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Connecting systematic and ecological studies using DNA barcoding in a population survey of Drosophilidae (Diptera) from Mt Oku (Cameroon)

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Abstract. The characters used in taxonomy to describe new species cannot always be used to identify species in population surveys involving large samples. We used DNA barcoding to validate the taxonomic status of the morphospecies used in an ecological study involving 11 000 individual African drosophilids which had been determined without dissection. Some taxonomic information had been lost by not discriminating between rare species or by mistakenly splitting a morphologically variable species into two groups. However, the original ecological dataset provided a reliable picture of species diversity and the conclusions based on the original dataset are still supported by the molecular data.

Keywords. Barcode, *Drosophila, Zaprionus*, *Sophophora*, Cameroon.

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Introduction

Reliable procedures to evaluate species diversity are required given the global loss of biodiversity and its consequences for environmental policies. However, environmental studies generally need datasets involving samples containing large numbers of individuals in which species cannot always be determined using the wealth of information provided by taxonomists when describing new taxa, since their methods cannot routinely be applied to a large number of specimens. This occurs frequently in insects, for example in beetles (Oliver & Beattie 1996), mayflies (Bauernfeind & Moog 2000) and chironomids (Carew *et al.* 2013), in which species often need to be distinguished using cryptic characters like male genitalia and internal parts observed through dissection. This is especially challenging in tropical environments

in which many species remain to be described. As a compromise, environmental studies often use operational taxonomic units (OTU) at a lower resolution than the species level, and assume they are sufficiently informative for analysis. These OTUs retain biological meaning as long as each consists of a monophyletic unit containing species sharing morphological, physiological and behavioral characters. The accepted risk is a loss of resolution of the study, since two related species can diverge in a number of characters. This approximation cannot, however, be assessed as long as the ability of the researcher to recognize species has not been evaluated using molecular analysis, inter-specific crosses or examination of museum types.

A useful tool to clarify this point is DNA barcoding, which was designed as a reliable technique for identifying species (Hebert *et al*. 2003; Monaghan *et al*. 2005; Janzen *et al*. 2009; Lukhtanov *et al*. 2009) and detect cryptic species (Hebert *et al*. 2004; Yassin *et al*. 2008). It may also be used for biodiversity assessment in groups where taxonomic information is incomplete or difficult to use (Blaxter *et al*. 2005; Passmore *et al*. 2006; Frezal & Leblois 2008) or for large inventories (Telfer *et al*. 2015).

Here we apply DNA barcoding to a sample derived from a study carried out on a drosophilid community from the Central African montane forest of Mt Oku in Cameroon (Prigent *et al*. 2013). Drosophilids, an important model in evolutionary biology (Powell 1997; Ashburner *et al*. 2004; Markow & O'Grady 2005), are also considered a useful marker in ecosystem dynamics studies (Parsons 1991; Davis & Jones 1994; Mata *et al*. 2008). They cover a large diversity of habitats, and species assemblages vary according to environmental changes in time and space (Krijger & Sevenster 2001; Avondet *et al*. 2003; Tidon 2006; Prigent *et al*. 2013). Standardized traps can be used to collect them efficiently in large numbers.

The study took place from October 2008 to October 2009, in the montane forest of Mt Oku, on the Cameroon volcanic line (Prigent *et al*. 2013). About 11 000 specimens were caught using banana traps over a year at different altitudes between 2200 m and 2800 m, and preserved in 70% ethanol until further determination. Drosophilid identification involves a large number of characters including relative size and shape of body parts, color pattern, wing venation, chaetotaxy and form of the male genitalia. The male genitalia are, in fact, the main structure used to discriminate between taxa. These minute organs usually need to be dissected and are often difficult to examine in ethanol-fixed individuals. Also, in a large survey it is not logistically feasible to dissect every specimen. Moreover, several hundred species are known in Africa (537 valid species according to the TaxoDros database; www.taxodros.uzh.ch) and species were described with varying precision. Old descriptions often lack detail (Coquillett 1902; Adams 1905; Séguy 1933). Some revisions of African taxa include new descriptions for formerly described species within a group (Tsacas 1972, 1980a), but no comprehensive comparative survey is available for the complete African fauna. The most important works providing a key were carried out on the drosophilid faunas of Ivory Coast (Burla 1954b), South Africa (Tsacas 1990) and Malawi (Chassagnard *et al*. 1997). Identification remains difficult, since *Drosophila* includes several species complexes, each containing morphologically similar species (Tsacas 1980b; Tsacas & Lachaise 1981; Tsacas & Chassagnard 1994; Tsacas 2002).

The material was therefore sorted on the basis of visible characters. Morphospecies (i.e., OTUs) were then defined as groups that could be separated from other groups by their external morphology. Determining females is a real challenge given the prevalent use of male genitalia as taxonomic criteria. Females might be expected to share characters with conspecific males. However, coloration patterns are sexually dimorphic in many species and in several species from the very speciose *montium* subgroup (or group, see Da Lage *et al*. 2007), females are dimorphic for abdominal color patterns. Sorting the specimens resulted in 62 recognizable morphospecies that were assigned, where possible, to known species according to published descriptions of external morphology (Prigent *et al*. 2013). Sample size

was highly variable across species. One of them, *Zaprionus vittiger* Coquillett, 1901, made up 81% of the catches, whereas many other species were rare. The spatial and temporal distribution of dominant species was a clear illustration of the high dependence of the drosophilid community on climatic factors. It was therefore essential to assess the taxonomic reliability of this study.

Using DNA barcoding confirmed the correct determination of dominant species. We detected some problems in the correct identification of cryptic species and in the allocation of females to the right species in groups of related species. The delimitation of species remained uncertain in some species due to low divergence between them. Mitochondrial introgression has been reported in some species and thus cannot be excluded in our sample. The conclusions of the environmental study are not modified, given the co-occurrence and low abundance of the species involved in these uncertainties. DNA barcoding actually helped in clarifying temporal or distributional patterns of cryptic species. It should allow comparison with further studies.

Material and methods

We used specimens collected in the framework of the ecological study carried out on Mount Oku (Prigent *et al.* 2013) along with an additional sample, involving the same morphospecies, collected at Koh Kesoten, a locality close to the sampling place. We extracted DNA from 125 individuals using the Qiagen® DNA extraction kit. Whole photographs of the individuals were taken under a Leica M165C binocular stereo microscope with coupled numerical imaging, and the legs, wings and genitalia were kept apart as vouchers. The rest of the body of the flies was used for DNA extraction. Forty additional extractions were made from one leg, allowing us to preserve the rest of the specimen as a voucher. All of the material will be deposited at the Muséum national d'Histoire naturelle (MNHN), Paris, when fully identified. Amplification and sequencing were carried out using already published primer sequences for COI (Bouiges *et al*. 2013). Barcoding was applied to both males and females for each morphospecies, when they were available. Each sequence was blasted against the NCBI sequence database. All available drosophilid COI sequences were downloaded (more than 3700 sequences, including more than 600 species mostly from *Drosophila* and closely affiliated genera), aligned with our data and analyzed using MEGA5.

The molecular phylogeny of Drosophila has been thoroughly studied using a number of markers (e.g., Pelandakis & Solignac 1993; Remsen & O'Grady 2002; Da Lage *et al.* 2007; van der Linde *et al.* 2010), including whole genomes (Drosophila 12 genomes consortium 2007). These studies were searching deep nodes in order to identify the main groups in this very large genus. Their results were of relatively little help to us. There were two reasons for this. First, there was little overlap between the species used in these studies and those from our sample, and thus they could not be used as barcode standards. Second, the purpose of a taxonomic study like ours is not to identify deep nodes but shallow ones. This is a technical issue. Hajibabaei *et al.* (2007) addressed the question of the best method being used depending on the kind of study being carried out. These authors stated that "the analysis of DNA barcoding data is usually performed by a clustering method, such as distance-based neighbor-joining (NJ)", whereas the construction of phylogenetic trees is carried out "by using optimality criteria such as Maximum Likelihood, Maximum Parsimony, or Bayesian analysis". Austerlitz *et al.* (2009) compared several phylogenetic and classification methods in analyses using known experimental datasets or data obtained from simulations using known parameter values. They confirmed the good performance of methods aimed at identifying closely related groups, such as nearest neighbor and neighbor-joining. For this reason, we analyzed our data using neighbor-joining, but we also ran a maximum-likelihood analysis on the same data, using the same software (MEGA5) in order to assess congruency between two methods that are widely divergent in their principles of analysis. We used a Kimura-2-p substitution model for neighbor-joining, and a GTR + G + I model for maximum likelihood.

Results

Sequencing success

We obtained 111 sequences out of 165 specimens (by body or leg DNA extraction). Our success in DNA amplification and sequencing was very heterogeneous across samples, but was not linked to time, seasonality or sample size (Fig. 1). We obtained a COI sequence for 52 of the 62 morphospecies (Table 1). Six of the ten unsuccessful assays were OTUs represented by only one individual. Both male and female specimens were successfully sequenced in 40 morphospecies. Only one sex was available in seven other morphospecies (Prigent *et al*. 2013). In the five remaining morphospecies only one sex was successfully sequenced, although both sexes were available.

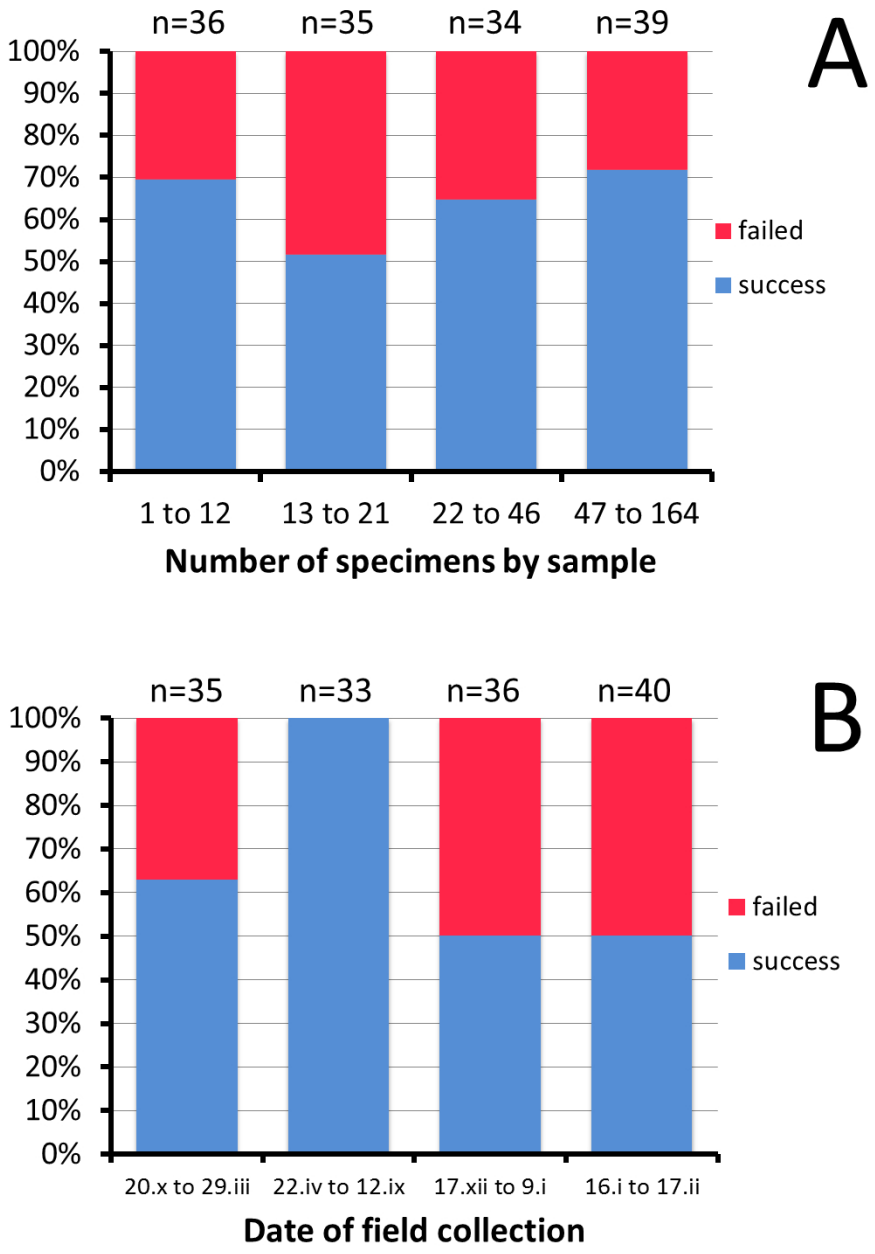

Fig. 1. Percent success and failure in obtaining a COI sequence from specimens. **A**. Total number of flies in the sample. **B**. Date of field collection (month indicated in lowercase Roman numerals). n = number of specimens used.

Table 1. List of the morphospecies recognized in Prigent *et al*. (2013) and their taxonomic interpretation based on DNA barcode analysis. Classification: the position of the taxon in the classification of Drosophilidae; the *loiciana* and the *nutrita* species complexes were established by Tsacas (2002) and Tsacas & Chassagnard (1994), respectively. The *adamsi*, *brachytarsa* and *dyaramankana* species groups are not formally defined; they are simply putative groups according to Prigent *et al.* (2013). Morphospecies: the identification of the taxon given in table 1 of Prigent *et al*. (2013). Closest relative: as determined by blasting against the NCBI database. Percent similarity (% simil.): with the closest relative. Relatedness: with the closest sister taxon or sister group either from the sample or from the NCBI database under neighbor-joining analysis; in parentheses the best bootstrap score observed over several analyses. COI barcode: GenBank accession numbers. n.a. = not available. [continued on next 3 pages]

Classification		Morphospecies	Closest relative	% simil.	Relatedness (bootstrap value)	COI barcode
Genus *Drosophila*						
Subgenus *Dorsilopha*	1	*D. busckii*	*D. busckii*	100	*D. busckii* (100)	KX771077–78
Subgenus *Drosophila*						
immigrans group	2	*D. immigrans*	*D. immigrans*	100	*D. immigrans* (100)	KX771079
loiciana complex	3	*D. allochroa*	*D. falleni*	90	*Scaptodrosophila* (45)	KX771080–81
	4	*D.* aff. *allochroa*	n.a.	n.a.	n.a.	n.a.
nutrita complex	5	*D.* aff. *nutrita*	*D. erecta*	90	"*dyaramankana* group" (34)	KX771082
ungrouped	6	*D.* aff. *nitida*	*D. munda*	91	*quinaria* group (50)	KX771083–84
	7	*Drosophila* sp. 1	*Scaptomyza frustulifera*	90	*D. fraburu* (35)	KX771085–86
"*adamsi* group"	8	*D. adamsi*	*D. punctatonervosa*	92	*D. punctatonervosa* (88)	KX771087–88
	9	*D.* aff. *adamsi*	*D. punctatonervosa*	92	same as 8	KX771089–90
	10	*D. acanthomera*	*D. mojavensis*	89	Hawaiian *Drosophila* (35)	KX771091–92
"*brachytarsa* group"	11	*D.* aff. *brachytarsa*	*D. nigella*	91	"*brachytarsa* group" (89)	KX771093
	12	*Drosophila* sp. 2	n.a.	n.a.	n.a.	n.a.
	13	*Drosophila* sp. 3	n.a.	n.a.	n.a.	n.a.

Classification		Morphospecies	Closest relative	% simil.	Relatedness (bootstrap value)	COI barcode
	14	*Drosophila* sp. 4	*D.* sp JFG-2010	91	"*brachytarsa* group" (89)	KX771094–95
	15	*Drosophila* sp. 5	*D. haleakalae*	89	"*brachytarsa* group" (89)	KX771096–97
"*dyaramankana* group"	16	*D. dyaramankana*	*Leucophenga* sp. HWC-2012l	90	"*dyaramankana* group" (21)	KX771098–99
	17	*D.* aff. *dyaramankana* sp. 1	n.a.	n.a.	n.a.	n.a.
	18	*D.* aff. *dyaramankana* sp. 2	*Leucophenga* sp. HWC-2012f	88	"*dyaramankana* group" (21)	KX771100
Subgenus *Sophophora*						
dentissima group	19a	*D. lamottei* (♂)	*D. erecta*	92	*dentissima* group (79)	KX771101
	19b	*D. lamottei* (♀)	*D. subpulchrella*	93	*dentissima* group (79)	KX771102
	20	*D.* aff. *matilei*	*D. bocki*	89	*dentissima* group (79)	KX771103
fima group	21	*D.* aff. *kulango*	n.a.	n.a.	n.a.	n.a.
	22	*D. microralis*	*D. mayri*	91	*fima* group (100)	KX771104–05
	23	*D.* aff. *microralis*	*D. mayri*	91	same as 22	KX771106–07
melanogaster group						
melanogaster subgroup	24a	*D. erecta* (♂)	*D. erecta*	99	*D. erecta* (100)	KX771108
	24b	*D. erecta* (♀)	*D. yakuba* / *D. santomea*	100	same as 27	KX771109
	25	*D.* aff. *orena*	n.a.	n.a.	n.a.	n.a.
	26a	*D. teissieri* (♂)	*D. yakuba* / *D. santomea*	99	*D. teissieri* (81)	KX771111
	26b	*D. teissieri* (♀)	*D. yakuba* / *D. santomea*	100	same as 27	KX771110
	27	*D. yakuba*	*D. yakuba* / *D. santomea*	100	*D. yakuba* (95)	KX771112–13
montium subgroup	28a	*D. bakoue* (♂)	*D. nikananu*	96	*montium* subgroup (99)	KX771114
	28b	*D. bakoue* (♀)	*D. suzukii*	92	same as 19a	KX771115
	29a	*D. bocqueti* (♂)	*D. burlai*	98	near *D. burlai* (99)	KX771116
	29b	*D. bocqueti* (♀)	*D. simulans*	92	*dentissima* group (79)	KX771117

Classification		Morphospecies	Closest relative	% simil.	Relatedness (bootstrap value)	COI barcode
	30a	*D.* aff. *bocqueti* (♂)	*D. burlai*	98	same as 29a	KX771118
	30b	*D.* aff. *bocqueti* (♀)	*D. tsacasi*	97	same as 34a	KX771119
	31	*D. burlai*	*D. burlai*	99	*D. burlai* (99)	KX771120
	32a	*D.* aff. *chauvacae* sp. 1 (♂)	*D. burlai*	99	same as 31	KX771121
	32b	*D.* aff. *chauvacae* sp. 1 (♀)	*D. burlai*	98	same as 29a	KX771122
	33	*D.* aff. *chauvacae* sp. 2 (♂)	*D. burlai*	100	same as 31	KX771123
	34a	*D.* aff. *megapyga* sp. 1 (♂, ♀ 1)	*D. tsacasi*	97	*montium* subgroup (99)	KX771124–25
	34b	*D.* aff. *megapyga* sp. 1 (♀ 2)	*D. tsacasi*	96	*D. diplacantha* (32)	KX771126
	35a	*D.* aff. *megapyga* sp. 2 (♂)	*D. tsacasi*	97	same as 34a	KX771127
	35b	*D.* aff. *megapyga* sp. 2 (♀)	*D. nikananu*	96	same as 28a	KX771128
	36	*D. nikananu*	*D. nikananu*	100	*D. nikananu* (100)	KX771129
	37a	*D. seguyi* (♂)	*D. tsacasi*	96	*montium* subgroup (99)	KX771130
	37b	*D. seguyi* (♀)	*D. tsacasi*	97	same as 34a	KX771131
Genus *Lissocephala*	38	*L.* aff. *diola*	*D. parabipectinata*	91	*Sophophora* (57)	KX771132
Genus *Microdrosophila*	39	*M.* aff. *mamaru*	*Z. davidi*	91	within *Zaprionus* (46)	KX771133
Genus *Scaptodrosophila*	40	*S. latifasciaeformis*	*Stegana* sp. TL-2012	90	*D. allochroa* (45)	KX771134
	41	*S.* aff. *nicolae* sp. 1	*Parastegana femorata*	90	*Phortica sobodo* (11)	KX771135–36
	42	*S.* aff. *nicolae* sp. 2	*Phortica* sp. 1 HC-2014	89	*Phortica sobodo* (7)	KX771137–38
	43	*Scaptodrosophila* sp. 1	*D. trapezifrons*	88	*Phortica sobodo* (11)	KX771139–40
	44	*Scaptodrosophila* sp. 2	*Phortica* sp. 1 HC-2014	89	same as 42	KX771141–42
	45	*Scaptodrosophila* sp. 3	*Phortica foliiseta* / *D. jambulina*	91	*D. immigrans* (76)	KX771143
Genus *Scaptomyza*	46	*Scaptomyza* sp. 1	n.a.	n.a.	n.a.	n.a.

Classification		Morphospecies	Closest relative	% simil.	Relatedness (bootstrap value)	COI barcode
Genus *Zaprionus*						
armatus group	47a	*Z. spineus* (♂)	*Z. campestris*	95	*Z. campestris* (99)	KX771144
	47b	*Z. spineus* (♀)	*Z. litos*	93	within *Zaprionus* (46)	KX771145
inermis group	48a	*Z. badyi* (♂)	*Z. davidi*	91	within *Zaprionus* (46)	KX771147
	48b	*Z. badyi* (♀)	*Z. campestris*	91	same as 50a	KX771146
	49	*Z. inermis*	n.a.	n.a.	n.a.	n.a.
	50a	*Z. momorticus* (♂)	*Z. campestris*	91	within *Zaprionus* (46)	KX771149
	50b	*Z. momorticus* (♀)	*Z. taronus*	91	within *Zaprionus* (46)	KX771148
	51a	*Z. tuberculatus*	*Z. tuberculatus*	99	*Z. tuberculatus* complex (99)	KX771150–51, -55
	51b	*Z. tuberculatus*	*Z. sepsoides*	100	*Z. sepsoides* complex (99)	KX771153–54, -56–58
	51c	*Z. tuberculatus*	*Z. mascariensis*	99	*Z. mascariensis* (99)	KX771152, -59
neglectus group	52	*Z. neglectus*	n.a.	n.a.	n.a.	n.a.
vittiger group	53	*Z.* aff. *camerounensis*	n.a.	n.a.	n.a.	n.a.
	54a	*Z. davidi*	*Z. davidi*	99	*Z. davidi* (99)	KX771160, -62–63
	54b	*Z. davidi*	*Z. proximus*	98	*Z. proximus* (99)	KX771161, -64–65
	55	*Z. indianus*	*Z. africanus*	98	*Z. africanus* (86)	KX771166–68
	56	*Z. koroleu*	*Z. camerounensis*	95	*Z. camerounensis* (87)	KX771169–70
	57	*Z.* aff. *lachaisei*	*Z. lachaisei*	99	*Z. lachaisei* (99)	KX771171–72
	58	*Z. ornatus*	*Z. megalorchis*	99	*Z. ornatus* (99)	KX771173–74
	59	*Z.* aff. *ornatus*	*Z. taronus*	99	same as 60a	KX771175–76
	60a	*Z. taronus*	*Z. taronus*	99	*Z. taronus* (99)	KX771178
	60b	*Z. taronus*	*Z. spinipilus*	95	*Z. vittiger* (66)	KX771177
	61a	*Z. vittiger*	*Z. spinipilus*	99	*Z. vittiger* (100)	KX771179–83, -85
	61b	*Z. vittiger*	*Z. spinipilus*	95	same as 60b	KX771184
	62	*Z.* aff. *vittiger*	*Z. spinipilus*	99	same as 61a	KX771186–87

Species identification using DNA databases

All COI sequences were blasted against the NCBI sequence database (Table 1). Percent divergence from the closest sequence ranged from 0 to 12% (Fig. 2). The distribution of matches was bimodal, with a first peak around 0–1% divergence and a second one around 8–9%, meaning that some of our taxa were present in the database, whereas others belonged to groups which were virtually absent from it.

Our identification of species (Table 1) was correct with 100% sequence similarity for *Drosophila busckii* Coquillett, 1901, *D. immigrans* Sturtevant, 1921, *D. yakuba* Burla, 1954 and *D. nikananu* Burla, 1954; and with 99% sequence similarity for *D. erecta* Tsacas & Lachaise, 1974, *D. burlai* Tsacas & Lachaise, 1974, *Zaprionus tuberculatus* Malloch, 1932, *Z. davidi* Chassagnard & Tsacas, 1993, *Z. ornatus* Séguy, 1933, *Z. taronus* Chassagnard & Tsacas, 1993 and *Z. vittiger*. Note that *Z. megalorchis* Chassagnard & Tsacas, 1993 is a synonym for *Z. ornatus*, and *Z. spinipilus* Chassagnard & McEvey, 1992 is a synonym for *Z. vittiger* (Yassin & David 2010; Yassin *et al*. 2010).

Some of our morphospecies were actually a pool of several cryptic species, as shown by the fact that some individuals matched the reference sequence, while some others were substantially divergent. This occurred in the *Z. tuberculatus* and *Z. davidi* morphospecies. Some specimens of *Z. tuberculatus* matched *Z. sepsoides* Duda, 1939 (100% similarity) or *Z. mascariensis* Tsacas & David, 1975 (99%

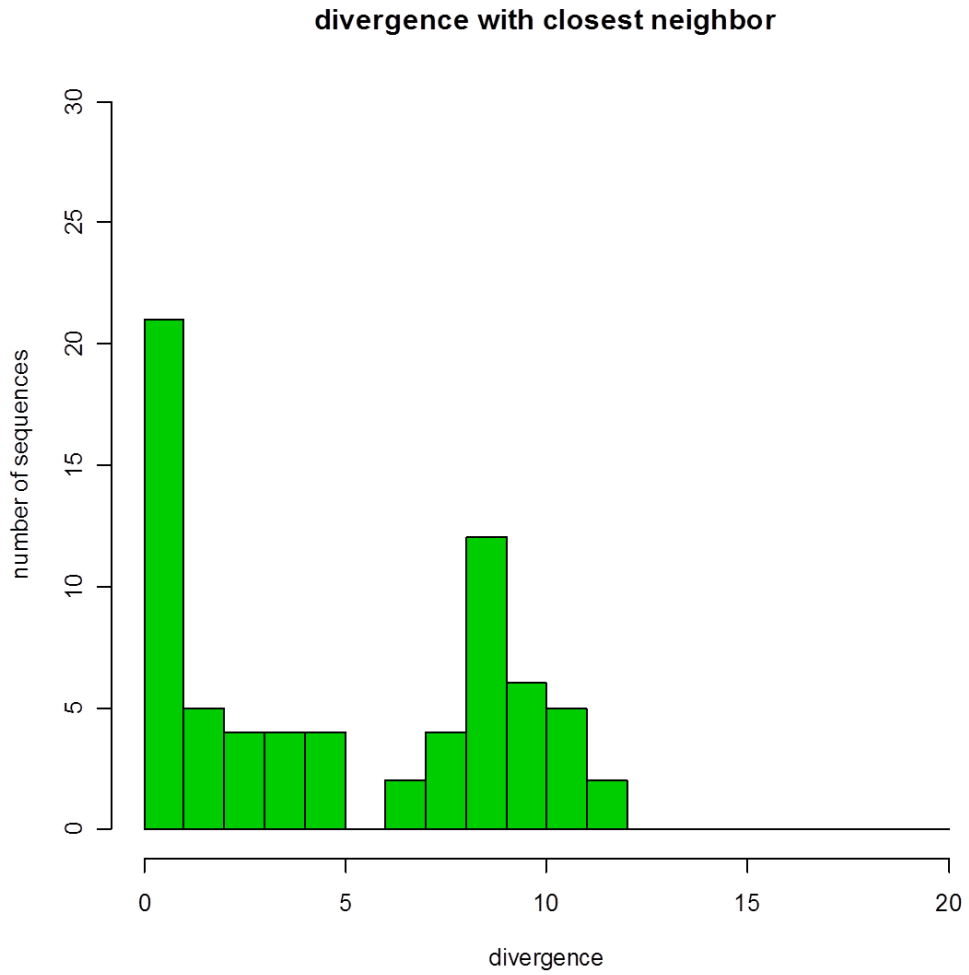

Fig. 2. Percent divergence of the morphospecies DNA barcode from the closest neighbor found in the barcode database.

similarity), two related species known to be hardly distinguishable from *Z. tuberculatus* (Tsacas *et al*. 1977). Half of the sequences of the *Z. davidi* morphospecies matched *Z. proximus* Collart, 1937 (98% similarity), suggesting the presence of a second species. Our results also suggested that *D.* aff. *chauvacae* sp. 1 and sp. 2 are in fact *D. burlai* (99–100% similarity), that *Z. indianus* Gupta, 1970 could be *Z. africanus* Yassin & David, 2008 (98% similarity), that *Z.* aff. *lachaisei* is *Z. lachaisei* Yassin & David, 2010 (99% similarity), that *Z.* aff. *ornatus* is *Z. taronus* (99% similarity), and that *Z.* aff. *vittiger* is *Z. vittiger* (99% similarity).

Misidentification was observed for females of the *melanogaster* subgroup. While males of this subgroup are easily determined using genitalia, females are morphologically very similar to each other and their identification requires dissection (Yassin & Orgogozo 2013). Introgression of mitochondrial DNA across species boundaries has been documented between several related species, including *D. yakuba*, *D. teissieri* Tsacas, 1971 and *D. santomea* Lachaise & Harry, 2000 (Llopart *et al.* 2005; Bachtrog *et al.* 2006). In this group, females thought to belong to *D. erecta* and *D. teissieri* matched the *D. yakuba* sequence (100% similarity) and the sequence of the *D. teissieri* male matched that of its sister species *D. yakuba* (99% similarity). The differences in abdominal color pattern used to distinguish morphospecies overlapped the polymorphism of *D. yakuba* and were thus useless for species identification.

In all remaining morphospecies, similarity with available data was so low that it was irrelevant for this study. For example, the best match of *Drosophila dyaramankana* Burla, 1954, a drosophiline, was with the steganine *Leucophenga sp*. (90% similarity), and *Drosophila* sp. 5 matched several Hawaiian *Drosophila* (89%). Overall, two thirds of our morphospecies corresponded to poorly represented taxa in the COI database, either in terms of species or in terms of higher taxonomic units.

Phylogenetic relatedness

A neighbor joining tree using the whole dataset (not shown) proved to be unreliable for deep nodes, since major groups from the known phylogeny of drosophilids were disrupted and showed incorrect connections. This was expected given the relatively poor information provided by the short barcode fragment, which was designed for identifying purposes by searching the closest neighbors of unidentified taxa. Moreover, the genus *Drosophila* is a very large taxonomic ensemble which is fragmented into smaller "groups" and "subgroups", involving some endemism. In addition, some distinct genera, including *Zaprionus*, are actually internal branches of the *Drosophila* tree. Most groups are not represented in Africa, and a reliable topology of drosophilids can hardly be inferred from a geographically limited sample.

A neighbor-joining tree was constructed for the species of *Zaprionus* and *Microdrosophila* aff. *mamaru* (Burla, 1954) (Fig. 3) and a second for the *Sophophora* lineage of *Drosophila* and *Lissocephala* aff. *diola* Tsacas & Lachaise, 1979 (Fig. 4). A maximum-likelihood tree was run on the same datasets. A remarkable result is that in each analysis, the two trees were fully congruent for all nodes supported by a 50% bootstrap value or more. The two series of bootstrap values are shown on Figs 3 and 4 (on the neighbor-joining tree), except for one case where the two values were very close, although above and below this threshold: 48% and 52%, respectively.

Below we present and discuss the results obtained for each group of taxa separately.

The most abundant genus in the Mt Oku forest is *Zaprionus* (Fig. 3), of which we found 16 morphospecies. COI sequences were obtained for 13 of them. Nine of them belonged to the *vittiger* species group. The *Z. indianus* morphospecies departed from *Z. indianus* and was related to *Z. africanus*, although with a node supported only by an 84–86% bootstrap value in phylogenetic analysis (Fig. 3). The *Z. davidi* morphospecies was split into two different clusters, as seen above, one of them being the true *Z. davidi*, the other being closer to *Z. proximus*. *Zaprionus* aff. *ornatus* clustered with *Z. taronus*, but was

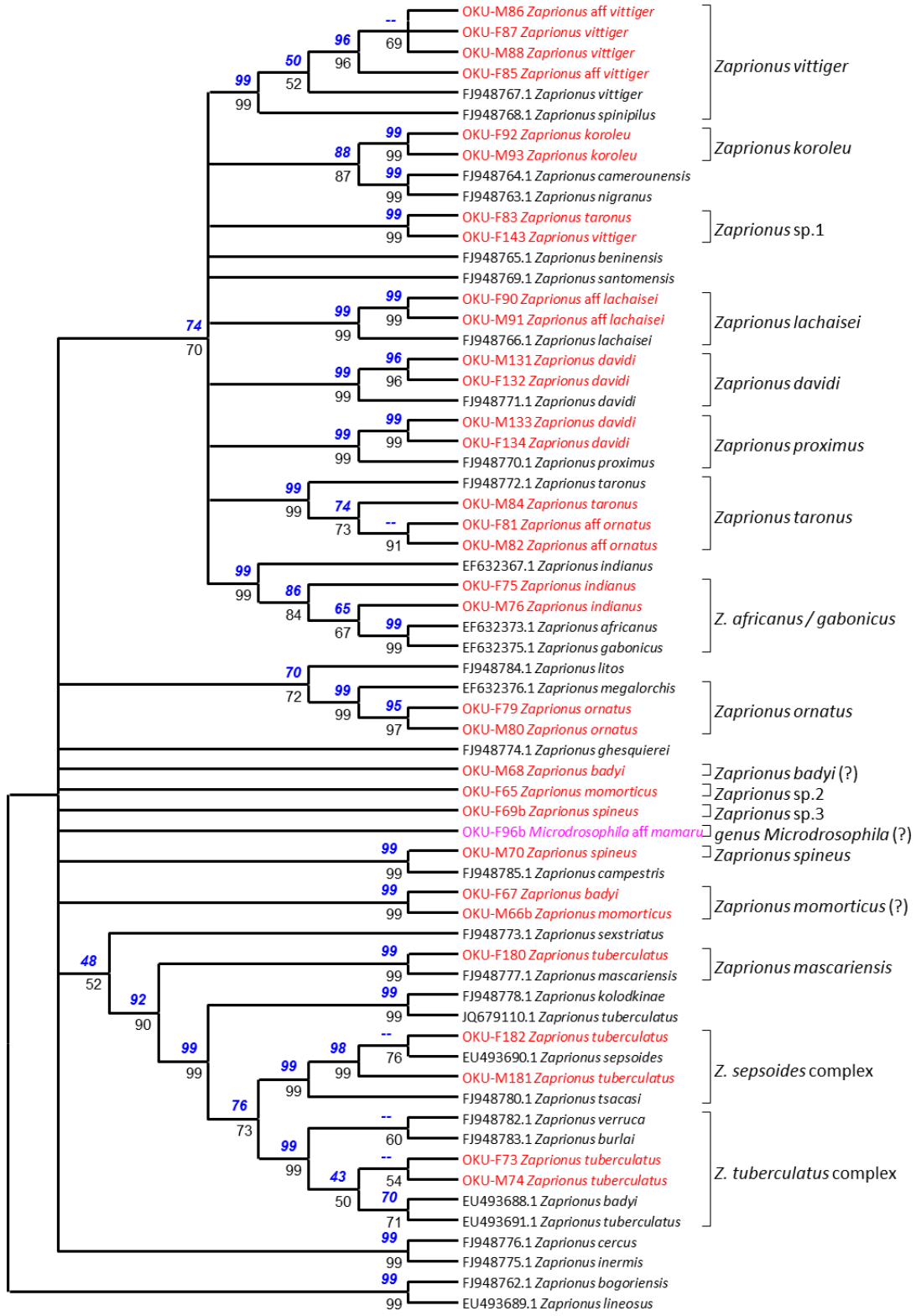

Fig. 3. Phylogenetic analysis of the genus *Zaprionus* and *Microdrosophila* aff. *mamaru* (Burla, 1954). This tree is the neighbor-joining tree. The maximum likelihood tree gives the same topology. Nodes with a bootstrap value lower than 50% were merged. Bootstrap values were calculated over 1000 repeats. Above nodes: bootstrap values for maximum likelihood using a GTR+G+I model. Below nodes: bootstrap values for neighbor-joining using the Kimura-2p distance.

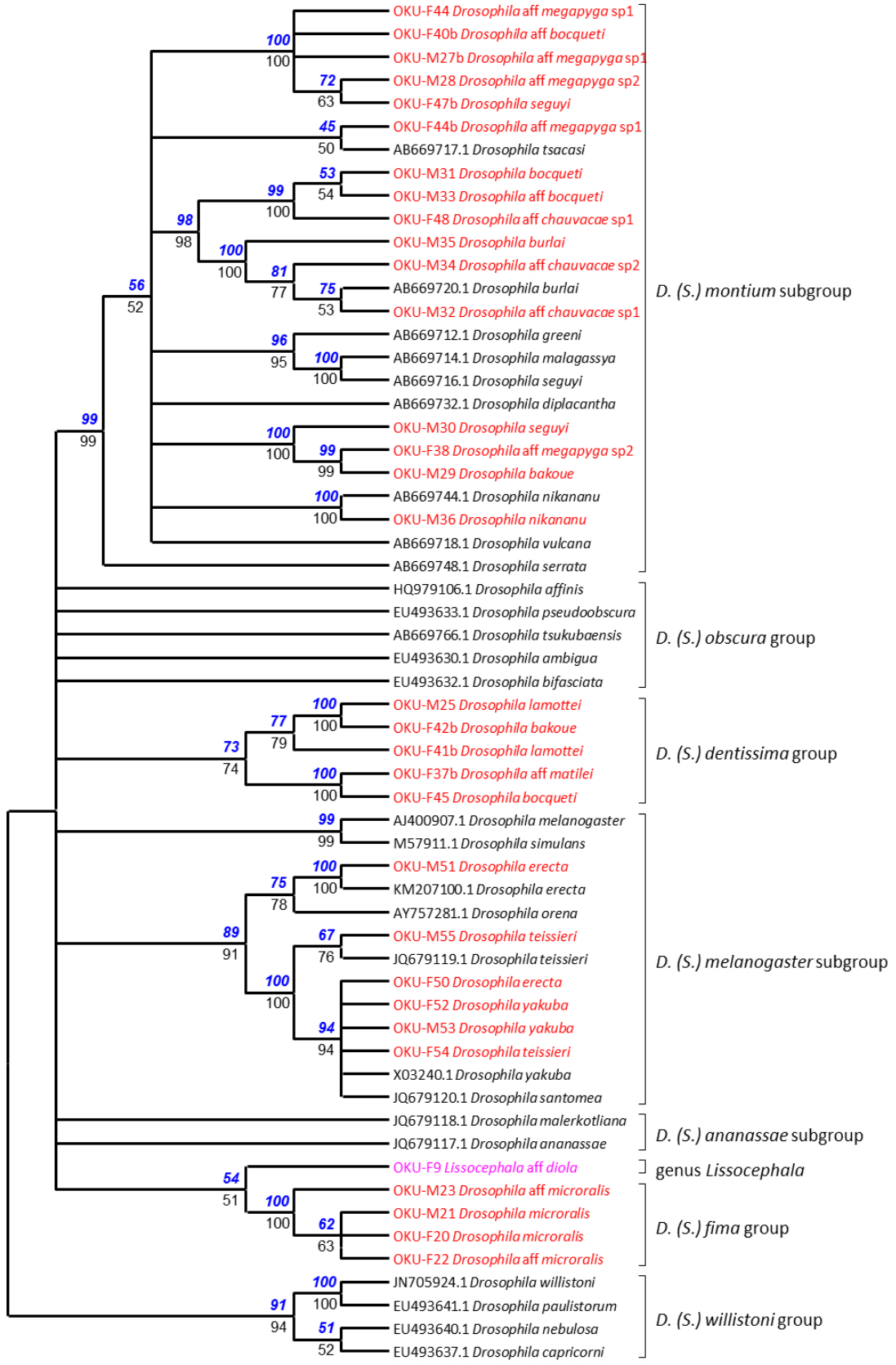

Fig. 4. Phylogenetic analysis of the subgenus *Sophophora* and *Lissocephala* aff. *diola* Tsacas & Lachaise, 1979. Conventions as for Fig. 3.

not closely related to the reference sequence for *Z. ornatus*. *Zaprionus* aff. *lachaisei* matched the true *Z. lachaisei*. Two specimens misidentified as *Z. taronus* and *Z. vittiger* clustered together ("*Zaprionus* sp. 1") and matched the *Z.* aff. *vittiger* sequence of Bouiges *et al*. (2013). The *Z.* aff. *vittiger* morphospecies of Prigent *et al*. (2013) branched within sequences from *Z. vittiger*, suggesting they belong to the same species. Our two sequences for *Z. koroleu* Burla, 1954 made up a new lineage related to *Z. camerounensis* Chassagnard & Tsacas, 1993 and *Z. nigranus* Yassin *et al.*, 2008. This taxon, however, is different from *Z. beninensis* Chassagnard & Tsacas, 1993, which is considered a synonym of *Z. koroleu* (Yassin & David 2010).

Z. tuberculatus belongs to a closely related group of species within the *inermis* group. Our sequences for *Z. tuberculatus* were split into three clusters corresponding to *Z. tuberculatus, Z. sepsoides* and *Z. mascariensis* (Fig. 3). This was expected, since our ecological study made no attempt to distinguish these three species, which can only be recognized by dissection. Our results indicate that the three of them were present in the study. The three remaining morphospecies, *Z. spineus* Tsacas & Chassagnard, 1990, *Z. momorticus* Graber, 1957 and *Z. badyi* Burla, 1954, gave no consistent results. The sequences for male and female for each of them were separate in the tree, suggesting that each individual belonged to a different species. Only two sequences clustering together indicated that the dark male of the *Z. momorticus* morphospecies belonged to the same species as the yellowish female of the *Z. badyi* morphospecies. Moreover, our two sequences for the *Z. badyi* morphospecies lie outside the *tuberculatus* cluster, in which the database reference for *Z. badyi* is found.

For the *Sophophora* subgenus of *Drosophila,* morphospecies from Mt Oku clustered into four distinct lineages (Fig. 4). They correspond to the *melanogaster* subgroup, the *montium* subgroup (both in the *melanogaster* group), and to two lineages for which no COI sequence has hitherto been available: the *dentissima* group and the *fima* group. The two morphospecies recognized for the *fima* group probably belong to the same species, *D. microralis* Tsacas, 1981. In the *dentissima* group two morphospecies were recognized, *D. lamottei* Tsacas, 1981 and *D.* aff. *matilei* Tsacas, 1975. Two females that we thought belonged to the *montium* subgroup were actually members of the *dentissima* group. The three *D. melanogaster* subgroup species from the Mt Oku forest, *D. erecta*, *D. teissieri* and *D. yakuba*, were correctly identified.

We used the sequences of the *montium* subgroup found in databases and found that African species cluster in a sub-section of the tree (data not shown), also including the sequences of this group from Mt Oku. The *D. burlai*, *D.* aff. *chauvacae* sp. 1 and *D.* aff. *chauvacae* sp. 2 morphospecies clustered with the *D. burlai* database sequence (Fig. 4). Another cluster contained *D. bocqueti* Tsacas & Lachaise, 1974 and an undetermined morphospecies. The *D.* aff. *megapyga* sp. 1 and *D.* aff. *megapyga* sp. 2 morphospecies made up a third cluster. The *D. seguyi* Smart, 1945 and *D. bakoue* Tsacas & Lachaise, 1974 morphospecies made up a fourth cluster. Six morphospecies were represented by both males and females. In five morphospecies, all females formed clades apart from the males we identified as conspecific. Thus, their determination was erroneous. Of two females sequenced for the *D.* aff. *megapyga* sp. 1 morphospecies, one was wrong and the other correct. Thus, females of similar morphology belonged to different species, confirming that the females of this group are difficult to determine.

Among the remaining species, identification was correct in *D. immigrans* and *D. busckii*, but unsettled in other species, since sequences did not generally cluster with available sequences. We did find some unexpected associations where OTUs clustered with supposed unrelated taxa. These were those between *Microdrosophila* aff. *mamaru* and the genus *Zaprionus* (Fig. 3) and between *Lissocephala* aff. *diola* and the *fima* group of the subgenus *Sophophora* (Fig. 4). However, the bootstrap values were always very low (40–60%). Thus, these results may be artefacts resulting from the lack of appropriate barcode references and from the low resolving power of DNA barcodes when divergence is high.

The remaining morphospecies confirmed this conclusion. The closest relatives in the tree were connected with very low support. For example, *D. acanthomera* Tsacas, 2001 clustered with a group of Hawaiian species with a support of only 35%. None of the *Scaptodrosophila* morphospecies clustered with a species of this genus which had already been sequenced except for *Scaptodrosophila* sp. 3. This OTU grouped either with *S. riverata* Singh & Gupta, 1977 (45%) or with *D. immigrans* (76%). At such a low level of discrimination and as a result of homoplasy, branching patterns and bootstrap values above the specific level are highly dependent on the size and on the composition of the sample of species used to construct the tree. They are unreliable and do not deserve further consideration.

Consequences for the Mt Oku ecological survey

Our ecological survey included 62 morphospecies (Prigent *et al*. 2013). Barcodes were obtained for 52 of them. The clustering of the specimens by their barcode suggests that nine morphospecies were duplicates of another species in the sample, as a probable result of color polymorphism within biological species. This does not substantially affect the conclusions of the survey, since the two incorrectly split subsamples occurred together in all cases. Moreover, such cases did not generally involve numerically important species. The fusion of *D.* aff. *adamsi* with *D. adamsi* Wheeler, 1959 and that of *D.* aff. *microralis* with *D. microralis* add up to a sample size of 20 individuals. Similarly, the *Z.* aff. *vittiger* sample size represents only 1.1% of the *Z. vittiger* catches. The most important changes occurred within the *montium* subgroup. Pooling the *D.* aff. *chauvacae* sp. 1, *D.* aff. *chauvacae* sp. 2 and *D. burlai* morphospecies made up a total of 101 individuals. In the same way, pooling *D.* aff. *megapyga* sp. 1 and *D.* aff. *megapyga* sp. 2 made up 77 individuals. In view of the total collection of 10 839 specimens, these changes had minor consequences for the statistics of abundance.

The COI barcode also showed that some morphospecies actually included several species. This was expected in some well-documented cases, for which external morphology is known to be insufficient to distinguish closely related species. DNA barcoding confirms the presence of two species in each of the *Z. davidi*, *Z. vittiger* and *Z. taronus* morphospecies, and of three species in the *Z. tuberculatus* morphospecies. This was an accepted lack of precision of the ecological survey, since these species were too numerous to allow for the dissection of all specimens.

Barcoding a stratified sample of morphospecies allowed us to check hypotheses as to underlying species assemblages in species complexes. The ecological distribution of the *Z. tuberculatus* morphospecies (which is made up of three biological species) had shown two distinct peaks in altitudinal distribution with an absence of flies at intermediate elevations (Prigent *et al*. 2013). We wondered whether this was due to habitat heterogeneity or to heterogeneity in the specific composition of the samples, given our inability to discriminate the three biological species by external morphology. The sequenced specimens from an elevation of 2300 m belonged to the three species; those from 2700 m belonged to two of them. This suggests that the gap in their distribution probably results from habitat heterogeneity, rather than from taxonomic confusion.

The *Z. indianus* morphospecies can similarly include three species in this geographical area. It shows a bimodal distribution around the year. A specimen collected in October belongs to the same species as two specimens collected in April. Thus, barcoding does not suggest that distribution heterogeneity results from species heterogeneity, even though appropriate sampling would be necessary to reach a firm conclusion.

Finally, barcoding allowed us to validate the determination of 43 morphospecies. Nine cryptic species had to be added to this count (Table 1), making up a total number of 52 barcoded species.

Discussion

This study took place on the Cameroon volcanic line, which belongs to an endangered biodiversity hotspot (Myers *et al*. 2000). This concentration of species has been interpreted as resulting from successive cycles of extension and regression of the forest having taken place during the Quaternary (Maley 1996; Plana 2004). During dry periods montane forests were restricted to montane refuges and to gallery forests. During rainy periods they could extend more widely and connect with each other to make up a continuous forest. Drosophilids are an important component of this rich biodiversity. This study, together with an earlier one (Prigent *et al*. 2013), was designed to record the response of drosophilid species to physical factors contributing to climate variation, including changes in habitat according to season and altitude. In this study the validity of the recognized morphospecies was tested using barcoding.

Consistent with the principle of DNA barcoding, the study was very informative when closely related reference sequences were present in DNA banks and almost intractable when an entire natural group of species was not represented. This occurred on several occasions, since African *Drosophila* have not been as well studied as those from most other areas. Overall the classification of our morphospecies was correct in the *Zaprionus* genus and the *Sophophora* subgenus of *Drosophila*. It was also correct for the two cosmopolitan species, *D. busckii* and *D. immigrans*. Morphospecies classification was more challenging in the *Drosophila* subgenus and in other genera, probably due to the fact that none of these species were included in the barcode database, and due to a lack of information on related taxa. Four taxa from our study were not represented in the database. The *fima* group belongs to the subgenus *Sophophora* but its branching position was uncertain due to a low bootstrap value. Similarly, the *dentissima* group was associated with the *melanogaster* subgroup (although with a low bootstrap value of 40–41%, Fig. 4) even though the *dentissima* group is considered to be distinct from the *melanogaster* group (Tsacas 1980). This result is, however, in agreement with the proposition of raising the *ananassae* and *montium* species subgroups to species groups (Da Lage *et al.* 2007). A barcode in the genus *Lissocephala* was sequenced for the first time and branched within the subgenus *Sophophora*, an unexpected observation needing confirmation, since this genus is generally given a basal position in Drosophilinae based on morphology (Throckmorton 1975) or molecular data (Harry *et al.* 1996, 1998; Yassin 2013). A species which we identified as belonging to the genus *Microdrosophila*, based on Burla's (1954b) key to drosophilids from Ivory Coast, branches in the genus *Zaprionus*, an unexpected result since the morphology of this morphospecies differs from that of *Zaprionus*, which is very homogeneous across species. The lack of definition of barcoding in some drosophilid genera may result from the relatively poor record of African drosophilids in databases, a requirement for such studies (Davison *et al*. 2009). Thus, the lack of similar works constitutes an extrinsic limitation of our study. Since *Drosophila* taxonomy relies heavily upon male genitalia, and since most species are sexually dimorphic in color patterns, our results illustrate the poor correspondence between males and females across a number of species. Several members of the *melanogaster* group show dimorphic females with black and yellow forms (Burla 1954a), presumably evolved through sexual selection (Yassin *et al*. 2016). Furthermore, females from different species of the *montium* subgroup are practically indistinguishable. Hence, some females thought to belong to the *montium* subgroup eventually appeared to actually belong to the *dentissima* group.

The main conclusion of this study is that DNA barcoding is a great help in making a link between taxonomic and ecological studies. This provides a means to assess our ability to identify species despite the methodological constraints brought about by the quantitative requirements of ecological studies. The results of our former ecological study (Prigent *et al*. 2013) were practically unaffected by the fact that nine morphospecies represented excessive splitting, and that nine true species had not been recognized. The misidentified species happened to be rare ones. As a rule, abundant species were correctly determined. In three cases we had consciously pooled groups of two or three related species

under a common heading ("*Zaprionus tuberculatus"*, "*Z. indianus"* and "*Z. davidi"*), since these taxa could be distinguished only through dissection, and since these groups made up very important sample numbers (Prigent *et al*. 2013). We also knew from previous studies that the ecological similarity of the species within each of these groups would not substantially affect the data. Our barcoding results confirm these assumptions. This positive outcome must, however, be viewed as a warning for possibly less favorable cases.

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