochondrial lineages have been shown to originate from maternally inherited ancestors, much as new loci are formed by a gene duplication event. These divergent maternal and paternal lineages can coexist within species, yielding species-level polyphyly in gene trees that include alleles from both. To date, such instances have generally been taxonomically restricted to various bivalve mollusks (e.g., Rawson & Hilbish 1995), so this phenomenon is not known to present a general cause of polyphyly. Recent results from humans (Bromham et al. 2003), however, illustrate that other taxa may also be affected.

Literature Patterns

Attempting to elucidate the actual causes of polyphyly in the studies from our survey is beyond the scope of the present review. However, some observations on authors' tendencies in reporting potential causes are worth noting. First, 24% of papers with polyphyletic gene trees offered no discussion of this pattern. Second, of those that evaluated polyphyly, 50% specifically suggested faulty taxonomy as one plausible explanation, introgressive hybridization was invoked in 32% of papers, and incomplete lineage sorting was cited in 30%. Inadequate phylogenetic information and unrecognized paralogy received mention in only a few papers each. Third, closer inspection of a subset (~one half) of the polyphyletic papers found that in 56% of these only one or another of three major causes (taxonomy, introgression, sorting) received any mention at all; two causes were mentioned in 25%, and all three in only 16% of the studies.

Although it is encouraging that most authors find polyphyly a worthy subject of comment, these patterns suggest that a fully pluralistic appreciation of its causes has yet to take root. The general disregard of nuclear copies of mtDNA as a possible explanation is especially concerning (but see Weckstein et al. 2001). The equivalent invocation of introgression and incomplete sorting and the considerably greater frequency of taxonomic explanations may reveal the biases of biologists or illuminate the relative importance of different causes. We recommend that future studies seek the most accurate and informative interpretations by systematically considering the full range of alternative explanations in accumulating datasets.

CONSEQUENCES FOR EVOLUTIONARY INFERENCE

Erroneous Estimates

If undetected, species-level polyphyly compromises evolutionary inferences based on gene trees that are erroneously assumed to accurately depict species trees (Funk 1996, 1999) (Figure 2). The extent of these problems will depend on several aspects of polyphyly, among them: (*a*) how commonly polyphyletic species occur in the study taxon, (*b*) how "polyphyletic" a given species is, i.e., how many

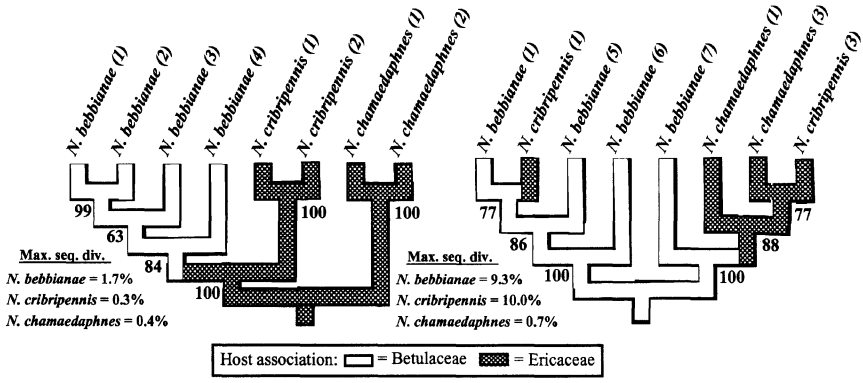

Figure 2 Erroneous evolutionary interpretations due to polyphyly. Each tree depicts the reconstructed relationships among mitochondrial haplotypes collected from different subsets of individuals representing three *Neochlamisus* leaf beetle species (data from Funk 1999). Haplotypes from different individual beetles are indicated by different numbers. Results illustrate how drastically estimates of phylogenetic relationship, character evolution, and genetic divergence can vary as a function of the particular individuals sampled when study species are highly polyphyletic. Bootstrap values indicate strong support of each data set for a different topology.

distinct allelic lineages are represented, (*c*) how genetically and phylogenetically diverse these allelic lineages are, and (*d*) how evenly alleles are distributed among these lineages. The likelihood that evolutionary inferences will vary dramatically according to the individuals sampled will be greatest when polyphyletic species bearing many, diverse, and equally frequent allelic lineages are common. In this context, the consequences of polyphyly may be more or less severe, on average, according to the particular cause. Incomplete lineage sorting, for example, is less likely to involve highly divergent allelic lineages than is an ancient duplication event or introgression between distantly related taxa. Some important inferential problems resulting from polyphyly are described below.

First and most basically, the phylogenetic relationships depicted by a gene tree may vary according to the particular individuals sampled when sequences from one or a few specimens are used as exemplars of polyphyletic species (Ballard 2000, Barraclough & Nee 2001, Funk 1999, Melnick et al. 1993, Omland et al. 1999, Smouse et al. 1991, Zink et al. 1998) (Figure 2). Systematists generally agree that multiple exemplars should be included at the level below the taxonomic rank of interest, and this should also apply to the species level (Ballard 2000, Barraclough & Nee 2001, Graybeal 1995, Omland et al. 1999, Wiens 1999; see also Lanyon 1994). Intensive sampling is more likely to document the underlying polyphyly and alert the systematist that something is amiss. Second, inferred times and rates of evolutionary divergence may be considerably inflated or deflated (depending on the cause of polyphyly) when alleles are sampled from polyphyletic

species (Ballard 2000, Funk 1999, Melnick et al. 1993; also see Edwards & Beerli 2000) (Figure 2). Third, when gene trees are used to reconstruct the evolutionary history of particular traits, polyphyly presents especially egregious problems because a single misplaced taxon can have major effects on character transformations throughout the tree (Omland 1997; also see Graybeal 1995) (Figure 2). Fourth, polyphyly may compromise population genetic and phylogeographic studies. This may occur, for example, when sampling of related species is insufficient to identify the heterospecific (e.g., introgressive) origin of divergent haplotypes in the focal species, leading to mistaken conclusions about demography and evolutionary processes that are based on allelic frequencies and relationships (Redenbach & Taylor 2002, Tegelström 1987; also see Ballard 2000). Fifth, mitochondrial polyphyly complicates the identification of species-diagnostic molecular characters for practical issues of management and conservation, such as identifying endangered species (Baker et al. 1996, Dalebout et al. 1998) and defining evolutionarily significant units (e.g., Moritz 1994, Paetkau 1999).

Intriguing Insights

Although species-level polyphyly can be quite problematic if undetected, when recognized it can provide informative clues that motivate future work (Funk 1996, 1998; Harrison 1998; Omland 1997). For example, appropriately sampled mitochondrial surveys can be an efficient means of detecting initial evidence for gene flow and insights on its direction, geographical and biological correlates, and participating taxa. Such observations can direct workers to informative investigations of hybrid zones, mechanisms of reproductive isolation, or the reexamination of species limits. The revised taxonomic and phylogenetic assessments of species-level taxa that are provoked by polyphyly may provide more stable classifications and more accurate historical inferences, for example, on character evolution (Omland 1997, Omland et al. 2000). The discovery of morphologically cryptic or unusually variable species may prompt studies on the evolutionary causes of convergence/stasis or polymorphism, respectively. Findings consistent with incomplete sorting may lead to studies of demography and speciation rates. Patterns consistent with budding speciation may identify taxa that contribute to ongoing debates on speciation mechanisms (Harrison 1991, Turelli et al. 2001). Even nuclear copies of mtDNA are now being exploited for novel evolutionary insights (Bensasson et al. 2001).

THE IMPORTANCE OF SAMPLING: PATTERNS AND PROSPECTS

Detecting polyphyly requires the sampling of multiple individuals of the polyphyletic species as well as other species with which it shares related alleles. As noted earlier, however, the phylogenetic and phylogeographic traditions initially

adopted sampling strategies that sometimes precluded polyphyly detection. These strategies reflect the tendencies of these traditions to deemphasize intraspecific and interspecific sampling, respectively, in their studies of closely related taxa. These different sampling strategies in turn reflect the differing interests of these scientific disciplines—phylogenetic structure versus microevolutionary process—as well as practical constraints on data collection.

Yet sampling is clearly important (Ballard 2000, Funk 1999, Hedin & Wood 2002, Omland et al. 1999). The more individuals sampled, the greater the likelihood of detecting polyphyly and when the interspecifically shared alleles that cause polyphyly are rare, very intensive sampling may be required to document this pattern (Wiens & Servedio 2000). For a given sampling intensity, polyphyly detection should generally be increased by dividing samples across the phenotypic, geographic, and phylogenetic diversity of individuals and species that might plausibly share allelic lineages. An ideal study would thus include all species believed a priori to be closely related (e.g., congeners), maximize the geographic diversity of samples and the number of samples collected from areas of sympatry between study species, and sample broadly from known sources of biological variation (subspecies, ecotypes, morphological variants, etc.).

Our literature survey provided empirical data on the distribution of sampling patterns between 1990 and 2002, beginning with some of the earliest molecular systematic studies using DNA sequence data. Because we included only those studies theoretically capable of detecting polyphyly (those including multiply sampled species), our estimates of sampling intensity may be somewhat upwardly biased. This survey shows a regular increase and possible plateau in the frequency of such studies (yearly frequencies from 1990 to 2002 = 3, 6, 7, 12, 22, 28, 39, 36, 53, 76, 96, 116, and 89, respectively). Two patterns are worth emphasizing here (Figure 3). First, although studies vary greatly in sampling intensity, the majority included no more than a few individuals and sampling localities per study species. Second, median levels of four sampling parameters (total number of congeneric species and individuals sampled, mean number of individuals and localities sampled per study species) that might be expected to correlate positively with polyphyly detection have not notably increased over the 13 years of this survey (Figure 3). While it is certainly true that investigations treating multiple well-sampled species are becoming more common, our survey suggests that many studies continue to adhere to an implicit early established standard of acceptable sampling.

The collection of mtDNA sequences is no longer nearly as onerous as during the early years of our survey. However, the high-volume automatic sequencing and declining costs that have allowed the genomics revolution have not yet translated into generally and considerably improved sampling in mitochondrial studies of closely related animal species. The contribution of improved sampling to the detection of polyphyly is indicated by our survey, which shows that species observed to be polyphyletic were represented, on average, by significantly more conspecific

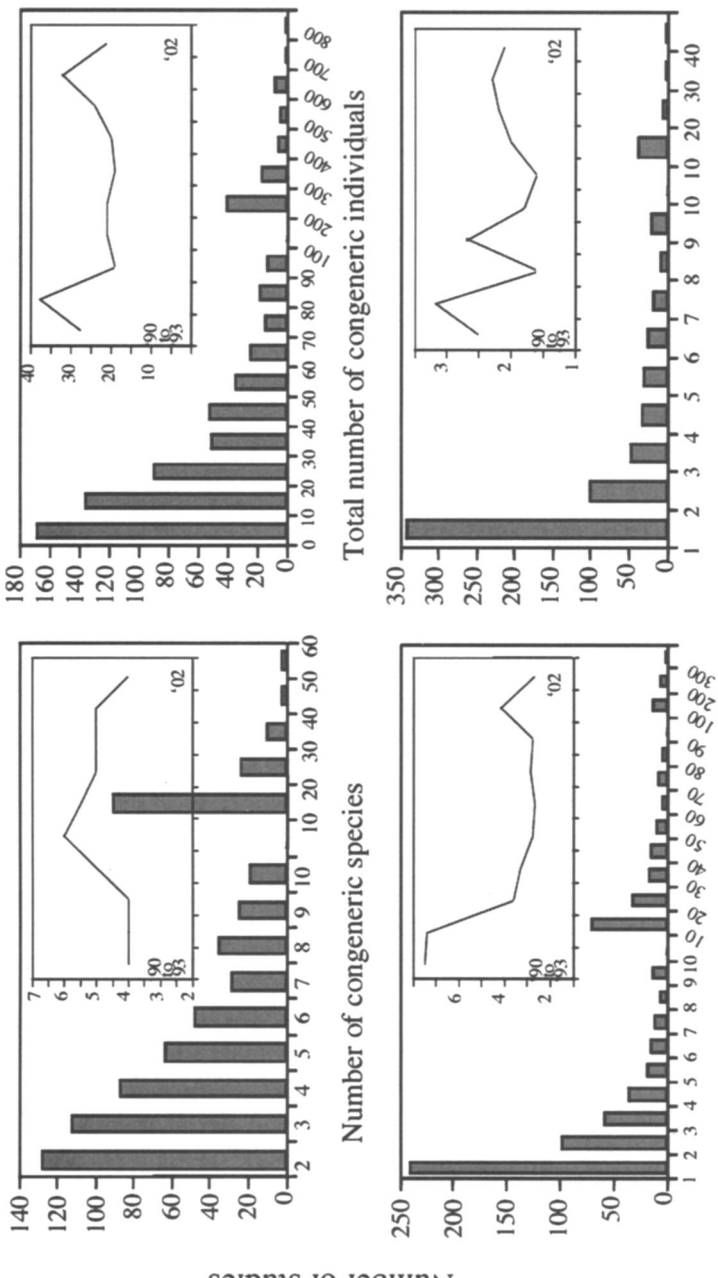

Figure 3 Sampling patterns from the literature survey. Each of four plots presents two kinds of information for a different aspect of sampling intensity. First, each plot illustrates the distribution of sampling intensity across all studies in the survey. Second, each illustrates temporal variation in the median level of sampling intensity across the 13 years of the survey (inset). Median values for the 1990–1993 period were based on pooled data, and each following year was plotted separately. Higher median values in the early 1990s partly reflect the higher proportion of (less resource-intensive) restriction analysis studies conducted at this time.

individuals (22.6 versus 10.7), collection localities (6.1 versus 3.8), and congeneric individuals (49.1 versus 26.9) than other species (one-way ANOVA, $P < 0.001$ in all cases).

We recommend that improved and diversified sampling be embraced as a timely and important goal that might allow a highly productive merger of the phylogenetic and phylogeographic traditions. Specifically, we encourage workers to collect and simultaneously analyze phylogeographic data from multiple closely related species and their infraspecific variants (also see Barraclough & Nee 2001, Hey 1994). These investigations might be facilitated by the increased exploitation of museum material and sequence data from public databases and by increased investment in the field work necessary to obtain diverse material. Investigations in this new tradition of "congeneric phylogeography" will improve the likelihood of detecting and appropriately interpreting critical patterns of intraspecific and interspecific allelic variation to the benefit of systematic and population biology alike.

CONCLUSIONS AND FUTURE DIRECTIONS

This review describes the diverse causes and consequences of species-level polyphyly and represents the first large-scale empirical survey of polyphyletic species and sampling patterns in mitochondrial studies of closely related animal taxa. We demonstrate polyphyly to be a common, statistically supported, and taxonomically general aspect of mitochondrial gene trees. We find that sampling intensity has not increased concurrently with the increasing ease of collecting mitochondrial sequence data. We call for the combination of phylogenetic and phylogeographic approaches to sampling in a new tradition of congeneric phylogeography. Similar surveys of nuclear loci might be informative, although a greater diversity of loci and many fewer studies may as yet make numerical comparisons difficult. Surveys of, and comparisons with, the botanical literature might be particularly informative as polyphyly is suspected to be more common in plants and has been embraced as a fundamental aspect of phylogenetic variation by the botanical community (Crisp & Chandler 1996, Rieseberg & Brouillet 1994). Increased attention to sampling and the interpretation of polyphyly across genes and taxa will provide improved insights in systematics, population genetics, and evolutionary biology in general.

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