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DNA Taxonomy: How many DNA Sequences are needed for solving a Taxonomic Problem? The Case of two Parapatric Species of Louse Flies (Diptera: Hippoboscidae: *Ornithomya* Latreille, 1802)

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> Abstract

Using molecular and morphological data, we investigate the validity of two hippoboscid species, *viz. Ornithomya fringillina* (Curtis) and *O. chloropus* (Bergroth) that are parapatric in northern Europe and had previously been synonymized. We study four morphological characters that had been proposed as diagnostic for species separation, but only one, relatively weak character (a gena marking), has discontinuous variation across the putative species. In order to collect more evidence for deciding on the species status, we sequenced an approximately 810 bp long region of COI for 13 specimens from sympatric populations. The signal from the sequences suggests that *O. fringillina* and *O. chloropus* are different species because the interspecific genetic distances between the taxa are twenty times larger than the intraspecific variability of *O. fringillina*. We argue that even a small number of sequences can yield significant information on taxonomic issues as long as the specimens are predominantly collected for (1) those species/populations whose status is difficult to resolve based on morphological information and (2) those specimens that come from sympatric populations of the "problematic" species. (3) We also suggest that the status of a rare species can be adequately addressed with very few sequences as long as the intraspecific variability of more common, close relatives have been adequately assessed.

> Key words

Diptera, *Ornithomya*, DNA taxonomy, DNA barcoding, COI, species boundaries, genetic distance.

Introduction

The use of DNA sequences for taxonomic and identification purposes is currently extensively discussed in the biological literature (Ferguson 2002; Hebert et al. 2003; Lipscomb et al. 2003; Meier et al. 2006; Tautz et al. 2003; Will & Rubinoff 2004). This discussion has covered many major problems and opportunities associated with DNA taxonomy and DNA barcoding, but much less attention has been paid to the seemingly lesser topic of how many and which DNA sequences are needed for making useful contributions to taxonomy. Yet, this issue is of considerable importance. It has been estimated that 40% of all beetle species have only been collected once (see Seberg 2004) and it is safe to assume that a significant number of these species are only known from a single specimen. In this paper, we will argue that even a relatively small number of DNA sequences can sometimes generate enough evidence to influence taxonomic decisions (see also Memon et al. 2006). However, the specimens that should be sequenced need to be carefully selected according to three criteria that we are promoting here. First, sequences should be predominantly collected for

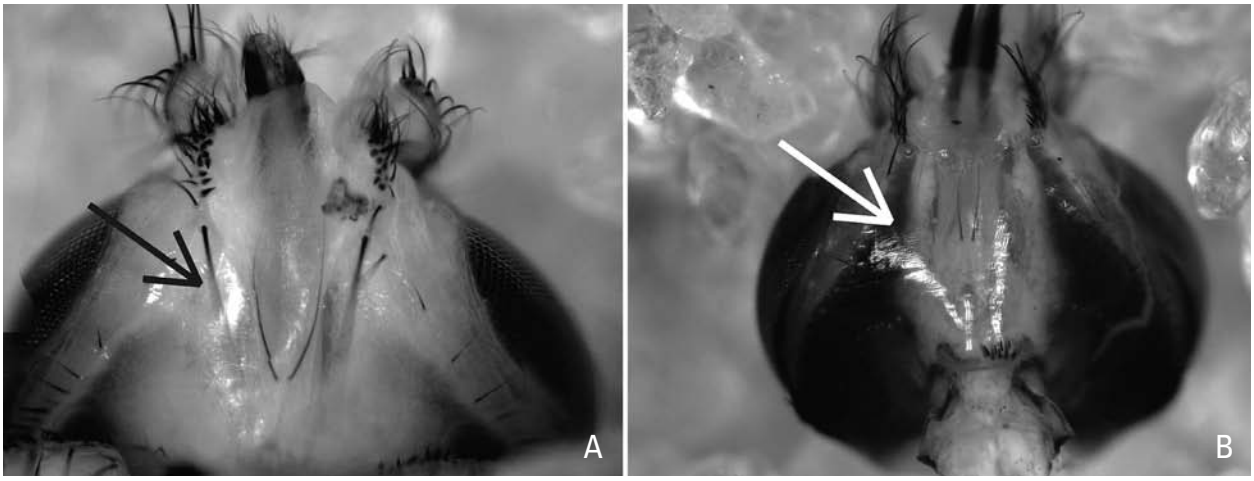

Fig. 1. Ventral view of head. Arrows indicate the area of the gena marking. **A**: *Ornithomya fringillina* (specimen fr10). **B**: *Ornithomya chloropus* (specimen ch1).

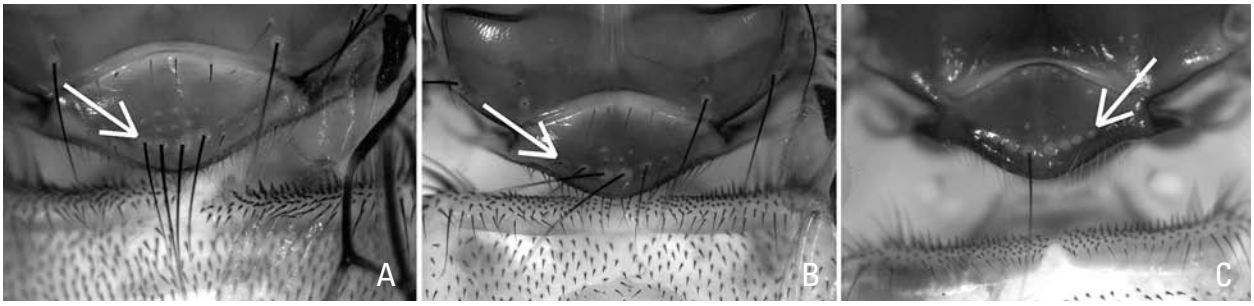

Fig. 2. Dorsal view of scutellum. Arrows indicate bristles or sockets. **A**: *Ornithomya fringillina* (specimen fr10). **B**: *Ornithomya fringillina* (specimen fr2). **C**: *Ornithomya chloropus* (specimen ch1).

those species whose validity is in doubt based on existing information. Second, if the putative species are parapatric, the sampled specimens should come from sympatric populations. Extensive sampling across the ranges of all populations is desirable, but only of marginal importance for determining the species status of parapatric populations. Third, it is similarly not necessary to extensively document the genetic variability of all species in a species complex. Instead, it is sufficient for deciding on species status to document whether there is a significant difference between the populations. This can be accomplished with very little data as long as the few sequences known for a rare "species" fall outside of the genetic variability of those species that are more common and whose genetic diversity can be more completely documented.

We here use a taxonomic problem within the louse-fly genus *Ornithomya* (Hippoboscidae: Calyptratae: Diptera) to illustrate our points of view. Identifying *Ornithomya* specimens to species is often not very difficult, because discrete morphological characters distinguish most species. The exception is *O. fringillina* and *O. chloropus*, for which taxonomists have only been able to find continuous and/or coloration characters that, depending on opinion, may or may not justify treating these taxa as separate species. Settling taxonomic disagreements in such cases can only be accomplished in two ways. One solution is to scrutinize large numbers of specimens in order to accurately assess those characters that have been proposed as having diagnostic value. A second solution is to explore new character systems that may indicate species boundaries (e.g. DAMGAARD 2005; USENER & COGNATO 2005). In this paper we explore the benefits of applying both methods to the *O. fringillina* and *O. chloropus* problem.

The genus *Ornithomya* currently consists of about 25 species, all of which as adults are obligate, blood-sucking ectoparasites on birds. The four species occurring in Northwestern Europe have been studied in detail in Scandinavia (HILL et al. 1964) and Britain (HILL 1962a,b, 1963). However, despite all study it remains controversial whether *O. fringillina* and *O. chloropus* are conspecific (e.g. BEQUAERT 1954; BEQUAERT & LECLERCQ 1947; JOHNSEN 1948) or distinct (e.g. HILL 1962a, 1964; HUTSON 1984; THEODOR & OLDROYD 1964). The two nominal species have almost identical habitus, but four morphological characters have been proposed for separation (Tab. 1). These are: (a) wing length (*O. fringillina*: 3.5–4.5 mm; *O. chlo-*

ropus: 4.5–5.5 mm); (b) number of scutellar bristles (*O. fringillina*: 4; *O. chloropus*: 6); (c) markings on the ventral side of the head (*O. fringillina*: absent or small; *O. chloropus*: present, reaching jugular bristles); and (d) shape and size of the setulose area on the wing membrane (*O. fringillina*: less setose; *O. chloropus*: more setose; HILL et al. 1964; HUTSON 1984).

The two nominal species are parapatric (HILL 1962b). *Ornithomya chloropus* has a more northern distribution than *O. fringillina* with both species occurring sympatrically in Denmark. As the flies are ectoparasites of birds, one possible isolation mechanism could be differences in host choice. However, although there is a tendency for *O. chloropus* and *O. fringillina* to infest different species of birds (HILL 1962b), some hosts, mainly passerines, are shared. One notable difference in the life history of the two species is the duration of the pupal stage under laboratory conditions. The mean duration for *O. fringillina* is 271 days, but 371 days for *O. chloropus* (HILL 1963).

Here, we test the validity of the proposed diagnostic morphological characters and collect new DNA sequence data (cytochrome c oxidase subunit I, COI) from specimens collected from sympatric populations.

Materials and methods

We studied 13 specimens that were collected during bird-ringing campaigns conducted in Denmark in August 2001, 2002 and 2003. Specimens were removed manually from their hosts and placed immediately in individual vials containing 96% alcohol. These campaigns yielded 13 specimens from eight Danish localities and three host species (Tab. 1). For all specimens we examined the diagnostic, morphological characters from two identification keys that are applicable to the faunas of Great Britain, Fennoscandia and Denmark (HILL et al. 1964; HUTSON 1984). In addition, we sequenced approximately 830 bp from the 3´half of the mitochondrial gene encoding *cytochrome c oxidase subunit I* (COI), corresponding to position 2184–3013 in *Drosophila yakuba* Burla (GenBank accession no. NP006902). The primers used for PCR-amplification and cycle sequencing were C1-J-2183 and TL2-N-3014 (see SIMON et al. 1994). DNA was extracted from legs and coxae using the QiaAmp tissue kit protocol (QIAGEN Inc., Santa Clara, California) and eluted in 200 µl AE buffer using a single centrifugation step. PCR amplification and cycle sequencing was carried out as described in DAMGAARD & SPERLING (2001). The sequences were edited and aligned in Sequencher (Gene Codes Corporation, Ann Arbor, Michigan), the alignment was indel-free, and uncorrected genetic distances were calculated using PAUP* (SWOFFORD 2004).

Results

Of the 13 specimens studied, the published keys immediately identified one specimen as *O. fringillina* (fr11) and two specimens as *O. chloropus* (ch1, ch2) based on all four morphological characters (see Tab. 1). The remaining ten specimens (fr1 through fr10) could not be unambiguously determined based on the identification keys. Most characters from the key have continuous alternatives in the couplets and we found that only the markings on the gena is discontinuous and allowed for an assignment of the specimens to either *O. fringillina* or *O. chloropus*. The remaining characters were inconclusive: The number of bristles varied from 2 to 6 or from 4 to even 8 depending on whether weak bristles were counted. Six specimens had the correct number for *O. fringillina* (4: fr1, fr4, fr7, fr8, fr10, fr11) and two had the correct number for *O. chloropus* (6). Figures 1 and 2 demonstrate the variability of the aforementioned characters. Five specimens had the correct wing lengths for *O. fringillina*, while the other six specimens fell into the range for *O. chloropus* (fr1, fr3, fr5, fr6, fr7, fr10). Seven specimens had the size and shape of the setose area on the wing that is supposed to be diagnostic for *O. fringillina*, while the other four were either intermediate or corresponded to the pattern described for *O. chloropus*.

Leading and trailing edges of the sequences for the 13 specimens were pruned and the rest of the sequences unambiguously aligned by eye (810 bp). Based on this alignment 68 nucleotide sites were variable. Of these, 61 substitutions (54% transitions) were unique to the two specimens assigned to *O. chloropus*, 5 were in the 1st position, 1 in the 2nd and 55 in the 3rd position. 7 substitutions (100% transitions) were shared between *O. chloropus* and one or more of the *O. fringillina* specimens, three of these were in the 1st position and 4 were in the 3rd position. When translated to amino acids using MacClade 4.03 (MADDISON & MADDISON 2001) and the *Drosophila* mtDNA code, *O. chloropus* had two unique amino acid changes relative to the amino acid sequence for *O. fringillina*. One individual of *O. chloropus* had a unique amino acid substitution. GenBank accession numbers are given in Table 1. Table 2 summarizes the genetic distances between the sequenced specimens. Specimens fr1 through fr11 are regarded as *O. fringillina* and ch1 and ch2 as *O. chloropus*. Variability within *O. fringillina* ranges from 0% to 0.4%. Variability within *O. chloropus* was 0.3%. However, variability between *O. chloropus* and *O. fringillina* ranged from 8.0% to 8.7%. These values were then compared to the distribution of intraspecific and interspecific values published for more than 1300 Diptera sequences (MEIER et al. 2006)

Discussion

1. The *O. fringillina*/*chloropus* problem

We had outlined that there are two general approaches to resolving a particular taxonomic problem. One was based on studying additional specimens and the other based on studying a different character system. With regard to the *Ornithomya fringillina* and *O. chloropus* problem the former approach yields mixed results. Of the four morphological characters previously used to discriminate the two species, only the markings on the ventral side of the gena provide a discontinuous character separating specimens into two groups, which could tentatively be regarded as *O. fringillina* and *O. chloropus*. Unfortunately the markings fade with time and thus this character is unreliable for old and ethanol-preserved specimens. The number of scutellar bristles at first appears to be a good character, but upon close scrutiny it exhibits continuous variation as some specimens have five (HILL et al. 1964) instead of the normal four or six bristles (see Fig. 2). In addition it is unclear whether weak bristles should be counted and if not how they could be distinguished from strong bristles. Wing lengths are similarly continuous across the 13 specimens that we collected and the exact size of the setose area on the wings is very difficult to determine objectively. Based on the genal marking, one may thus suspect that *O. fringillina* and *O. chloropus* are indeed two species. However, at least some taxonomists may consider coloration differences insufficient to justify this conclusion although the lack of specimens with intermediate conditions for this character suggests that the populations are not interbreeding.

Fortunately, the second approach to solving the taxonomic problem based on sampling a new character system yields more conclusive results. The COI sequences for the 13 specimens clearly cluster into two different groups. These two clusters correspond to the clusters that are also delimited by the only morphological character with a discontinuous distribution (gena marking). Both kinds of data thus point to the conclusion that *Ornithomya fringillina* and *O. chloropus* are two different species. The genetic variability within the species is relatively low (<1%), while the interspecific variability exceeds 8%. Such large genetic distances are very unusual within species. A survey of COI sequences in Diptera by MEIER et al. (2006) revealed that 95% of all intraspecific variability was below 2.31% and that the probability of observing an 8% difference within a species was below 0.7%. Thus, the best explanation for the observed differences between *O. fringillina* and *O. chloropus* is that they are separate species. A recent phylogenetic analysis of Hippoboscoidea furthermore revealed that *O. fringillina* and *O. chloropus* are not even sistergroups (PETERSEN et al. 2007) thus implying that the morphological similarities between the species are due to unusually slow morphological evolution. With regard to COI, PETERSEN et al. (2007) established that for *O. fringillina* the closest distances were observed for *O. biloba* (5.9%) and *O. avicularia* (6.2%) while *O. chloropus* is only the third best match. Interestingly, both *O. avicularia* and *O. biloba* are widely distributed within Europe and thus parapatric with *O. fringillina* and *O. chloropus*. Yet, there is no doubt about their status as valid species.

2. How many sequences are needed to solve a taxonomic problem?

In the Introduction we raised the question whether a small number of sequences can yield sufficient information for influencing taxonomic issues. Our study is based on only 13 DNA sequences. Yet, we would argue that the evidence generated by these sequences is significant enough to unambiguously support that *O. fringillina* and *O. chloropus* are two separate species (see also MEMON et al. 2006). In sequencing only 13 specimens we deviate from standard approaches to DNA taxonomy in that we only generated a fraction of the molecular data that is normally produced. The reason is not that we consider additional data useless. Instead, we strongly believe that the use of DNA sequences in routine taxonomic research can only become common if time- and cost-effective approaches can be embraced, and taxa with few known specimens can also be covered. Ultimately, this requires that the number of specimens that are sequenced is kept relatively low. Proponents of DNA barcoding originally even proposed a single sequence per species (HEBERT et al. 2003). But we are here arguing that this one-sequence-fits-all-species approach is undesirable, because there are more frugal sampling schemes that can yield more useful information for solving existing taxonomic problems. We recommend that study specimens are selected according to the following three criteria:

2.1. Focus on species with taxonomic problems

Most *Ornithomya* species can be distinguished based on morphological characters. We thus strongly believe that a first-stop approach to using DNA sequences in taxonomy should focus on those species and species-complexes that cannot be resolved with existing data. In our case, there was a need to focus on *O. fringillina* and *O. chloropus*. Our approach to using DNA sequences differs from DNA barcoding in that it

Tab. 1. Locality information, GenBank accession number, host data, and morphological information on the specimens examined. All listed localities are in Denmark.

Specimen	Species	Host	Locality	Bristles on scutellum	Markings on gena	Wing length	Setulae on wing
fr1: DQ217755	*O. fringillina*	*Passer montanus*	Store Dalby	2 + 2 weak	fringillina-type	4.8	fringillina-type
fr2: DQ217765	*O. fringillina*	*Passer montanus*	Vipperød	4 + 2 weak	fringillina-type	4.5	chloropus-type
fr3: DQ217756	*O. fringillina*	*Passer montanus*	Uldum	4 + 1 weak	fringillina-type	4.7	intermediate
fr4: DQ217757	*O. fringillina*	*Passer montanus*	Hesselballe	2 + 2 weak	fringillina-type	4.5	intermediate
fr5: DQ217758	*O. fringillina*	*Passer montanus*	Fåborg	4 + 1 weak	fringillina-type	4.7	fringillina-type
fr6: DQ217759	*O. fringillina*	*Emberiza citrinella*	Kornum	4 + 2 weak	fringillina-type	4.9	fringillina-type
fr7: DQ217760	*O. fringillina*	*Emberiza citrinella*	Kornum	4	fringillina-type	4.8	fringillina-type
fr8: DQ217761	*O. fringillina*	*Sylvia communis*	Kornum	4	fringillina-type	4.4	intermediate
fr9: DQ217762	*O. fringillina*	*Sylvia communis*	Kornum	4 + 2 weak	fringillina-type	4	fringillina-type
fr10: DQ217763	*O. fringillina*	*Passer montanus*	Hinnerup	4	fringillina-type	5	fringillina-type
fr11: DQ217764	*O. fringillina*	*Passer montanus*	Hinnerup	4	fringillina-type	4.4	fringillina-type
ch1: DQ217766	*O. chloropus*	*Passer montanus*	Kornum	6 + 2 weak	chloropus-type	5.3	chloropus-type
ch2: DQ217767	*O. chloropus*	*Passer montanus*	Kornum	6 + 2 weak	chloropus-type	5.2	chloropus-type

Tab. 2. Uncorrected genetic distances for COI for the specimens. fr1 through fr11 are *Ornithomya fringillina* and ch1 and ch2 are *Ornithomya chloropus*.

	fr1	fr2	fr3	fr4	fr5	fr6	fr7	fr8	fr9	fr10	fr11	ch1
fr1												
fr2	0%											
fr3	0.4%	0.4%										
fr4	0.4%	0.4%	0.3%									
fr5	0.3%	0.3%	0.1%	0.1%								
fr6	0.4%	0.4%	0.3%	0.3%	0.1%							
fr7	0.3%	0.3%	0.1%	0.1%	0%	0.1%						
fr8	0.3%	0.3%	0.1%	0.1%	0%	0.1%	0%					
fr9	0.4%	0.4%	0%	0.3%	0.1%	0.3%	0.1%	0.1%				
fr10	0.3%	0.3%	0.1%	0.1%	0%	0.1%	0%	0%	0.1%			
fr11	0.1%	0.1%	0.3%	0.3%	0.1%	0.3%	0.1%	0.1%	0.3%	0.1%		
ch1	8.4%	8.4%	8.3%	8.3%	8.2%	8.0%	8.2%	8.2%	8.3%	8.2%	8.3%	
ch2	8.7%	8.7%	8.6%	8.6%	8.4%	8.3%	8.4%	8.4%	8.6%	8.4%	8.6%	0.3%

appears unnecessary to us to target "uncontroversial" species for molecular work. Note also that a standard DNA barcoding approach to the genus *Ornithomya* would have required one sequence per species and thus almost twice as many sequences as used here (25 instead of 13). Yet, it would have yielded much less information for resolving a real taxonomic issue in *Ornithomya*. Our approach to using DNA sequences is also different from approaches to DNA taxonomy. DNA taxonomists would have collected and/or sampled specimens for multiple species throughout their ranges. This is both expensive and time-consuming and we find such procedures difficult to justify for routine investigations. Although such sampling could potentially reveal additional phenomena such as putatively cryptic species (Bickford et al. 2007).

2.2. Focus on sympatric populations

Many species concepts require that taxonomists assess whether two specimens come from the same or two

different populations (e.g., Biological Species Concept: MAYR 2000; Phylogenetic Species Concept *sensu* Wheeler & Platnick: WHEELER & PLATNICK 2000; Hennigian Species Concept: MEIER & WILLMANN 2000). Such data can be collected for species with sympatric or parapatric populations. However, the specimens have to originate from areas of sympatry. Studying the genetic diversity of species across their entire range may be of phylogeographic interest, but it yields little useful data for resolving species-level problems. With regard to *O. fringillina* and *O. chloropus*, the critical data had to come from a country like Denmark where both species can be found on the same host and at the same locality. Unfortunately, the maternally inherited COI is not the best choice for directly assessing gene flow (WILL & RUBINOFF 2004). However, in our case, standard population genetic models incorporating lineage sorting and genetic drift predict that it is very unlikely that one population of a species has the kind of large genetic variability that we observed for *O. fringillina* and *O. chloropus* (MOORE 1995).

2.3. Species complexes with rare and common species

Forty percent of all beetle species have only been collected once (see SEBERG 2004); i.e., there is little use for DNA sequences in taxonomy if collecting DNA sequences is only useful once numerous specimens for each species are sampled. But fortunately, such extensive sampling may not be needed. It is quite common that one of the species involved in a taxonomic problem is rare while the other(s) are common. Under this circumstance, we would argue that it is sufficient to properly assess the genetic diversity of the common species and use whatever little data are available for the rare species to test whether its sequences fall within the range observed for the common species. This is clearly not the case for *O. chloropus* and we can thus conclude that *O. fringillina* and *O. chloropus* are unlikely to be conspecific.

Conclusions

Our use of DNA sequences differs from other approaches in that the sequence information is used as additional evidence (DAYRAT 2005; ROE & SPERLING 2007; WILL et al. 2005). We are using existing morphological and distributional information for selecting those specimens that are most likely to contribute to the understanding of species boundaries within a given taxonomic group with a taxonomic problem. Furthermore, all data are evaluated simultaneously; i.e., our research is rooted in the tradition of an integrative taxonomy that uses all available data.

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