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Critical Comment: What We Don’t Recognize Can Hurt Us: A Plea for Awareness About Cryptic Species

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We Don’t Recognize Can Hurt Us: A Plea for Awareness About Cryptic Species

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ABSTRACT: We conducted an extensive literature review on studies that have used DNA sequences to detect cryptic species of parasites during the last decade. Each literature citation that included the term “cryptic” or “sibling” species was analyzed to determine the approach used by the author(s). Reports were carefully filtered to retain only those that recognized the existence of cryptic species centered on the use of DNA sequences. Based on analysis of these papers, we comment on the different ways that parasite cryptic species are discovered in studies focusing on different aspects of the host–parasite relationship, or disciplines, within parasitology. We found a lack of methodological and theoretical uniformity in the discipline for defining and delimiting cryptic species, and we draw attention to the need for standardizing these approaches. We suggest that cryptic species, in the strict sense, are always provisionally cryptic, in that the possibility does exist that new morphological studies or techniques will reveal previously unknown diagnostic structural differences which will permit rapid and practical morphological diagnosis. To avoid future taxonomic confusion, we recommend that parasitologists describe (and formally name) cryptic species following standard taxonomic practice.

Parasitologists discover and describe new species of parasites with regularity, and DNA-based taxonomic methods are increasingly used to complement these observations. Undoubtedly, the routine discovery of new parasite species reinforces the belief that parasitism is one of the most successful and common modes of life on earth, a belief which is also illuminated by our increased understanding of parasite ecology, evolution, and biogeography (Brooks and McLennan, 1993). The discovery of cryptic species in nature (morphologically indistinguishable, but genetically distinct species) has attracted the attention of systematists, ecologists, and evolutionists. Implicit in their discovery are potential methods for detecting and delimiting cryptic species, and emphasis herein is given to the difference between cryptic species prospecting (methods to detect putative cryptic species) and delimitation (testing that we have cryptic species).

Cryptic species have significant implications for evolutionary theory, biogeography, and conservation planning (e.g., Beheregaray and Caccone, 2007; Bickford et al., 2007; Plejnenner and Schwenk, 2007; Trontelj and Fieser, 2009). The term “cryptic species” does not have a uniform meaning as applied by different investigators either within, or outside of, parasitology. In the strictest sense, species are cryptic when no morphological differences (qualitative structural, meristic, or morphometric) are known with which to diagnose them. Such species are most often recognized based on analysis of genetic evidence that falsifies the hypothesis of a single species. However, many investigators use a much less strict concept with respect to morphological uniformity. For example, some will apply the term “cryptic species” to taxa that can be diagnosed based on morphology, but not easily so, such that the species have a high degree of morphological similarity; this may lead to misidentification. This view reflects aspects of a definition provided by Bickford et al. (2007), “…two or more distinct species that are erroneously classified (and hidden) under one species name,” in that such cryptic species are often discovered within what was formerly recognized as a single species (typically discovered using genetic data), but then a posteriori analysis of the delimited species may reveal diagnostic morphological differences that were previously unrecognized. In the strictest sense, species that once were not recognized as distinct, based on morphological evidence, may become so. However, application of the term “cryptic species” is often continued based on either taxonomic history or, more justifiably, because the species cannot be readily or practically diagnosed based on morphology due to their similarity and nature of the diagnostic characters. This raises the issue of, “How similar do species need to be (if they are diagnosable based on morphology) to qualify as cryptic?” In the strictest interpretation of cryptic species, once these species can be diagnosed based on morphology, they are no longer cryptic; however, from a purely practical standpoint, they may remain “hidden” during routine examination. Thus, it is useful to distinguish between cryptic species sensu stricto (no morphological differences are known) versus a functional definition of cryptic species (for which the application of the term is defined by the systematist).

A bibliographic search for a 10-yr period (from January 1999 to November 2009) in the ISI Web of Knowledge (http://apps.isiknowledge.com) yielded 3,913 records (or “cryptic species reports”) with the search term “cryptic species.” If the search term “sibling species” is used, the number of records for the same period is 2,313. Interpretation of such results requires caution, however, because these search terms also recover records (e.g., “cryptic” biological phenomena involving species, e.g., cryptic behavior, cryptic coloration, and cryptic host-specificity. In addition, papers on cryptic species are not exclusively based on molecular data. For example, Deuff et al. (2004) recently described a cryptic species of spinturnicid mite, a parasite of chiropterans, by associating biologic, biometric, and morphologic criteria together with the host’s ecotology. Nevertheless, the number of such bibliographic records has increased every year since 1999, when 132 reports were published; in 2008, there were 513 records, and from just January to November 2009, 576 had been published.

When the same 10-yr period was searched in the ISI Web of Knowledge (ISI), using the terms “cryptic species” and “parasites” (and also “sibling species” and “parasites,” as both terms have been used for the same phenomenon in our discipline), with a focus on the major groups of parasitic organisms (protists, helminths, and arthropods), 464 records were recovered (“cryptic species reports”). Although the number of published studies reporting on cryptic species of parasites is increasing, it is not increasing at the same rate as in free-living taxa (Tables I, II). Careful filtering of these 464 records obtained from the ISI (plus manual searches of the major parasitological journals to retrieve additional records not recovered during the ISI search) yielded 68 reports where cryptic species of parasites (or sibling species) also reference DNA sequences. Some papers refer to the potential presence of cryptic species, but no sequence data were presented, and these papers were not considered in Table III, e.g., Bolek et al. (2009). These 68 “filtered” papers include the recognition (or at least the potential recognition) of cryptic species among protists (apicomplexans, diplomonadids, trypanosomatids, and trichomonads), helminths (monogeneans, digeneans, cestodes, nematodes, and acanthocephalans), and arthropods (ticks, lice, and crustaceans) and also include different host groups including humans, livestock, and wildlife. Interestingly, only 17 of the 68 papers reported that voucher specimens had been archived in a parasite collection. During the decade of the 1990s, several papers were published revealing the presence of cryptic parasite species, although most of these studies were based on interpretation of multilocus protein electrophoretic data with a focus on assessing levels of genetic divergence between taxa and, in some cases, distinguishing among species (and discovering cryptic species) based on genetic distance criteria (e.g., Chilton et al., 1992; Pozio et al., 1992; Beveridge et al., 1993; Naselli et al., 1993).

This review of the literature published during the last decade was conducted to identify reports that detected parasite cryptic species by using sequence data from 1, or more, genes. The review was then used to assess the extent to which such species are found among parasitic organisms. Meta-analyses of publication records for cryptic species have concluded that the discovery of cryptic species is likely to be non-random with regard to taxon and biome (Bickford et al., 2007), but they have not established if cryptic species are more common in particular habitats, latitudes, or taxonomic groups. If cryptic species are not randomly distributed, but are influenced by ecological, historic, and abiotic factors,
The definition of "cryptic species" usually includes 2 elements, i.e., species that are morphologically indistinguishable, or practically so, and genetically distinct lineages that are considered to represent separate species. Parasitologists have recognized cryptic species in different parasite groups following this definition, with the first molecular approaches employing protein electrophoretic data and testing whether or not genetic (allelic) population data were consistent with expectations of single, genetically distinct lineages that are considered to represent separate species. From published papers, it is often difficult to discern if these same data can also be used to recognize the existence of cryptic species and the radiation of cryptic species complexes have important potential impacts on understanding and developing parasite evolutionary theory, historical biogeography, and ecology (Hoberg and Brooks, 2008). Here, we evaluate the extent to which cryptic species have been reported among parasites, and we also analyze the approaches by which these species have been discovered. This evaluation has also revealed a lack of uniformity in what parasitologists mean by "cryptic species," coupled with an absence of consistency regarding the theoretical and methodological underpinnings by which these species are discovered. For instance, most authors apply this terminology when they are incapable of distinguishing species that are morphologically very similar, i.e., "hidden." Some have used "cryptic" in other contexts, including "cryptic variation," "cryptic host-specificity," "cryptic host-associated divergence," or for the hypothesis that parasites may reveal the "cryptic phylogeographic history of their hosts" (Hoberg, 1995), an idea subsequently adopted for applications of molecular data (Nieberding et al., 2004).

The definition of "cryptic species" usually includes 2 elements, i.e., species that are morphologically indistinguishable, or practically so, and genetically distinct lineages that are considered to represent separate species. Parasitologists have recognized cryptic species in different parasite groups following this definition, with the first molecular approaches employing protein electrophoretic data and testing whether or not genetic (allelic) population data were consistent with expectations of single, panmictic species (e.g., Bullini et al., 1978; Nascenti et al., 1979; Baverstock et al., 1985). During the last decade, nucleotide sequence-based methods have replaced earlier molecular approaches, e.g., native proteins, RFLPs, and RAPDs, and a common practice has been to obtain gene sequences to characterize levels of genetic variation over geographic space; however, these same data can also be used to recognize the existence of cryptic species. From published papers, it is often difficult to discern if investigators were deliberately prospecting for cryptic species (sensu Blouin, 2002; Criscione et al., 2005; Vilas et al., 2005), or if their discovery was accidental. Regardless, evidence suggests that cryptic species are relatively common among parasitic organisms (Table III). These reports show that approximately 128 cryptic species of parasites were discovered in the last decade, although not all of these were characterized as such in the original publications and very few were formally described (and named). These cryptic species contribute a small proportion of the relatively large number of new species descriptions published annually in parasitological and general zoology journals. Most descriptions of new parasite species are still based solely on morphological characters. However, to provide for the needed integration of morphological and molecular data in parasite systematics, it is strongly advisable to collect and preserve specimens for both morphological and molecular characterization. This recognizes the importance of genetic data, which should be less affected by host-induced phenotypic variation, for parasite species delimitation. The modern, integrated approach should assist efforts to solve taxonomic problems, even though relatively few formal descriptions of new parasite species incorporate molecular data. Modern taxonomic practices also require the preservation and deposition of voucher specimens in established parasite collections.

**POTENTIAL PROBLEMS IN RECOGNIZING CRYPTIC SPECIES**

From the literature review, we identified some problems inherent with the recognition of cryptic species of parasites. Before addressing the different approaches to identify these species, we first discuss some of these problems. One difficulty is that there has been no agreement on what constitutes appropriate discovery methods, or analytical approaches, to test hypotheses of such species. It is noteworthy that some authors are clearly discovering cryptic species, but the term cryptic species (or even sibling species) is not used in their papers (see, for example, Felici et al., 2000; Iwagami et al., 2000). Another problem regarding the lack of uniformity in the way parasite cryptic species are found is related to the observation that their discovery is often peripheral to the main focus of the investigation. Only occasionally has an article title included reference to cryptic parasite species (see, for example, Macnish et al., 2002; Miura et al., 2005; Miller and Cribb, 2007; Razo-Mendivil et al., 2010). More frequently, the presence of cryptic species is a collateral finding resulting from genetic analyses of other aspects of parasite evolution such as phylogeography, population genetic structure, or phylogenetic analysis.
that includes samples of many individuals (Criscitone and Bloquin, 2004; Johnson et al., 2007; Bouzid et al., 2008). It is likely that, in many such cases, parasitologists (and experts) have been识别ed in the morphological diversity of the “cryptic” taxa. Another complication in parasitology is that the concept of cryptic species has been applied to larval forms when larvae are morphologically indistinguishable, but show high genetic differentiation (e.g., Donald et al., 2004, 2007; Miura et al., 2005; Palm et al., 2008). However, there are many instances where larvae or juveniles from different species are morphologically indistinguishable, as is the case with nematodes, yet their respective adult stages are morphologically distinct. Without linking these indistinct larval stages to their adult stage, no complete morphological comparison is possible and, therefore, there is no basis for describing as “cryptic” any species that are incompletely known or characterized. Without such a linkage, it would be possible to conflate larval genetic differentiation as evidence for one or more new species when the distinct adult stages of the parasites may have already been described and named.

Another important, potential shortcoming is the absence of an underlying species concept when researchers are delimiting species (including cryptic species) in nature. The advantages and disadvantages of different species concepts are beyond the scope of this review, but there are many recent discussions of this subject (e.g., Wheeler and Meier, 2000; Brooks and McLennan, 2002). Although systematists may disagree on what is the optimal species concept, they would probably agree that it is important to understand what you are looking for (have a species concept) before trying to find or delimit species (Adams, 1998, 2001). Usually, it is not explicit in parasite taxonomic papers what species concept is being followed (although see Nadler et al., 2000; Zietara and Lumme, 2003), and clearly this should be communicated, and the choice of concept defended, because use of different concepts can lead to different decisions for the same data (Adams, 1998). Most papers recovered in the literature search (Table III) used sequence divergence levels to assess conspecificity through application of a genetic yardstick, but this approach, although useful for species prospecting (Villas et al., 2005), has been criticized as inappropriate for species delimitation for both practical and theoretical reasons (Nadler et al., 2000; Nadler, 2002). Assessments of sequence divergence have sometimes been accompanied by an evolutionary (phylogenetic) analysis to assess reciprocal monophyly, usually in the form of a neighbor-joining, maximum parsimony, maximum likelihood, or a Bayesian tree. These approaches generally conform to the recommendation by Adams (1998), which was that testing the hypothesis of lineage independence (species) in any particular case requires phylogenetic interpretation of data and the potential for failure to recover such lineages.

Other potential complications follow from how specimens are typically collected and processed. Parasites are collected from their hosts during fieldwork, and specimens are initially distinguished (sorted) based on a morphological species concept, i.e., a distinction is made among specimens and they are allocated to species level (morphospecies) based on existing morphological diagnoses. In some cases, depending on the taxonomic complexity of the group, parasites are only readily separated as morphotypes, but not to morphospecies. For some parasite groups, information on host-specificity and infection localization (tissue site) is also used as diagnostic evidence, when particular species of parasites are only known from a particular host species or from specific predilection sites within hosts. The pervasive influence of host species and traditional morphological diagnostic methods on species status is evident from a landmark study on reptile malaria. Perkins (2000) presented evidence that Plasmodium azurophilum, a parasite of lizards in the eastern Caribbean, involves 2 cryptic species; she supported her conclusion with data for defining these species based on similarity, biologic, and phylogenetic species concepts. Sequence data from the mitochondrial cytochrome b (Cyt b) gene showed that this lizard apicomplexan morphospecies was, in fact, 2 cryptic species (Perkins, 2000). These reproductively isolated species are indistinguishable by light microscopy, but 1 species undergoes schizogony only in erythrocytes and the other only in white blood cells. Bensch et al. (2004) suggested that avian haemosporideans have substantial potential for undiscovered cryptic species. This argument was made by McMonigle et al. (2006) to contrast morphologic versus molecular identification of these parasites, i.e., parasites were identified to species based on morphology and partial sequencing of the mitochondrial Cyt b gene. These data were analyzed by 3 species concepts (morphologic, genetic, and phylogenetic) and, as interpreted by these authors, the morphological species concept requires grouping the parasites by similarity, the genetic species concept by genetic distance, and the phylogenetic species concept by monophyly. They reported that, with 1 exception, the result of assessing parasite monophyly had identical sequences for all infections (or differed only by a few synonymous substitutions) and were monophyletic by all tree reconstruction methods (maximum parsimony, maximum likelihood, and Bayesian inference). Only 1 species did not follow this pattern, representing instead 2, or more, genetically distinct clades that were inferred to be cryptic species. It should be noted that the phylogenetic species concept is applicable to an analysis of all types of data (morphologic, molecular, combined evidence), and we do not necessarily advocate using different concepts for different types of data.

APPROACHES FOR RECOGNIZING CRYPTIC SPECIES

The correct identification of parasite species, cryptic or not, is at the core of our understanding of biodiversity and the impact of parasitism in nature. Cryptic species simply exacerbate the problem of correctly assessing biodiversity. In addition, research on the ecology or systematics of parasites may take different paths, depending entirely on the research objectives of the investigator. Cryptic species of parasites are being discovered by taxonomists, especially those conducting molecular prospecting (sensu Bloquin, 2002), but also by researchers specializing in areas such as population genetics, life cycles, and veterinary or medical parasitology. Cryptic species may be particularly common in cases where their discovery and diagnosis are important for diseases involving agriculture (plant or animal health), companion animals, and humans. Additionally, cryptic species are known to have major implications in the implementation of effective control and surveillance programs targeted for parasites of medical and veterinary importance, or their vectors (e.g., Conn et al., 1997; Cepicka et al., 2005; Sajunatha et al., 2007). A factor that has led to lack of uniformity in the way cryptic parasite species are recognized in nature is the expertise and focus of research groups. Naturally, this leads to different starting points for research and different actions regarding recognition of cryptic species (Fig. 1). Traditional alpha taxonomy mainly uses morphological attributes to characterize organisms at various levels of the taxonomic hierarchy. Such investigations might be used to estimate phylogenetic relationships among previously identified species, or to test the hypothesis of conspecificity for morphospecies (or morphotypes) with independent genetic data, thus potentially revealing the existence of previously unrecognized evolutionary lineages (species) or, conversely, finding molecular data consistent with conspecificity. Independent genetic evidence of conspecificity can also provide a framework for documenting levels of intraspecific morphological variability (including polymorphism among males, as in certain Ostertagiae) or, in the extreme, to demonstrate that 2 parasite morphospecies (even those formerly assigned to different genera) represent the same species (Stevenson et al., 1996; Dallas et al., 2006; Desseives et al., 2000; Bell and Sommerville, 2002; Li and Liao, 2003). Quite often, parasitologists observe a parasite species with a broad host range, geographical distribution, or both, that is accompanied by morphological variation (Hoberg et al., 1999). Typically, this variation is interpreted as occurring within the same species; for example, as normal population-level genetic variation, or perhaps as morphometric (size) differences correlated with the host species. Molecular tools offer the possibility to test the null hypothesis that this would represent variation within a single species.

Cryptic species are found by different approaches that are based on the null hypothesis that researchers are dealing with a single species (Fig. 1). Often, parasitologists must deal with widespread species, defined in terms of both host (2006) or geographic distribution range, or both, where morphological characters do not provide evidence for separate morphospecies and where morphological variability is low among members of different geographic populations. A single parasite species might be found in multiple host species that occur in sympathy, but also may be found associated with multiple host species in several localities. Given this wide
Table III. Published reports (data reported from January 1999 until November 2009) where DNA sequence data from nuclear or mitochondrial genes (or both) is used to reveal and diagnose, or to simply suggest the possibility of, cryptic species as a result of a diagnostic study, molecular prospecting, or phylogeographical analysis.

<table>
<thead>
<tr>
<th>PY*</th>
<th>Parasite group</th>
<th>Molecular marker(s)†</th>
<th>Reference‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>Teladorsagia boeoarcticus</td>
<td>NADH-4</td>
<td>Hoberg et al. (1999)</td>
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<td></td>
<td>Cylicostephanus minutus</td>
<td>ITS1, ITS2</td>
<td>Hung et al. (1999)</td>
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<td>2000</td>
<td>Macraciaria/Monorchis</td>
<td>ITS1</td>
<td>Jousson et al. (2000)</td>
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<td>Opecoeioides columbellae</td>
<td>ITS1</td>
<td>Jousson and Bartoli (2000)</td>
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<td></td>
<td>Plasmodium azurophilum</td>
<td>Cyt b</td>
<td>Perkins (2000)</td>
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<td></td>
<td>Cryptosporidium parvum</td>
<td>HSP70</td>
<td>Sulaiman et al. (2000)</td>
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<td></td>
<td>Contracaecum osculatum</td>
<td>ITS1, ITS2</td>
<td>Zhu et al. (2000)</td>
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<tr>
<td>2001</td>
<td>Trypanosoma spp.</td>
<td>ITS1</td>
<td>Sehgal et al. (2001)</td>
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<td></td>
<td>Parampholephala spp.</td>
<td>ITS1</td>
<td>Haukisalmi et al. (2001)</td>
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<td>2002</td>
<td>Intestinal Nematoda in cattle (pairs of congeners)</td>
<td>ITS1, ITS2, NADH-4, COI</td>
<td>Blouin (2002)</td>
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<td>Gyrodactylus ruginoides</td>
<td>ITS1, 5.8S, ITS2</td>
<td>Huyse and Volckaert (2002)</td>
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<td></td>
<td>Columbicollia/Physconelioidei</td>
<td>COI</td>
<td>Johnson et al. (2002)</td>
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<td>Hymenolepis nana</td>
<td>ITS1, COI</td>
<td>Macnish et al. (2002)</td>
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<td></td>
<td>Bolbophorus sp.</td>
<td>18S, ITS1, ITS2, 28S, COI</td>
<td>Overstreet et al. (2002)</td>
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<td>Iodes holocyclus</td>
<td>ITS2</td>
<td>Shaw et al. (2002)</td>
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<td>Pseudoterranova decipiens</td>
<td>ITS1, ITS2</td>
<td>Zhu et al. (2002)</td>
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<td>Teladorsagia circumcincta</td>
<td>b-tubulin, ITS2, NADH-4</td>
<td>Leigelm et al. (2002)</td>
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<td></td>
<td>Gyrodactylus spp.</td>
<td>18S, ITS1, 5.8S, ITS2, 28S</td>
<td>Zietara and Lumme (2003)</td>
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<td>Contracaecum ommorphini</td>
<td>Cyt b</td>
<td>Mattiucci et al. (2003)</td>
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<td>Megathyloecoides giganteum</td>
<td>28S</td>
<td>Rosas-Valdez et al. (2004)</td>
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<td>Derogenes aspina, Plagiopus shawi, Nanophyetus salmnicola</td>
<td>NADH-1, ITS1</td>
<td>Criscione and Blouin (2004)</td>
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<td>Larval digenea (Opecoelidae)</td>
<td>ITS2, 16S</td>
<td>Donald et al. (2004)</td>
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<td>Parampholephala omphalodes</td>
<td>COI</td>
<td>Haukisalmi et al. (2004)</td>
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<td>2005</td>
<td>Progymotaenia exeri, P. macropodis, P. zschokkei</td>
<td>COI</td>
<td>Hu et al. (2005)</td>
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<td>COI, ITS1</td>
<td>Miura et al. (2005)</td>
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<td>Pairs of closely related digeneans and cestodes</td>
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<td>Vilas et al. (2005)</td>
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<td>ITS1, 28S</td>
<td>Wu et al. (2005)</td>
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<td>Tetrarichomonas gallinarum</td>
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<td>Cepicka et al. (2005)</td>
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<td>Echinococcus shiquicus</td>
<td>COI, NADH-1, atp6, Cyt b, rRNA, elp</td>
<td>Xiao et al. (2005)</td>
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<td>ITS1, ITS2</td>
<td>Li et al. (2005)</td>
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<td>2006</td>
<td>Plasmodium, Haemoproteus, Leucocytozoon</td>
<td>Cyt b</td>
<td>Martinsen et al. (2006)</td>
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<td>Leucocytozoon toddi</td>
<td>Cyt b</td>
<td>Sehgal et al. (2006)</td>
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<td>Marques et al. (2007)</td>
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<td>Teladorsagia circumcincta</td>
<td>5 microsatellites</td>
<td>Grillo et al. (2007)</td>
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<td>Columbicola spp.</td>
<td>COI, 12S, EF-1α</td>
<td>Johnson et al. (2007)</td>
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<td>Soboliphyme bataure</td>
<td>NADH-4</td>
<td>Koehler et al. (2007)</td>
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<td>Hysterohlyciunr aduncum</td>
<td>ITS1, 5.8S, ITS2</td>
<td>Klippe et al. (2007)</td>
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<td>Krone et al. (2007)</td>
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<td>Steinauer et al. (2007)</td>
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<td>ITS1, 5.8S, ITS2</td>
<td>Kellermanns et al. (2007)</td>
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<td>Tentacularia coryphaenae</td>
<td>28S</td>
<td>Palm et al. (2007)</td>
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<td>Geomyloecus spp.</td>
<td>COI, EF-1α</td>
<td>Light and Hafner (2007)</td>
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<td>2008</td>
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<td>ITS2, Cyt b, COI</td>
<td>Bouzid et al. (2008)</td>
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<td>Tania polyacantha, T. taeniiformis</td>
<td>COI, NADH-1</td>
<td>Lavikainen et al. (2008)</td>
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<td>Pseudoleptothorium sp.</td>
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<td>Gyrodactylus spp.</td>
<td>COI, ITS1, 5.8S, ITS2</td>
<td>Kuusela et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Anclyloma canum</td>
<td>COI</td>
<td>Miranda et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Mesonermis flumenanlis</td>
<td>COI</td>
<td>St-Onge et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Eimeria sp.</td>
<td>ITS2</td>
<td>Cantacesssi et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Contracaecum rudolphii</td>
<td>ITS1, 5.8S, ITS2</td>
<td>Farjallah et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Anisakids nematodes</td>
<td>Review</td>
<td>Mattiucci and Nascetti (2008)</td>
</tr>
<tr>
<td></td>
<td>Caligus elongatus</td>
<td>18S, COI</td>
<td>Oines and Schram (2008)</td>
</tr>
<tr>
<td></td>
<td>Anoplophaloides variabilis</td>
<td>COI</td>
<td>Haukisalmi et al. (2008)</td>
</tr>
</tbody>
</table>

(Table III continued)
array of possibilities, a parasite species may show within-species variation for some morphological traits (sometimes even diagnostic traits as originally defined in the differential diagnosis), or only show within-species variation in terms of body size. In some cases, diagnostic differences in parasite morphology have been shown to be a host-induced variation within a single species (Duffy et al., 1990). However, some types of morphological variation may be difficult to discern during the normal course of specimen examination. In other cases, insufficient sampling can result in a failure to detect cryptic species in widespread parasite species, as discussed by Hoberg et al. (2003) and Cook et al. (2005) for cestode (Arostrilepis) diversity in arvicoline rodents.

Tests of cryptic species require postulation of the null hypothesis (Ho), which is for a single species (Fig. 1). The alternate hypothesis is that the species, as currently conceived, is represented by 2, or more, species. In some cases, researchers have presented this alternate hypothesis (existence of a cryptic species complex) as the null hypothesis for a particular host–parasite system (e.g., Hoberg et al., 1999). Either way, proper recognition (and delimitation) of cryptic species in nature involves scientific hypothesis testing. In most instances, parasitologists begin from a molecular perspective, sequencing specimens from a wide host or geographic range (or even genetically characterizing many individuals from several populations). When appropriate analyses lead to the recognition of independent lineages (rejecting Ho), a more detailed morphological investigation can serve as “reciprocal illumination” (Hennig, 1966), perhaps providing structural evidence consistent with separate species or, alternatively, revealing no apparent morphological differences, thus providing for provisional recognition of cryptic species, i.e., the species remain in “taxonomic crypts” following discovery (Schliek-Steiner et al., 2007). This requirement for “reciprocal illumination,” or comparison between morphological and molecular evidence, is necessary if reliable inferences about the “cryptic” status of species are to be made (Jenkins et al., 2005; Kutz et al., 2007). For completeness, this extends to morphological examination of all life cycle stages (when the life cycle is known), particularly those that have the greatest likelihood of showing morphological differences. For example, examination of nematode larvae might cause investigators to conclude that 2 species are indistinguishable, when the adults are morphologically distinct. The research interests of the investigator shape the hypothesis-testing path (Fig. 1), and research outcomes can differ as a result. For example, one possible outcome is recognizing the presence of cryptic species based on phylogenetic analysis of molecular data, whereas a more desirable outcome for describing biodiversity would be the formal description (and naming) of the species. The discovery and delimitation of species using molecular data does not guarantee the discovery of diagnostic morphological features for these same species, even if such morphological differences exist. This leads to practical challenges involving the formal description of the species—if they are cryptic in the strict sense, e.g., providing a differential diagnosis in the absence of any known morphological differences—in that diagnosing such species depends upon molecular evidence.

Hypothesis testing for cryptic species requires acceptance or rejection of the null hypothesis. Testing the null hypothesis with molecular data has taken several different forms, including phylogenetic analysis for potential evolutionary structure (reciprocal monophyly of individuals falsifying the null) and population genetic analyses, e.g., patterns inconsistent with panmixia of individuals falsifying the null hypothesis. If the null proposal is accepted, then from the morphological perspective, any observed structural variation represents intraspecific differences among individuals and may be due to normal intraspecific genetic variation, or to phenotypic plasticity, including host-induced variability (e.g., Perez-Ponce de Leon, 1995). When the null hypothesis is accepted based on molecular data, some researchers have characterized observed intraspecific variability (geographic, morphologic, ecological, etc.) as strain variation (e.g., O’Mahony et al., 2004; Kawazoe et al., 2008). In some cases, the main impetus for cryptic species prospecting is that such species were previously discovered in close relatives, yet such findings are not necessarily good predictors for other species groups (Shaw et al., 2002; Kimbel et al., 2007; Koehler et al., 2007; Palm et al., 2007). Testing hypotheses of cryptic species can be affected by sampling error. For example, using too few characters, or molecular markers with low rates of substitution relative to the time scale of speciation, may fail to reject the null hypothesis when separate species are present (Nadler, 2002). This is one reason that, for species delimitation (rather than prospecting), multiple loci should be used. The absence of genetic differences at a single locus may not falsify the null hypothesis, but data from other loci could do so. For both prospecting and delimitation, faster-evolving loci should provide more appropriate data than would more-conservatively evolving ones (Blouin, 2002; Nadler, 2002). For phylogenetic analyses, corroborating the same pattern of reciprocal monophyly with multiple loci is particularly strong evidence of separate species (Nadler, 2002, 2005). Similarly, population genetic analyses of genetic structure will benefit from examination of multiple loci, because not every locus will reflect a pattern of variation consistent with a neutral genetic marker (Nadler, 1995). In this sense, there is a parallel between morphological and molecular approaches, i.e., both are provisional upon the available data and, in theory, both are improved by additional sampling of independent data, e.g., morphological characters and genetic loci. Clearly, investigations that are exclusively molecular, or only morphological, both have limitations. The most thorough studies include both types of information, with morphological conclusions being tested by genetic data and with molecular evidence for separate species being examined by a detailed morphological study of specimens representing each independent evolutionary lineage. Indeed, by design, some investigators propose using both sources of information to test the null hypothesis (e.g., Marques et al., 2007). Similarly, while describing a substantial radiation of sanguinicolid blood flukes from 3 families of marine fishes, Nolan and Cribb (2006) used a species-level
taxonomy based on a combined molecular, biological, and morphological approach. These authors recognized a priori that morphology alone may be insufficient for the unequivocal identification of parasite species; they used molecular methods to augment the traditional morphological approach, concluding that cryptic species are an increasingly important consideration for accurately characterizing parasite biodiversity.

If data analysis leads to the rejection of the null hypothesis (Fig. 1), several scenarios can result. From the morphology-based approach, finding diagnostic characters can lead to the traditional description of the new species, as is characteristic of comparative morphological analysis. Traditionally, the decision-making process of "rejecting the null hypothesis" (presence of a multiple species) has been made through comparisons of a taxonomic expert referencing differential diagnoses (and physical specimens) of other congeners. Most often, no explicit procedure is presented in concluding that a taxon merits recognition as a distinct species. Species diagnosed in this way do not reflect hypotheses testing, in any formal sense, given that there is no explicit methodology for analyzing data that leads to falsification. Instead, this approach is an implicit appeal to the experience and expertise of the taxonomist. Although many of these decisions may be correct, they do little to bolster confidence in the objectivity and consistency of species delimitation as a science (Adams, 1998; Nadler, 2002).

More recent systematic studies of parasites show that, for many taxonomic groups, molecular tools are very valuable and efficient resources, not only for initial "prospecting," but for providing additional characters useful for delimiting and diagnosing species. Most typically, molecular sequences are obtained for samples of individuals from within, or among, infrapopulations, and the null hypothesis is rejected because reciprocal monophyly is revealed for the taxa (individuals) based on a phylogenetic analysis of sequences; if the evolutionary lineages are distinguished by morphological characters, the species are described (and named) using both the sequence and morphological data (e.g., Curran et al., 2006; Pérez-Ponce de León et al., 2008). However, if only genetic data distinguish the evolutionary lineages (i.e., no correlated morphological differences are discovered), then falsification of the null hypothesis, based on molecular data in the absence of morphological differences, is indicative of cryptic species sensu stricto (see Overstreet et al., 2002; Rosas-Valdez et al., 2004; Chilton et al., 2007; the latter study was based on allozyme electrophoresis).

If the hypothesis-testing process leads to the rejection of the null proposal, from the perspective of the DNA-based approach, the existence of cryptic species sensu stricto means that the delimited species are either indistinguishable morphologically, or are so similar that they have not been distinguished. If the null hypothesis has been rejected, research generally takes 1 of 2 directions (Fig. 1). One, or more, cryptic species may be discovered and delimited, but without formal scientific description (e.g., Hung et al., 1999; Macnish et al., 2002; Cepicka et al., 2005; Wu et al., 2005; Miranda et al., 2008). Some authors, after recognizing the existence of cryptic species (but not necessarily delimiting them), discuss the implications of cryptic diversity or cryptic diversification (sometimes erroneously referred to as "cryptic speciation") for the investigation, which is typically focused on ecological and evolutionary questions rather than on systematics, per se (e.g., Jousson et al., 2000; Schgal et al., 2006; Grillo et al., 2007; Steinauer et al., 2007). In some cases, cryptic species have been recognized, but not described, with the rationale that there is no mechanism for the formal description of species based only on genetic data (Andrews et al., 1998). However, there is no rule in the International Code of Zoological Nomenclature (ICZN, http://www.iczn.org/iczn/index).
When morphological diagnosis of species is difficult, or species-level diversity is poorly understood, e.g., cryptic species complexes, for the accurate characterization of species-level diversity it is critically important to obtain molecular and morphological data from the same, individual specimens. More typically, different sub-samples of specimens are prepared using different procedures, one optimized for morphology and another for DNA (but this is less than ideal, particularly when there are few specimens from a host or if the sample may contain several morphologically similar congeneric species. Even when strong evidence is found to delimit 2, or more, species using molecular data, authors can be reluctant to propose nomenclatural changes based on molecular data alone, when morphological diagnosis is uncertain (see Nolan and Cribb, 2006; Marques et al., 2007). Commonly, more detailed morphological study is suggested as a necessary step to investigate the possibility of subtle morphological differences consistent with genetically recognized species, or even to emphasize other biological data such as growth requirements, metabolism, host preference, or geographical distribution. However, when cryptic species of parasites are discovered using a molecular approach, morphological re-examination of the specimens for diagnostic traits often follows, which are less amenable to molecular approaches, such as morphometric analysis or use of new data types, e.g., scanning electron microscopy. These and other approaches can lead to a formal description of cryptic species.

Some examples are available that illustrate how research on cryptic species can successfully progress from discovery through description. For example, feather lice species of *Columbidae* have been the subject of intensive investigation (e.g., Johnson et al., 2002; Johnson et al., 2003; Johnson et al., 2007; Bush et al., 2009; Malek, 2009); molecular phylogenetic analysis of 49 of the 80 species within the genus (Johnson et al., 2007) revealed a considerable divergence that was correlated with host associations, and this was used as evidence to delimit more than 30 cryptic species. In the initial paper describing their discovery (Johnson et al., 2007), it was indicated that additional morphological studies were required prior to taxonomic revision. This was followed by more detailed morphological evaluations and descriptions (Bush et al., 2009). Similarly, the nematode *Teladorsagia* was found to represent a species complex in Holarctic ruminants, based on reciprocal monophyly inferred from mitochondrial ND1/4 sequences and on estimates of sequence divergence and nucleotide diversity (Hoberg et al., 1999). These authors formally described the new species *Teladorsagia boreoarctica* as part of the original publication, reporting discovery of the cryptic species.

Researchers working with other groups of parasites have sometimes taken similar, integrated approaches to delimiting species and describing them. For example, Xiao et al., (2005) investigated taeniid cestodes of *Echinococcus* under the premise that potential cryptic species should be conspecific. These authors analyzed 1,200 bp of 5 mitochondrial and 1 nuclear gene to describe a new *Echinococcus* species (*Echinococcus shiquicus*) from the Tibetan fox and plateau pika in China. An investigation of the molecular systematics of larval tapeworms (*Mesocoeotidacea*) from dogs and coyotes (Crosbie et al., 2000) showed at least 3 distinct monophyletic groups; this information was expanded upon by Podgett et al. (2005), who used multiple genetic loci in combination with a morphometric analysis and an hypothesis-testing framework to demonstrate that these 3 clades within *Mesocoeotidacea* were distinct species. One of these species was conspecific with *Mesocoeotidacea* and, although none was characterized as “cryptic,” nevertheless, these authors illustrated the practical application of sequence data to test the hypothesis of lineage independence and species status for cestodes. Similarly, research on the ascaridoid nematode *Toxocara* revealed that original reports of *Toxocara canis* in Malaysian domestic cats were inaccurate; subsequent molecular studies showed that this parasite represented a separate species from *T. cati* and *Toxocara cati*, the common ascaridoid of domesticated cats (Zhu et al., 1998). Originally identified as a cryptic species (*Toxocara cf. canis*), further taxonomic study led to its description as *Toxocara multiceps* (Gasser et al., 2001). Subsequent molecular sequencing has provided additional molecular support for this distinction (Gasser et al., 2005; Jex et al., 2008). For trematodes, Miller and Cribb (2007) described cryptic species of digeneans in marine fishes from Australia. To test their morphologically based taxonomic approach, they sequenced 3 nuclear ribosomal regions (partial 28S, ITS1, and ITS2) and conducted a posteriori analysis, revealing that morphological diagnoses were consistent with genetic differences, thus leading to the recognition of cryptic species in the study.

Investigators working with monogeneans of the genus *Gyrodactylus*, where cryptic species seem to be very common, have proposed a seemingly novel approach for presenting species descriptions that involves both morphological and molecular data (Zietara and Lumme, 2003; Kuusela et al., 2008). With this approach, the definitive species recognition is based on the nucleotide sequence of nuclear ribosomal DNA (internal transcribed spacer 1 (ITS1) of the rDNA gene). On evaluating the number of nucleotide substitutions, the presence of indels, and on pair-wise divergence. A molecular diagnosis is presented, and morphometric and morphological diagnoses are established for each new species, with illustrations of the haplotype structures, along with all relevant biological and geographical data and information on deposition of specimens and sequence data. In a similar way, Nolan and Cribb (2006) presented a remarks section in their publication that included a molecular diagnosis immediately following the morphological description of each sanguinicolid digenean species they discovered in marine fishes.

Other investigations that may lead to the recognition of cryptic species are those of phyleogeographic and population genetic analyses (Fig. 1). These approaches usually begin without a taxonomic focus because their main goal is to investigate the population genetics or the phylogeography, of a single species. Most typically, mitochondrial genes are employed for phyleogeography whereas microsatellites, amplified fragment length polymorphisms (AFLPs), or other nuclear gene markers are used for estimating population genetic structure (Grillo et al., 2007; Stefka et al., 2009). Population-level analyses are useful for inferring which aspects of the host–parasite relationship and life history may have shaped the genetic structure of the species, including geography, host-specificity, and life cycles (e.g., Nadler, 1995; Criscione and Blouin, 2004; Bouzid et al., 2008; Glennon et al., 2008). In studies of population genetic structure, estimated parameters include measures such as F-statistics (and their haplotype equivalents), including \( F_{ST} \) and \( F_{IS} \), effective population size, nucleotide and haplotype diversity, parsimony networks, and estimated migration rates. Characteristics of the genetic markers (dominant vs. codominant expression) also influence the types of analyses that can be conducted. For example, direct assessments of interbreeding through the determination of heterozygote frequencies requires codominant markers. High levels of genetic structure, a reduction of heterozygotes relative to expected numbers, and parsimony networks with large numbers of inferred substitutions separating individuals, might be explained by the lack of genetic exchange that is characteristic of cryptic species, wherein individuals are separated by reproductive isolation rather than by geographic barriers to gene flow. For instance, Bouzid et al. (2008) studied 2 factors (geography and host specificity) that affected the genetic structure of *Ligula intestinalis*, a widespread tapeworm with larvae infecting freshwater fishes. They found different evolutionary mechanisms at the local and global geographical scales, based on sequences of nuclear and mitochondrial genes (ITS2, Cyt b, and cox1), for 109 tapeworms from 13 host species and 18 localities. These authors found genetically divergent and well-separated clusters in different geographical areas sampled globally, and reproductive isolation was apparent for clades distributed sympatrically and infecting the same definitive host, suggesting the likelihood of separate (cryptic) biological species (labeled as clades A and B in their study to represent these 2 different species), although specific taxonomic recommendations were not made.

Similarly, during early stages of their research, some authors interested in characterizing the genetic structure of parasites in order to address ecological and evolutionary questions uncover the presence of distinct evolutionary lineages that leads to recognition of cryptic species. For example, as part of a study to examine the effects of life cycles on the distribution of genetic variation within and among parasite populations with allomorphic and autogenic life cycles, Criscione and Blouin (2004) discovered 2 divergent mitochondrial ND1 lineages among the digenean *Deroegus aspina*. To test whether these 2 mtDNA haplogroups represented cryptic species, they sequenced the internal transcribed spacer 1 (ITS1) of their study. The rDNA gene is a common target for studies of cryptic taxa delimited by mtDNA and ITS1, a result indicating no (or minimal) introgression between the 2 lineages of *D. aspina* and, therefore, they considered them to be genetically distinct species (*D. aspina* A, and *D. aspina* B) and analyzed their genetic structures separately. A similar situation was found by Glennon et al. (2008) while studying host
specificity of monogeneans from rhinobatid rays in southern Australia. To
determine whether the 3 species of monogeneans found in the ray
Trygonorrhina leptura were restricted to this host, these authors used Cyt
b sequences and found that, not only were these monogeneans not strictly
host-specific, but that the genetic structure of Pseudoleptobotrium
apychotremae (found in 3 of the 5 rhinobatid species surveyed in
Australian coastal waters) was genetically homogeneous across most
localities. However, 1 population showed an unusually high Cyt b
divergence. Sequencing of the nuclear gene elongation factor 1-a
 corroborated the deep mtDNA divergence, suggesting that this clade
represents a cryptic species. In these cases, where a taxonomic approach is not the major focus of the
research, cryptic species are inferred, but typically no effort is made to re-
examine the specimens and to corroborate the molecular findings with
detailed morphological studies. Without such an effort, the “cryptic
status” of such taxa remains enigmatic, and such species often remain
undescribed and without a proper scientific name. In many cases, research
results may only suggest that unknown species are present, with
confirmation requiring sequencing of additional loci, more detailed
analyses for hypothesis testing, e.g., corroboration of lineage indepen-
dence, and suitable morphological studies to differentiate between cryptic
species and those that are morphologically diagnosable (or even previously
described). The desired outcome for newly discovered cryptic species is
that they are properly described and that the initial molecular studies that
led to their discovery are followed by detailed morphological studies that
have the potential to reveal unrecognized structural differences. Although
such cryptic species may be indistinguishable based on current morpho-
logical practice, it remains possible that future developments in
morphological tools, e.g., microscopy techniques, staining methods, etc.,
may provide methods that permit their diagnosis based on structural
features. In this sense, the strict cryptic status of such species remains
provisional, although the practical matter of diagnosing these species on a
routine basis may remain difficult—even following the discovery of structural
differences between species.

This emphasis on integrative molecular and morphological approaches
to parasite systematics raises the important issue of the lack of sufficient,
traditionally trained taxonomists to undertake the scope of research that
results from molecular investigations. Classically trained taxonomists have
a wealth of information on parasite morphology and natural history,
knowledge that is critical for the required comparative analyses of known
versus potentially new species and for the formal description of these
species. As pointed out by Baldwin et al. (1999), addressing this issue
requires not only efforts to strengthen traditional taxonomic expertise and
infrastructure, but changing attitudes among scientists themselves so that
systematists are considered as critical to university research as are the
faculty who study fundamental molecular processes using reductionist
approaches.

CONCLUSIONS AND FUTURE DIRECTIONS

The discovery of cryptic species has a direct impact on our assessment of
parasite biodiversity (Poulin and Morand, 2004). Increased precision of
cryptic species discovery and description will result if parasitologists follow a common, theoretical framework and methodology for finding and
delimiting cryptic species in nature. It is clear that research on cryptic
species has 3 steps. The first step is the recognition of potential cryptic
species, sometimes discovered through cryptic species prospecting. The second step is their delimitation through hypothesis testing (potential falsification of the null hypothesis of a single species), and the third step is that of formal description (and naming). As previously noted, the
recognition of parasite cryptic species is frequently achieved when taxonomy is not the major focus of the research. However, recognizing potential cryptic species, without actually delimiting and
describing them, will lead to increased taxonomic uncertainty that is
counterproductive to research progress and synthesis in parasite systema-
tics. This also holds true for other research areas such biogeography,
ecology, and evolutionary biology. Molecular research on potential
cryptic species has 3 steps. The first step is the recognition of potential
cryptic species, including cryptic species research, and voucher specimens should be preserved and deposited in established museum collections to facilitate future systematic
study, perhaps with improved techniques.

Unfortunately, it can be difficult to ascertain, from some published
texts involving potential cryptic species, if specimens have been
processed for morphological examination, or even if such specimens have
been properly preserved for that purpose. Properly archived specimens are essential for future work on cryptic species, including
archival material for molecular work. Several recent parasite survey and
inventory projects (e.g., The Beringian Coevolution Project or the
freshwater fish helminth parasite fauna in Mexico) serve as exemplars
for host and parasite sampling strategies, preservation for morphological
and molecular studies, archiving of specimens, and integrated molecular
and morphological investigations that have forced broader questions
concerning the distribution of parasite biodiversity (Hoberg et al. 2003;
Cook et al., 2005; Pérez-Ponce de León and Choudhury, 2010). The future of parasite systematics will be substantially complicated, if reports of
putative cryptic species accrue without proper delimitation or description
(and a valid name), because scientists will be required to deal with
organisms of uncertain status (real species or not?) as well as with their
labels, e.g., Species A, Species B, etc. Recently, Hoberg et al. (2009)
examined the critical role of permanent and well-supported museums or
natural history collections as foundations for systematic research in the
traditional sense, but also for all aspects of our discipline that depend
upon the meaningful reference to a parasite by scientific name. In
addition, museum collections and their associated (curated) data create an
empirical record that promotes our understanding of the biosphere
through time. For example, while studying a range of taxonomic
(nomenclatural) and biogeographic questions about pinworms and pikas from the American west, Hoberg et al. (2009) demonstrated the critical
importance of type and voucher specimens and the role of museum
repositories for documenting species diversity and changes in faunal
structure over time (see also Brooks and Hoberg, 2000; Hoberg, 2002).

Research on cryptic species of parasites is still in its infancy, but it is
very likely that these species are much more common than previously
thought. Data available thus far (Table III) are insufficient to permit
generalizations, as are those made by authors such as Bickford et al.
(2007) and Pfenniger and Schwenk (2007). For instance, the observation
that more cryptic species have been discovered among parasitic nematodes
(Table II) has to be considered in relation to the comparatively large
number of research groups worldwide investigating the molecular
systematics of anisakids. Researchers in Australia, Italy, and the United
States have demonstrated several instances of cryptic species among
anisakid nematodes, and because of their comparatively large size,
molecular methods (e.g., allozyme electrophoresis) have long been
practical for ascaridoid nematodes (for a review see Mattucci and
Nascetti, 2008). Within other parasite groups, few comparable efforts have
been made to discover cryptic species, so it is premature to search for
trends among different taxonomic groups. It has been suggested in a few
cases that certain host–parasite associations might show a larger number of
cryptic species, such as anoplocephalid and hymenolepidid tapeworms in
arvicoid rodents (Hoberg et al., 2003, but also see Haukisalmi et al.,
2004), roundworms (Cook et al., 2009), or in protostomiasis (Hoberg et al.,
2005). As discussed by Hoberg and Brooks (2008), it is the history and
structure of hosts and their parasites that can promote such species-level
diversity (including cryptic species complexes). The fact that parasites
contribute a small fraction to the total of “cryptic species reports” (sensu
Bickford et al., 2007; Pfenniger and Schwenk, 2007) does not establish that
cryptic species are less common among parasites than are free-living
organisms. The literature search for general patterns of cryptic species, as
discussed by Bickford et al. (2007) and Pfenniger and Schwenk (2007),
should be interpreted cautiously because these reports were not
individually verified for content. However, this may also reflect that
cryptic species research has proceeded at a slower rate within our
discipline.

We predict that there are very large numbers of parasite cryptic species
to be discovered and speculate that they may account for a substantial
fraction of parasite biodiversity in some clades. Host–parasite systems are
known to represent rich macroevolutionary mosaics, with empirical
studies indicating that host-switching and geographical dispersal of
parasites are more common phenomena than is strict (maximum)
cospeciation (Hoberg and Brooks, 2008). Predictions concerning parasite
diversity, including cryptic species, should contribute to the diversity
of macroevolutionary possibilities. Research programs on biodiversity
and cryptic species will be enhanced as more parasite taxonomists use
molecular approaches that include infrapopulation-level sampling of
individuals and analysis of multiple genetic loci. Molecular prospecting
studies will increase in frequency as molecular methodologies become

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LITERATURE CITED


