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Using DNA-barcoding to make the necrobiont beetle family Cholevidae accessible for forensic entomology

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ABSTRACT

The beetle family Cholevidae (Coleoptera: Staphylinoidea), sometimes viewed as the subfamily Cholevinae of the Leiodidae, consists of some 1700 species worldwide. With the exception of specialized cave-dwelling species and species living in bird and mammal nests and burrows, the species are generalized soil-dwellers that, at least in temperate regions, are mostly found on vertebrate cadavers. Although they have been regularly reported from human corpses, and offer potential because of many species' peak activity in the cold season, they have not been a focus of forensic entomologists so far. This is probably due to their small size and the difficulty in identifying the adults and their larvae. In this paper, we show that DNA-barcoding can help make this group of necrobiont beetles available as a tool for forensic research. We collected 86 specimens of 20 species of the genera *Catops*, *Fissocatops*, *Apocatops*, *Choleva*, *Nargus*, *Ptomaphagus*, and *Sciodrepoides* from the Netherlands and France and show that a broad "barcoding gap" allows almost all species to be easily and unambiguously identified by the sequence of the "barcoding gene" cytochrome *c* oxidase I (COI). This opens up the possibility of adding Cholevidae to the set of insect taxa routinely used in forensic entomology.

1. Introduction

Exposed vertebrate cadavers, including human ones, form a transient, but nutrient-rich food source that is exploited by many groups of invertebrate animals, especially insects. Numerous ecological studies (e.g., [1–5]) have investigated the abundance, species diversity, succession, and ecological interactions of insects on decaying cadavers. These studies have shown that the ecological preferences of many species are very narrowly circumscribed and that the insect community changes continually and in predictable ways. Hence, the composition of species and in particular their life-stages on a corpse allows forensic entomologists to make accurate pronouncements on the Postmortem Interval (PMI) and on other events surrounding the body since death [6]. Despite the increased popularity of forensic entomology, the potential of the insect assemblages on corpses has not been completely tapped. For example, although the carrion-inhabiting fauna includes members from a wide variety of arthropod taxa (e.g., Diptera, Coleoptera, Hymenoptera, Lepidoptera, and Acari [7]), cases where entomological evidence contributes to death investigations almost exclusively involve Diptera [8,9]. Coleoptera, for example, are much less popular, despite the fact that they are taxonomically and ecologically more diverse than Diptera. Among the Coleoptera inhabiting corpses are scavengers (e.g., Ptiliidae, Dermestidae) as well as predators (e.g., Histeridae and certain Silphidae), and groups that are specialized on early (e.g., *Nicrophorus* silphids), middle (e.g., many Staphylinidae), and late (e.g., Dermestidae) stages of decomposition [5,8,9]. Thus, potentially Coleoptera include species that could yield forensic information complementary to that obtained from Diptera. The reasons for the relative rarity of Coleoptera and many other necrobiont insect groups in forensic entomological practice probably include their taxonomic inaccessibility, especially where species-rich and small-bodied groups are concerned [10].

One such group is the family Cholevidae. These staphylinoid beetles, sometimes viewed as a subfamily (Cholevinae) of the

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Leiodidae, comprise some 1700 species world-wide [11]. All are small (mostly 2–6 mm in length), ovoid in body shape, brown, grey or black, and as adults distinguishable only by subtle differences in the proportions of antennal articles, pronotal shape, and the genitalia [12]; identification of the larvae is equally cumbersome (Zwick, personal communication). Although they include some groups that are highly derived cave-dwellers (such as the subfamily Leptodirinae) or specialised inhabitants of mammal nests and burrows (e.g., the genus *Choleva*), the majority of the species are found above-ground on decaying matter, most commonly animal cadavers, where they probably feed predominantly on fungal spores [13,14]. In temperate regions, they are among the dominant insect groups found on animal carcasses, both above-ground [5] and buried [15]. E.g., Kočárek [16] collected 586 specimens on beef heart in the Czech Republic, making them (in that study) the third most numerous beetle family after Silphidae and Staphylinidae. Also in the Czech Republic, Růžička [17] collected 8903 cholevid specimens on rotten fish, by which they were as common as silphid burying and carrion beetles, and in a Polish study [18], a cholevid species was by far the commonest on dead fish bait, almost twice as common as the next-ranking beetle, and overall, cholevids made up about one-quarter of the total (29,088 individuals). Cholevid larvae are also regularly found on human corpses. For example, Easton and Smith [19] found *Catops tristis* on a human corpse; Lefèbvre and Gaudry [20] in reviewing French forensic entomology cases, report four cases where Cholevidae were collected, although it is likely that they were reported under the old family name Silphidae in other cases; the Alaskan species *Catoptrichus frankenhaeuseri* was even first discovered on a human cadaver, in 1852 [21]; and forensic entomological specimens collected in the Netherlands during police investigations also regularly include Cholevidae: out of 25 cases with coleopterans present, 20% (all of which collected during winter in rural settings) contained cholevid larvae (J. Huijbregts, personal communication). Nevertheless, they do not figure prominently in the forensic literature, which is all the more surprising in view of their phenology: many species reproduce in autumn and complete their larval development during winter [22]. This means that cholevids are among the few insects that feed on corpses during the cold season, when most dipteran groups are absent, and hence could be a valuable tool for determining PMI in cold-season death investigations.

To help stimulate the use of Cholevidae in forensic investigations in the Netherlands, we have begun compiling a database on distribution, niche preferences, life cycle, and identification characteristics for the Dutch species. Because correct identification of both larvae and adults requires investigation of minute and often internal morphological characters by a specialist, we here report on the use of DNA-barcodes for the identification of Dutch cholevid species. Animal DNA-barcoding relies on the property of animal mitochondrial DNA (mtDNA) variation to coalesce rapidly during evolution [23]; much more rapidly, usually, than the time required for the evolutionary splitting of species. This means that mtDNA variants tend to be contained within species and are usually not shared between species [24]. This "barcoding gap" allows mtDNA sequences to be used reliably for the identification of species [25]. The gene of choice for DNA-barcoding is *cytochrome c oxidase I* (COI), and although problems remain [25–28], DNA-barcoding has been shown to be successful for accurate species-level identifications of insects [29,30], including those of forensic importance [31]. Here, we report on a first attempt to generate reliable DNA-barcodes for Dutch Cholevidae. We find that sequencing a 600-bp fragment of COI from 86 individuals allows them to be sorted quite accurately into 20 recognised, but morphologically very similar species.

2. Materials and methods

2.1. Trapping and preparing specimens

We used pitfall traps [32] to collect live specimens of Cholevidae. Glass jars were filled with a 3-cm-thick sand layer on which either a piece of chicken or a piece of Limburg or Münster cheese (or both) was placed. These cheese types are common substitutes for decaying meat [33], since they exude a similar mixture of volatiles, and can be used instead of meat if time in the field is limited and it would take too long for meat to have reached the appropriate stage of decay (especially during cold weather). The jar was covered with an iron lid into which 20–30 holes of 6 mm diameter had been punched (to prevent larger animals from accessing the bait) and buried in the soil to such a depth that the ground level was flush with the top of the lid. A plastic roof was placed 10 cm above the trap to prevent rain water from entering. Five traps were placed at each of nine localities in the Netherlands: oak forest on sandy soil at "Noordberg", Heelsum, Prov. Gelderland (51.58°N, 5.44°E); pine forest on sandy soil at "De Sysselt", Ede, Prov. Gelderland (52.02°N, 5.40°E); alder-willow woods on clay-peat soil in the polders west of IJsselstein, Prov. Utrecht

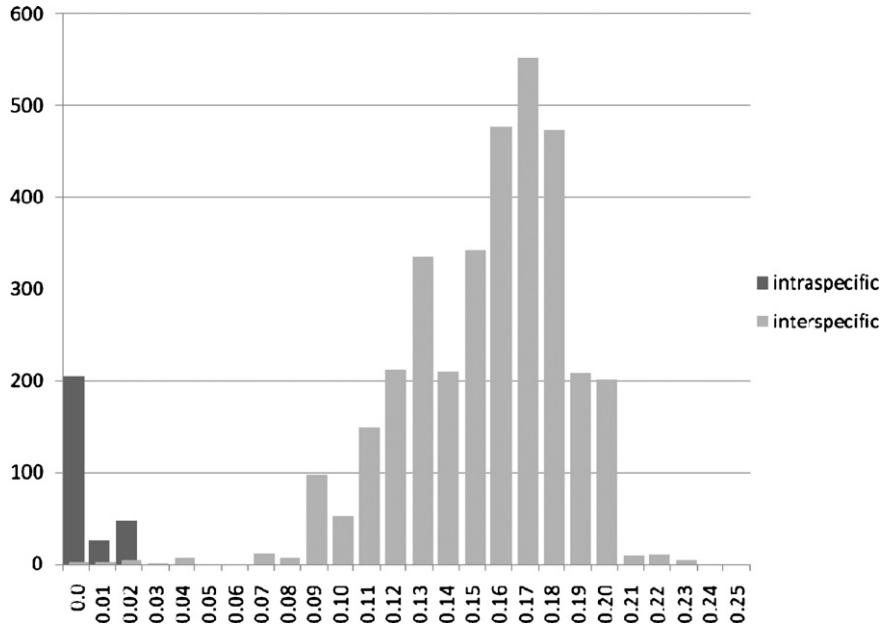

Fig. 1. Distribution of pairwise Kimura 2-parameter genetic distances between COI-sequences, revealing a distinct "barcoding gap".

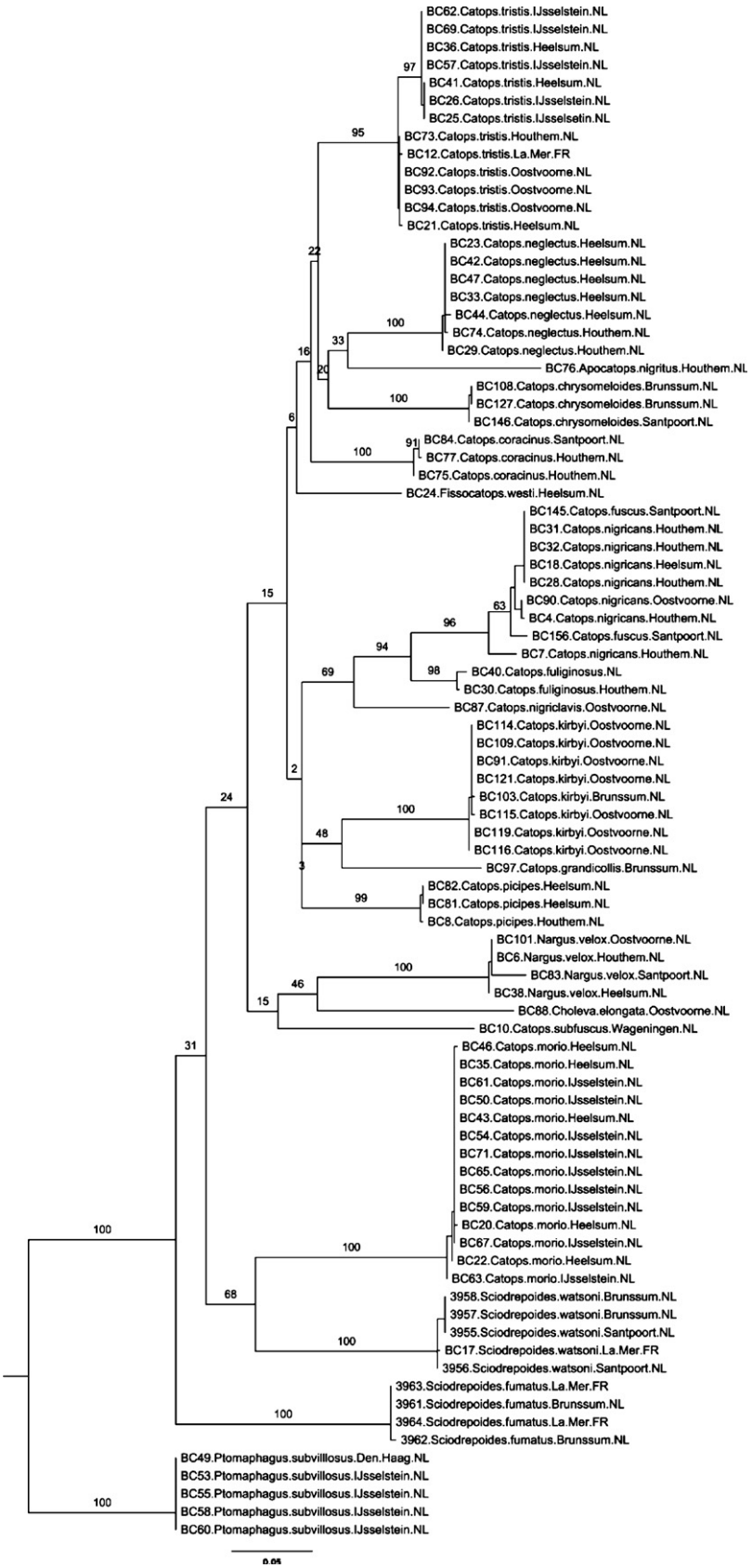

Fig. 2. Maximum likelihood phylogenetic reconstruction for the COI sequences. Bootstrap values are indicated on the branches (bootstrap values on intraspecific branches have only been given in selected cases).

(52.01°N, 5.05°E); deciduous forest on sandy soil in the dunes north of The Hague, Prov. Zuid-Holland (52.12°N, 4.30°E); oak/beech forest on sandy soil at "Mildenburg", Oostvoorne, Prov. Zuid-Holland (51.91°N, 4.08°E); low hawthorn and seabuckthorn vegetation at the shore of the "Oostvoornse Meer", Oostvoorne, Prov. Zuid-Holland (51.92°N, 4.07°E); moorland with birch woods on löss near Brunssum, Prov. Limburg (50.93°N, 6.00°E); old oak forest on sandy soil at "Midden-Heerenduin", Santpoort-Noord, Prov. Noord-Holland (52.44°N, 4.63°E); and oak/beech forest on löss soil on the north-facing slope of the Geul river in Houthem, Prov. Limburg (50.87°N, 5.79°E). The traps were maintained in the months of February and March 2009 (Heelsum, IJsselstein, Ede, Den Haag, Houthem) and 2010 (Oostvoorne, Brunssum, Santpoort) and emptied weekly. Captured cholevid specimens were placed in labeled vials with 100% ethanol immediately. In addition, we used specimens collected earlier in a similar manner in Heelsum in 1997, in Wageningen (Prov. Gelderland; 51.97°N, 5.67°E) in 1997, and in Faucogney-et-la-Mer (Vosges, France; 47.85°N, 6.57°E) on 10 May 1999, and specimens collected in winter nests of moles (*Talpa europaea*) near Oostvoorne (51.88°N, 4.06°E), in February 2010. For each specimen, the sex was determined based on the protarsal width [12]. From all male individuals, while remaining in 100% ethanol, the aedeagus was extracted and the species was identified with the keys in Schilthuizen and Vallenduuk [32]. Then, part of the abdomen and the legs on the left-hand side of the body were removed and stored in 100% ethanol. These tissues were used for further DNA work, while the rest of the specimen and its aedeagus were dry-mounted, labeled, and stored in the collection of museum Naturalis as voucher. Voucher specimens were provided with sample IDs BC-1–BC-156, collection reference numbers RMNH.INS63078–RMNH.INS63155, RMNH.INS63184–RMNH.INS63284, and photographed. These IDs and collection reference numbers were also used for the subsequent DNA-extracts, PCR-products, and sequences.

2.2. DNA extraction, PCR-amplification and sequencing

We used the QIAGEN DNeasy Blood & Tissue kit and followed manufacturer's instructions for DNA extraction. Two μl of each extract was used for determining DNA concentration and purity in a NanoDrop Bioanalyser. Thermocycling was carried out with the COI-primers L1490 (named LCO1490 on www.barcodinglife.org), GGTCAACAAATCATAAAGATATTGG and H2198 (named HCO2198 in www.barcodinglife.org), TAAACTTCAGGGTGACCAAAAAATCA of Folmer et al. [34] (and, in a few cases where L1490 and H2198 failed to amplify a product, with the internal primers 'Ron', GGATCACCTGATATAGCATTCCC, and 'Nancy', CCCGGTAAAATTAAAATATAAACTTC [35]), an annealing temperature of 50 °C, 40 cycles, and otherwise standard conditions. PCR-products were cleaned using the Promega-kit 'Gel and PCR Clean-Up System', and direct-sequenced on an ABI 3730 automated sequencer by Macrogen Corp. (Korea), using the PCR-primers as sequencing primers.

2.3. Sequence analysis

Sequences were assembled and edited for obvious reading errors in Sequencher 4.8 (Gene Codes Corp., Ann Arbor) and then aligned manually in BioEdit [36]. They were submitted to the Barcoding of Life Database (www.barcodinglife.org) under the project CHOLE ("Cholevinae of Forensic Importance") and are publicly available. Using Akaike's Information Criterion [37], the most appropriate nucleotide substitution model was determined among 88 candidate models in jModeltest 0.1.1 [38] (after removal of sequences with missing data). A maximum-likelihood phylogenetic tree was then reconstructed in PhyML 3.0 [39] using the selected substitution model (GTR) and 100 non-parametric bootstrap replicates. The phylogenetic tree was edited in FigTree 1.3.1 [40]. Intra- and interspecific variability was assessed by inspecting all pairwise Kimura's 2-parameter genetic distances using DNADIST 3.5c [41].

3. Results

We obtained 86 male individuals that could be unambiguously identified (by the first author) by their genitalia as belonging to altogether 20 species, including several species considered rare in the Netherlands, such as *Fissocatops westi*, *Catops neglectus*, and *Catops grandicollis*. After removal of bases on both the 5′ and the 3′ end because of poor trace readability and combination of forward and reverse sequences of all PCR-products, we obtained an alignment of 86 sequences. Of these, 80 had a full length of 600 bp, while six (BC-30, BC-49, BC-53, BC-55, BC-58, and BC-60) for which the internal primers Ron and Nancy had been used, were 394 bp long. In three sequences (BC-17, BC-46, and BC-67), between 19, 26, and 15 unknown nucleotides needed to be introduced, respectively, because of low signal and/or reading ambiguities. Fig. 1 shows the "barcoding gap" in the plot of genetic distances in all pair-wise sequence comparisons, differentiated for intra- and interspecific distances. Fig. 2 shows the maximum-likelihood tree (log likelihood = −4420.06), rooted with *Ptomaphagus subvillosus*, the only member of the subfamily Ptomaphaginae in the data set (all other species are Cholevinae). All species for which more than one sequence was available, form monophyletic groups, supported by bootstrap values between 95 and 100, with the exception of the sequences of the two species *Catops nigricans* and *Catops fuscus*, which are mixed within the same clade.

4. Discussion

Our results, which comprise the first DNA-barcoding study of cholevid beetles, show that COI sequences generally allow accurate identification of species in this family. Genetic distances between species are large (for the most part 9% or more), whereas within species they are small (4% or less). This "barcoding gap" (Fig. 1) is borne out in the phylogenetic reconstruction, which shows the grouping, with mostly high confidence (bootstrap values of 95% or more), of all individuals of the same species. Similar good barcoding accuracy was reported for certain other insect groups (e.g., [29]). However, we note that one species pair in our sample is not separable: *C. nigricans* and *C. fuscus*. Although taxonomically related, these two species are morphologically well-characterised and easily distinguishable [12], so it is not likely that they are in fact conspecific. Therefore, the COI results will need confirmation with nuclear markers. Despite this inability for the barcoding region to separate these particular two species, overall the initial results hold promise for successful DNA-based identification of these necrobiont beetles.

In two species did we find relatively deep intraspecific divergences: the sequences of *Catops tristis* form two clades that are up to 3.4% genetically diverged. Similarly, one sequence of *C. nigricans* from Houthem is separated from other conspecific sequences, including ones from the same locality, by a genetic distance of up to 3.7%. In other insects, such genetic distances are more often found associated with divergences *between* closely related species (e.g., [42]), and it is not impossible that these individuals do in fact belong to sibling, unrecognized species. Initial morphological inspection of the voucher specimens and their genitalia revealed no obvious differences, but this warrants further study. Alternatively, ancient polymorphisms may have been preserved in these species.

The common observation that the relatively high evolutionary rate of COI makes the gene less useful for obtaining phylogenetic resolution at the deeper taxonomic levels, is borne out by our phylogenetic reconstruction: very few groupings of two or more species are supported by substantial bootstrap values. Only the three species *C. nigricans*, *C. fuscus*, and *C. fuliginosus* (all members of the "*fuscus*-group" of Jeannel [12]) form a well supported clade. The generic taxonomy of the Cholevidae clearly requires further DNA-based study, for which more slowly evolving genes may prove useful.

In conclusion, we would like to emphasise that DNA-barcoding holds promise for the use of Cholevidae in forensic science. The initial data suggest that accurate DNA-based identification of these species, otherwise almost unidentifiable by the non-specialist, is relatively straightforward. This will facilitate the use of information derived from these beetles in death investigations. However, effective use would require an expert system of integrated information where species names and their DNA barcodes are coupled to data on life cycle, phenology, niche preferences, and geographic distributions, as are already available for some dipteran families [9]. The present study is a first step towards the development of such a system. Future work should expand the set of species and the intraspecific sampling, and should enhance

the already available data (e.g., [27,43,44]) on their ecology and life history.

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