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A comprehensive DNA sequence library is essential for identification with DNA barcodes

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Abstract

In this study we examine the possibility of utilising partial cox1 gene sequences as barcodes to identify non-biting midges (Diptera: Chironomidae). We analysed DNA from 97 specimens of 47 species in the genera *Cladotanytarsus*, *Micropsectra*, *Parapsectra*, *Paratanytarsus*, *Rheotanytarsus*, *Tanytarsus* and *Virgatanytarsus* with a main focus on *Micropsectra*, *Parapsectra* and *Paratanytarsus*. Our findings show that (1) cox1 is easily amplified from extracts from different life stages with the standard barcoding primers. (2) Although K2P-distances between con-specific sequences varied up to 4.9%, con-specifics clustered together with 91–100% bootstrap support in maximum parsimony analysis. This indicates that barcodes may be excellent tools to identify species that are already in a cox1 library. (3) Both neighbour joining and maximum parsimony failed to reconstruct monophyletic genera. Thus, if a well-matching cox1 sequence is not already available in the library, the prospects of approximately identifying an unknown taxon, even to the correct genus of subtribe Tanytarsina, are not good.

Keywords: DNA-barcoding; DNA library; Chironomidae; *Micropsectra*; *Paratanytarsus*; *Parapsectra*; Cox1; CO1

1. Introduction

The immature stages of the dipteran family Chironomidae are commonly the most diverse and abundant macroinvertebrates in freshwater ecosystems. Many species have specific habitat requirements and the species compositions in diverse fresh waters are often read as pointers to more or less distinct states of environmental gradients. Therefore, chironomids are frequently used by freshwater biologists to assess and monitor environmental conditions (e.g. Brodersen and Lindegaard, 1999; Verneaux and Verneaux, 2002; Aagaard et al., 2004) and to infer past environments from the usually abundant and species rich fossil larval head capsules in lake sediments (e.g. Velle et al., 2005). Unfortunately, the larvae and females of closely related species are usually difficult to distinguish by means of morphology, and species identification frequently depends on association of these life stages with identified pupal exuviae or adult males which tend to posses more species specific characteristics. Moreover, the larvae and females of numerous species, even in the relatively well-documented European fauna, remain unknown to science. Hence, environmental assessments and bio-monitoring of freshwater habitats presumably would have much to gain if the larvae and other life history stages could be more readily identified to species.

Relatively few authors have as yet reported the use of genetic markers as identification tools in studies of Chironomidae. Different molecular techniques and target genes have been used in those few studies (Asari et al., 2004; Carew et al., 2003, 2005; Ekrem and Stur, in press; Sharley et al., 2004; Willassen, 2005) and some of the results may be of limited interest as identification tools for practising

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freshwater ecologists, who probably prefer methods that are directly comparable and universally applicable for all Chironomidae taxa.

The Barcoding of Life initiative (Hebert et al., 2003a,b) has envisioned a standardized method to alleviate difficult species identifications by focusing sequencing efforts on one target gene, cytochrome *c* oxidase subunit 1 (cox1). Cox1 sequence clustering by neighbour joining (NJ) has been suggested as an effective and suitable way to recognise and identify animal species (Hebert et al., 2003a,b; Hebert et al., 2004a) and to discover cryptic taxa (Hebert et al., 2004b). This approach has been argued to be too imprecise for reliable species diagnoses in some cases, and character based identification systems have therefore been proposed as the preferred way to proceed with the Barcoding of Life Project (DeSalle et al., 2005).

Complete knowledge of the life stages of an organism is essential for a good understanding of its ecology, taxonomy, phylogeny and evolution. An acknowledged advantage of DNA-barcoding is the possibility to easily associate different life stages of the same species (Blaxter, 2004; Stoeckle, 2003). This is particularly valuable when taxa are difficult to rear in the laboratory, and several studies have recognised the benefit of short DNA sequences in associating immature stages with adult counterparts (e.g. Hebert et al., 2004b; Barrett and Hebert, 2005; Miller et al., 2005; Paquin and Hedin, 2004; Thomas et al., 2005; Vences et al., 2005a,b). Many chironomid species are both difficult to rear in the laboratory and unknown as larvae. Only one study has so far demonstrated the usefulness of DNA-barcodes to associate chironomid life stages in practice (Carew et al., 2005).

The goals of the present study were to (1) test the success of cox1 amplification in chironomids with the suggested general PCR primers for DNA-barcoding (LCO1490 and HCO2198) and (2) investigate whether partial cox1 gene sequences can be used to associate life stages and identify species of non-biting midges. We chose to focus on species of the Tanytarsini genera *Micropsectra*, *Parapsectra* and *Paratanytarsus* because the species comprised by these genera are well studied and presumably morphologically relatively distinct. Fresh material of numerous species was available from some of our latest field trips in Europe.

2. Materials and methods

2.1. Taxon sampling and identification

The taxa included in this study were selected with the aim of getting representatives of as many of the known species in *Micropsectra*, *Parapsectra* and *Paratanytarsus* as possible. Field work was mainly conducted in Europe, but some material from other geographical regions was made available to us by colleagues (Table 1). Most of the specimens sampled were adult males ($n = 81$, 85.3%), nine of which were reared from immature stages and thus have associated larval and/or pupal exuviae (Table 1). Six specimens were adult females that all but one could be identified to species by associated pupal skins. Two of the sampled specimens were pupae and six were larvae of which four only could be identified to genus level due to incomplete knowledge of larval taxonomy (*Micropsectra* sp. B, *Micropsectra* sp. C, *Paratanytarsus* sp., *Rheotanytarsus* sp.). Some of the species sampled in this study also could not be named since they were previously unknown to science and are pending formal descriptions (*Micropsectra* sp. A, *Parapsectra* sp. A, *Parapsectra* sp. B, *Tanytarsus* sp. B). More than one life stage was sequenced from six of the included species (Table 1). When possible, we included three specimens of each species, preferably from different locations. The species were identified by their original descriptions and by recent revisions (Stur and Ekrem, 2006; Säwedal, 1976; Reiss and Säwedal, 1981). We also examined types and other reference material in the Natural History Collections in Bergen, Norway and Zoologische Staatssammlung München, Germany. Voucher specimens are deposited in the Natural History Collections, Bergen Museum, University of Bergen, Norway and in the Museum of Natural History and Archaeology, Norwegian University of Science and Technology in Trondheim, Norway.

2.1.1. Extraction, amplification, sequencing and alignment

DNA extraction largely followed the standard protocol for the Qiagen DNeasy tissue extraction kit. We used only 120–170 µl elution buffer (depending on specimen size) to yield an appropriate concentration of DNA in the DNA template solutions. Each PCR was made with addition of 2 µl DNA template, 2.5µl 10 × PCR buffer (Qiagen, with ~15 mM $MgCl_2$), 2 µl of dNTPs in 10 µM concentration, 1 µl of each of the suggested standard barcode primers (Folmer et al., 1994) LCO1490 (5′-GGTCAACA AATCATAAAGATATTGG-3′) and HCO2198 (5′-TA AACTTCAGGGTGACCAAAAAATCA-3′) in 10 µM concentration, 1 U of Qiagen HotStar *Taq* DNA polymerase, and distilled water for a total reaction volume of 25 µl. The PCR had 5 cycles of 30 s annealing at 45 °C and 35 cycles of 30 s annealing at 51 °C in a typical step-up procedure on PTC-100 and PTC-200 PCR machines from MJ Research. The PCR products were purified using QIAquick PCR purification kit (Qiagen). Purified products were sequenced in both directions using BigDye (Perkin-Elmer) termination reactions and analysed on ABI377 or ABI Prism 3100 genetic analysers. Sequences were assembled and edited using Sequencher 3.1.1 (Gibbs and Cockerill, 1995) or BioEdit 7.0.5.2 (Hall, 1999), and aligned in BioEdit. Alignment of the nucleotide sequences was unproblematic since indels were absent and conceptual translation with the invertebrate mitochondrial code returned uninterrupted amino acid sequences that were identified as cox1 fragments with *blastp* search in GenBank. After trimming of uncertain bases at both ends, the aligned sequences were 654 bp long. An overview of species sequenced and their respective GenBank accession numbers is given in Table 1.

Table 1
List of analysed specimens with associated sample localities, voucher reference numbers and accession numbers

Taxon	Life stage	Locality	Voucher number	Accession number
Cladotanytarsus atridorsum (Kieffer, 1924)	Male adult	Norway, Aust Agder, Valle, Flåni, 29.VI.2001, T. Ekrem	To81	AM398682
Cladotanytarsus pallidus (Kieffer, 1922)	Male adult, pex, lex	Germany, Bavaria, Munich, Nymphenburger Park, Kleiner See, 13-17.VII.1999, T. Ekrem	To02	AM398683
Micropsectra appendica (Stur and Ekrem, 2006)	Male adult	Norway, Hordaland, Bergen, Espeland, at marine research station, 07.V.2003, E. Stur & T. Ekrem	To132	AM398684
Micropsectra appendica	Male adult	Norway, Hordaland, Bergen, Espeland, at marine research station, 07.V.2003, E. Stur & T. Ekrem	To133	AM398685
Micropsectra atrofasciata (Kieffer, 1911)	Male adult	Norway, Sør Trøndelag, Oppdal, Kongsvold, Blesbekken, 1350 m a.s.l., 9.IX.2004, T. Ekrem	To152	AM398686
Micropsectra attenuata (Reiss, 1969)	Male adult	Luxembourg, Diekirch, N Haerebierg, Rheocrene spring at Schmittenhaff, 27.VI.2002, T. Ekrem	To109	AM398687
Micropsectra attenuata	Male adult	Norway, Hordaland, Bergen, Fjellveien at Starefossen, 07.V.2003, E. Stur & T. Ekrem	To131	AM398688
Micropsectra attenuata	Male adult	Germany, Bavaria, Achmühle, spring brook, 03.V.2005, T. Ekrem	To302	AM398689
Micropsectra contracta (Reiss, 1965)	Male adult, pex, lex	Germany, Bavaria, Ramsau, Hintersee, 6.VII.1999, T. Ekrem	To06	AM398690
Micropsectra contracta	Male adult	Germany, Bavaria, Murnauer Moos, Fügsee, 27.VII.2005, T. Ekrem	To356	AM398691
Micropsectra contracta	Male adult	Germany, Bavaria, Murnauer Moos, Fügsee, 27.VII.2005, T. Ekrem	To357	AM398692
Micropsectra contracta	Male adult	Germany, Bavaria, Murnauer Moos, Fügsee, 27.VII.2005, T. Ekrem	To358	AM398693
Micropsectra insignilobus (Kieffer, 1924)	Male adult	Norway, Hordaland, Odda, Dyrskar, 7.VII.2001, T. Ekrem	To31	AM398694
Micropsectra insignilobus	Male adult	West Greenland, Kangerlussuaq,Tasersuatsiaq (Lake Ferguson), 09.VII.2002, C. Lindegaard	To182	AM398695
Micropsectra insignilobus	Male adult	West Greenland, Kangerlussuaq,Tasersuatsiaq (Lake Ferguson), 09.VII.2002, C. Lindegaard	To184	AM398696
Micropsectra junci (Meigen, 1818)	Male adult	Norway, Sogn og Fjordane, Aurland, Vestredalsvatnet, 21.VII.2001, T. Ekrem	To54	AM398697
Micropsectra junci	Male adult	Luxembourg, Diekirch, N Haerebierg, Rheocrene spring at Schmittenhaff, 06.VII.2003, leg. T. Ekrem	To154	AM398698
Micropsectra junci	Male adult	Luxembourg, Diekirch, N Haerebierg, Rheocrene spring at Schmittenhaff, 06.VII.2003, leg. T. Ekrem	To157	AM398699
Micropsectra kurobemaculata (Sasa and Okazawa, 1992)	Male adult	Japan, Honshu, Ibaraki, Mt. Tsukuba, 11.IX.2000, T. Ekrem	To07	AM398700
Micropsectra kurobemaculata	Male adult	Japan, Honshu, Ibaraki, Mt. Tsukuba, 11.IX.2000, T. Ekrem	To08	AM398701
Micropsectra logani (Johannsen, 1928)	Male adult	Norway, Vest Agder, Vennesla, Skjerkedalsbekken at Åmdal, 6.VII.2001, T. Ekrem	To33	AM398702
Micropsectra logani	Female adult, pex	West Greenland, Kangerlussuaq, Melt water from glacier, at waterfall, 5.VII.2002, C. Lindegaard	To187	AM398703
Micropsectra logani	Female adult, pex	West Greenland, Kangerlussuaq, Melt water from glacier, at waterfall, 5.VII.2002, C. Lindegaard	To188	AM398704
Micropsectra logani	Male adult	Norway, Bear Island, Spælvatnet, Malaise trap, 12.-19.VII.2002, O.K. Berg & A. Finstad	Bj50	AM398705
Micropsectra notescens (Walker, 1856)	Male adult	Germany, Bavaria, Wolfratshausen, Achmühle, light at string brook, 22.VI.2005, E. Stur	To315	AM398706
Micropsectra notescens	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. M. Kotrba	To324	AM398707
Micropsectra notescens	Male adult	Germany, Bavaria, Murnauer Moos, Limnocrene spring, 01.V.2005, T. Ekrem	To351	AM398708
Micropsectra notescens	Male adult	Germany, Bavaria, Murnauer Moos, Limnocrene spring, 01.V.2005, T. Ekrem	To352	AM398709
Micropsectra notescens	Female adult	Germany, Bavaria, Murnauer Moos, Limnocrene spring, 01.V.2005, T. Ekrem	To353	AM398710
Micropsectra notescens	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. M. Kotrba	To354	AM398711
Micropsectra pallidula (Meigen, 1830)	Male adult	Norway, Vest Agder, Vennesla, Skjerkedalsbekken at Åmdal, 6.VII.2001, T. Ekrem	To34	AM398712
Micropsectra pallidula	Male adult	Norway, Vest Agder, Vågsbygd, Kjosbekken at Storevann, 2.VII.2001, T. Ekrem	To74	AM398713

Micropsectra pharetrophora (Fittkau and Reiss, 1999)	Male adult, pex, lex	Germany, Bavaria, Berchtesgaden National Park Herrenroint, spring 24c, 24.VI.2000, E. Stur	To11	AM398714
Micropsectra pharetrophora	Female adult, pex	Germany, Bavaria, Berchtesgaden National Park Herrenroint, spring 24c, 24.VI.2000, E. Stur	To12	AM398715
Micropsectra polita (Malloch, 1915)	Male adult	West Greenland, Kangerlussuaq,Tasersuatsiaq (Lake Ferguson), 09.VII.2002, C. Lindegaard	To181	AM398716
Micropsectra polita	Male adult	West Greenland, Kangerlussuaq,Tasersuatsiaq (Lake Ferguson), 09.VII.2002, C. Lindegaard	To183	AM398717
Micropsectra radialis (Goetghebuer, 1939)	Male adult	Norway, Hordaland, Ulvik, Finse, Research Station, 17.VII.1999, E. Willassen	To35	AM398718
Micropsectra radialis	Male adult	Norway, Hordaland, Ulvik, Finse, Research Station, 17.VII.1999, E. Willassen	To37	AM398719
Micropsectra recurvata (Goetghebuer, 1928)	Male adult	Norway, Hordaland, Ulvik, Finse, Research Station, 17.VII.1999, E. Willassen	To38	AM398720
Micropsectra roseiventris (Kieffer, 1909)	Male adult	Norway, Hordaland, Øygarden, Turøy, 13.IV.2002, E. Stur	To84	AM398721
Micropsectra schrankelae (Stur and Ekrem, 2006)	Male adult	Norway, Sogn og Fjordane, Aurland, Aurlandsvatnet, 21.VII.2001, T. Ekrem	To42	AM398722
Micropsectra schrankelae	Male adult	Norway, Sogn og Fjordane, Aurland, Aurlandsvatnet, 21.VII.2001, T. Ekrem	To43	AM398723
Micropsectra schrankelae	Male adult	Norway, Sogn og Fjordane, Aurland, Aurlandsvatnet, 21.VII.2001, T. Ekrem	To44	AM398724
Micropsectra schrankelae	Male adult	Germany, Bavaria, Berchtesgaden National Park, Herrenroint, Spring 24d, 15.V-15.VI.2001, E. Stur & S. Wiedenbrug	To47	AM398725
Micropsectra schrankelae	Male adult	Luxembourg, Diekirch, N Haerebierg, Rheocrene spring at Schmittenhaff, 27.VI.2002, T. Ekrem	To108	AM398726
Micropsectra seguyi (Casas and Laville, 1990)	Male adult	Germany, Bavaria, Berchtesgaden National Park, Schapbach spring, 27.V.-14.VI.2005, F. Eder	To333	AM398727
Micropsectra seguyi	Male adult	Germany, Bavaria, Berchtesgaden National Park, Schapbach spring, 27.V.-14.VI.2005, F. Eder	To334	AM398728
Micropsectra sofiae (Stur and Ekrem, 2006)	Male adult	Germany, Bavaria, Berchtesgaden National Park, Schapbach spring, 23.IX.2001, E. Stur & S. Wiedenbrug	To92	AM398729
Micropsectra sofiae	Male adult	Germany, Bavaria, Berchtesgaden National Park, Schapbach spring, 29.IX.2001, E. Stur & S. Wiedenbrug	To145	AM398730
Micropsectra sofiae	Male adult	Luxembourg, Gutland, SW Kopstal, Rheocrene spring, 25.VI.2002, T. Ekrem & E. Stur	To166	AM398731
Micropsectra sofiae	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, B2, 30.VII.-15.VIII.2005, K. Aagaard et al.	Sø11	AM398732
Micropsectra sofiae	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, B1, 30.VII.-15.VIII.2005, K. Aagaard & et al.	Sø12	AM398733
Micropsectra sp. A	Male adult, pex	Switzerland, Berner Oberland, Grimselpass, Oberaars Dam, Stream at Berghaus, 14.VII.2005, T. Ekrem	To336	AM398734
Micropsectra sp. A	Female pupa	Switzerland, Berner Oberland, Grimselpass, Oberaars Dam, Stream at Berghaus, 14.VII.2005, T. Ekrem	To349	AM398735
Micropsectra sp. B	Larva	Switzerland, Berner Oberland, Grimselpass, Oberaars Dam, Stream at Berghaus, 14.VII.2005, T. Ekrem	To350	AM398736
Micropsectra sp. C	Larva	Norway, Sør Trøndelag, Agdenes, Rockpools at lighthouse, 6.VIII.2004, T. Ekrem & E. Stur	To361	AM398737
Parapsectra mendli (Reiss, 1983)	Male adult	Germany, Bavaria, Berchtesgaden National Park, Herrenroint spring 308, 14.-28.VI.2005, R. Gerecke	To335	AM398738
Parapsectra nana (Meigen, 1818)	Male adult	Norway, Hordaland, Vaksdal, Bolstadfjorden at Straume, Sweep net, 17.V.2003, E. Stur & T. Ekrem	To135	AM398739
Parapsectra sp. A	Male adult	Norway, Hordaland, Ulvik, Finse, at Research Station, 17.VII.1999, E. Willassen	To39	AM398740
Parapsectra sp. A	Male adult	Norway, Hordaland, Ulvik, Finse, at Research Station, 17.VII.1999, E. Willassen	To40	AM398741
Parapsectra sp. A	Male adult	Norway, Sogn og Fjordane, Aurland, Vestredalsvatnet, 21.VII.2001, T. Ekrem	To55	AM398742
Parapsectra sp. A	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, C2, 04.-10.VII.2005, K. Aagaard et al.	Sø04	AM398743
Parapsectra sp. B	Male adult	Germany, Bavaria, Murnauer Moos, Rollischsee, 26.V.2005, E. Stur & T. Ekrem	To307	AM398744
Paratanytarsus austriacus (Kieffer, 1924)	Male adult, pex, lex	Germany, Bavaria, Ramsau, Hintersee, 6-12.VII.1999, T. Ekrem	To04	AM398745
Paratanytarsus austriacus	Male adult	Germany, Bavaria, Murnauer Moos, Fügsee, 27.VII.2005, T. Ekrem	To355	AM398746

(*continued on next page*)

Table 1 (*continued*)

Taxon	Life stage	Locality	Voucher number	Accession number
Paratanytarsus austriacus	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, A1, 10.-19.VII.2005, K. Aagaard et al.	Sø01	AM398747
Paratanytarsus austriacus	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, A1, 10.-19.VII.2005, K. Aagaard et al.	Sø02	AM398748
Paratanytarsus austriacus	Male adult	Norway, Bear Island, Spælvatnet, Malaise trap, 12.-19.VII.2002, O.K. Berg & A. Finstad	Bj55	AM398749
Paratanytarsus austriacus	Male adult	Norway, Bear Island, Spælvatnet, Malaise trap, 12.-19.VII.2002, O.K. Berg & A. Finstad	Bj62	AM398750
Paratanytarsus bituberculatus	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To318	AM398751
Paratanytarsus dissimilis (Johannsen, 1905)	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To316	AM398752
Paratanytarsus grimmii (Schneider, 1885)	—	Australia, Greater Melbourne Area (Carew et al., 2005)		AY752669
Paratanytarsus grimmii	Female adult, pex	Norway, Bergen, University of Bergen, Dept. of Zoology, Wet Lab., I.2001, G. A. Halvorsen	To18	AM398753
Paratanytarsus hyperboreus (Brundin, 1949)	Male adult	Norway, Sogn og Fjordane, Aurland, Aurlandsvatnet, 21.VII.2001, T. Ekrem	To45	AM398754
Paratanytarsus intricatus (Goetghebuer, 1921)	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To317	AM398755
Paratanytarsus laetipes (Zetterstedt, 1850)	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To319	AM398756
Paratanytarsus laetipes	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To320	AM398757
Paratanytarsus laetipes	Male adult	Germany, Thuringia, Hainich National Park, Silbersee, 18.VI.2005, leg. E. Stur & T. Ekrem	To321	AM398758
Paratanytarsus natvigi (Goetghebuer, 1931)	Male adult	Norway, Sogn og Fjordane, Aurland, Aurlandsvatnet, 20.VII.2001, T. Ekrem	To50	AM398759
Paratanytarsus setosimanus (Goetghebuer, 1933)	Male adult	Norway, Sør Trøndelag, Agdenes, Rockpools at lighthouse, 6.VIII.2004, T. Ekrem & E. Stur	To359	AM398760
Paratanytarsus setosimanus	Female pupa	Norway, Sør Trøndelag, Agdenes, Rockpools at lighthouse, 6.VIII.2004, T. Ekrem & E. Stur	To360	AM398761
Paratanytarsus setosimanus	Prepupa	Norway, Sør Trøndelag, Agdenes, Rockpools at lighthouse, 6.VIII.2004, T. Ekrem & E. Stur	To362	AM398762
Paratanytarsus tenuis (Meigen, 1830)	Male adult	Norway, Hordaland, Kvam, Tørvikvatn, 24.VII.2001, G. A. Halvorsen	To61	AM398763
Paratanytarsus tenuis	Male adult	Norway, Hordaland, Kvam, Tørvikvatn, 24.VII.2001, G. A. Halvorsen	To63	AM398764
Paratanytarsus sp.	Larva	Norway, Sør Trøndelag, Agdenes, Rockpools at lighthouse, 6.VIII.2004, T. Ekrem & E. Stur	To363	AM398765
Rheotanytarsus pentapoda (Kieffer, 1909)	Male adult, pex, lex	Norway, Vest Agder, Søgne, Søgneelva, 5.VII.2001, T. Ekrem	To41	AM398766
Rheotanytarsus sp.	Larva	South Africa, Western Cape Province, Vogelgat Nature Reserve, Main Waterfall, 04.I.2005, T. Ekrem & E. Stur	To176	AM398767
Tanytarsus brundini	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, A2, 10.-19.VII.2005, K. Aagaard et al.	Sø05	AM398768
Tanytarsus brundini	Male adult	Norway, Sør Trøndelag, Brekken, Sørlende, B1, 30.VII.-15.VIII.2005, K. Aagaard et al.	Sø13	AM398769
Tanytarsus curticornis (Kieffer, 1911)	Male adult	Norway, Aust Agder, Valle, Flåni, 29.VI.2001, T. Ekrem	To82	AM398770
Tanytarsus mendax (Kieffer, 1925)	Male adult, pex, lex	Germany, Bavaria, Munich, Nymphenburger Park, Kleiner See, 24-28.VII.1999, T. Ekrem	To01	AM084268
Tanytarsus mendax	Male adult, pex, lex	Germany, Bavaria, Munich, Nymphenburger Park, Kleiner See, 13-19.VII.1999, T. Ekrem	To05	AM084269
Tanytarsus sp. A	—	Australia, Greater Melbourne Area (Carew et al., 2005)		AY752686
Tanytarsus sp. B	Male adult	Brazil, Sao Paulo State, Populina, Posto Amaral, Rio Grande, 14.VII.2002, A.R. Calor	To94	AM398771
Virgatanytarsus aboensis (Harrison, 2004)	Female adult, pex	South Africa, Western Cape Province, Franschhoek, tributary to Berg River, 5.I.2005, T. Ekrem & E. Stur	To198	AM398772
Virgatanytarsus aboensis	Male adult, pex, lex	South Africa, Western Cape Province, Franschhoek, tributary to Berg River, 5.I.2005, T. Ekrem & E. Stur	To304	AM398773
Virgatanytarsus aboensis	Larva	South Africa, Western Cape Province, Franschhoek, tributary to Berg River, 5.I.2005, T. Ekrem & E. Stur	To310	AM398774

Pex, associated pupal exuviae; lex, associated larval exuviae. Acronyms in voucher numbers: "To", sample processed by Torbjørn Ekrem; "Bj", sample from Bear Island ("Bjørnøya"); "Sø", sample from Sølendet Natural Reserve.

2.1.2. Analysis

Neighbour joining (NJ) and maximum parsimony analyses were conducted using PAUP* 4.10b (Swofford, 1998). Neighbour joining analysis was based on the Kimura 2-parameter (K2P) substitution model for easier comparison with other DNA-barcode studies. Initial maximum parsimony (MP) analysis used 100 random replicates in heuristic searches with TBR branch swapping and the *multrees* option in effect. Extensive searches used 1000 random replicates, keeping only 400 trees equal or longer length than 2416 steps to visit more 'tree-islands' without considerably increasing analysis time. Bootstrapping was performed with 1000 bootstrap replicates in NJ analyses and 250 bootstrap replicates on 100 heuristic search replicates in parsimony analyses. Identical sequences were deleted to increase speed of parsimony bootstrap analysis. Tree graphics were produced using TreeView (Page, 1996).

3. Results

Partial cox1 gene sequences were sequenced from 97 specimens of 47 species (Table 1), covering approximately 50% of the European species of *Micropsectra* and *Parata-nytarsus* (Stur and Ekrem, 2006; Sæther and Spies, 2004). The standard barcode primers worked well on templates from all tested species except for *Micropsectra calcifontis* (Stur and Ekrem, 2006). We observed no difference in amplification or sequencing success with extracts from different life stages.

Micropsectra sp. A, *Parapsectra* sp. A, *Parapsectra* sp. B and *Tanytarsus* sp. B are as yet undescribed species. We find it interesting to observe that a relatively small survey still reveals several morphospecies that have remained unnoticed in one of the best sampled regions in the world.

There were 273 variable sites in the 654 bp character matrix (41.7%), most of which occurred in the third codon position (Table 2). The sequences were heavily AT-biased, in particular in third position which showed a combined average composition of 87.3% (Table 2). Thus, it was not a surprise that plotting of third position transitions against K2P distances (not shown) indicated saturation of 3rd codon position transitions.

There were no identical gene sequences between species, thus all species were separable by genetic distance and character state differences (Figs. 1 and 2). Sequences of several specimens were identical within species, and genetic distances were usually considerably greater between than within morphological species (but see comments below on *Micropsectra notescens*). However, there was no clear-cut gap between intra- and interspecific genetic distance variation (Fig. 3). Average intra- and interspecific P-distances were 0.87% and 14.7%, respectively. K2P divergence varied from 0% to 4.9% within species with a maximum divergence average (coalescent depth) of 0.9%. Between species, the variation was from 5.1% to 25.2% (16.2% on average). Nevertheless, con-specific cox1 gene sequences mostly grouped with 100% bootstrap support in NJ and maximum parsimony analyses (91% for *Micropsectra sofiae*) (Fig. 2).

The NJ and MP consensus trees (Figs. 1 and 2) did not depict monophyletic genera and corresponded poorly with the authors' current knowledge on Tanytarsini phylogenies (Ekrem and Willassen, 2004; Stur and Ekrem, 2006). The MP analyses generated 381 different trees of equal length (2415 steps, CI 0.20, RI 0.68), and the strict consensus tree conflicted with the NJ tree in many respects. Nevertheless, all species except *M. notescens* were monophyletic.

The specimens identified as *M. notescens* divided into two separate clusters. Nucleotide sequences of these specimens differed by maximum 13.1% (K2P-distances) and in up to 78 nucleotide sites. The substitutions on all but one codon site (pertaining to the non-polar amino acids isoleucine and valine) are synonymous. These diverging specimens were collected from three populations in Germany, two in southern Bavaria and one in Thuringia, but representatives of both clusters were found in sympatry at the locality in Murnauer Moos (Table 1). The morphologically defined *M. notescens* appeared polyphyletic in the NJ and parsimony trees. We suspected that saturation of phylogenetic signal might affect the outcome of both NJ and MP trees, and therefore conducted separate analyses on a matrix confined to *Micropsectra* sequences only. This made *M. notescens* paraphyletic with *Micropsectra contracta* (Figs. 4 and 5).

4. Discussion

4.1. Technical feasibility of barcode identification in chironomids

One goal for this study was to test the effectiveness of the 'universal' barcode primers (LCO1490 and HCO2198) on chironomids. Primer design is critical for the success of large scale DNA-barcoding (Hajibabaei et al., 2005), and the method would probably be too awkward for routine applications if a large battery of special primers were

Table 2
Variable sites and average nucleotide composition in the analysed cox1 gene sequences

Nucleotide pos.	% Variable sites	% Adenine	% Cytosine	% Guanine	% Thymine
1st	22.5	28.9	18.1	26.3	26.7
2nd	0.4	14.2	26.2	17.0	42.6
3rd	98.6	42.6	10.0	2.7	44.7
All	41.7	28.6	18.1	15.3	38.0

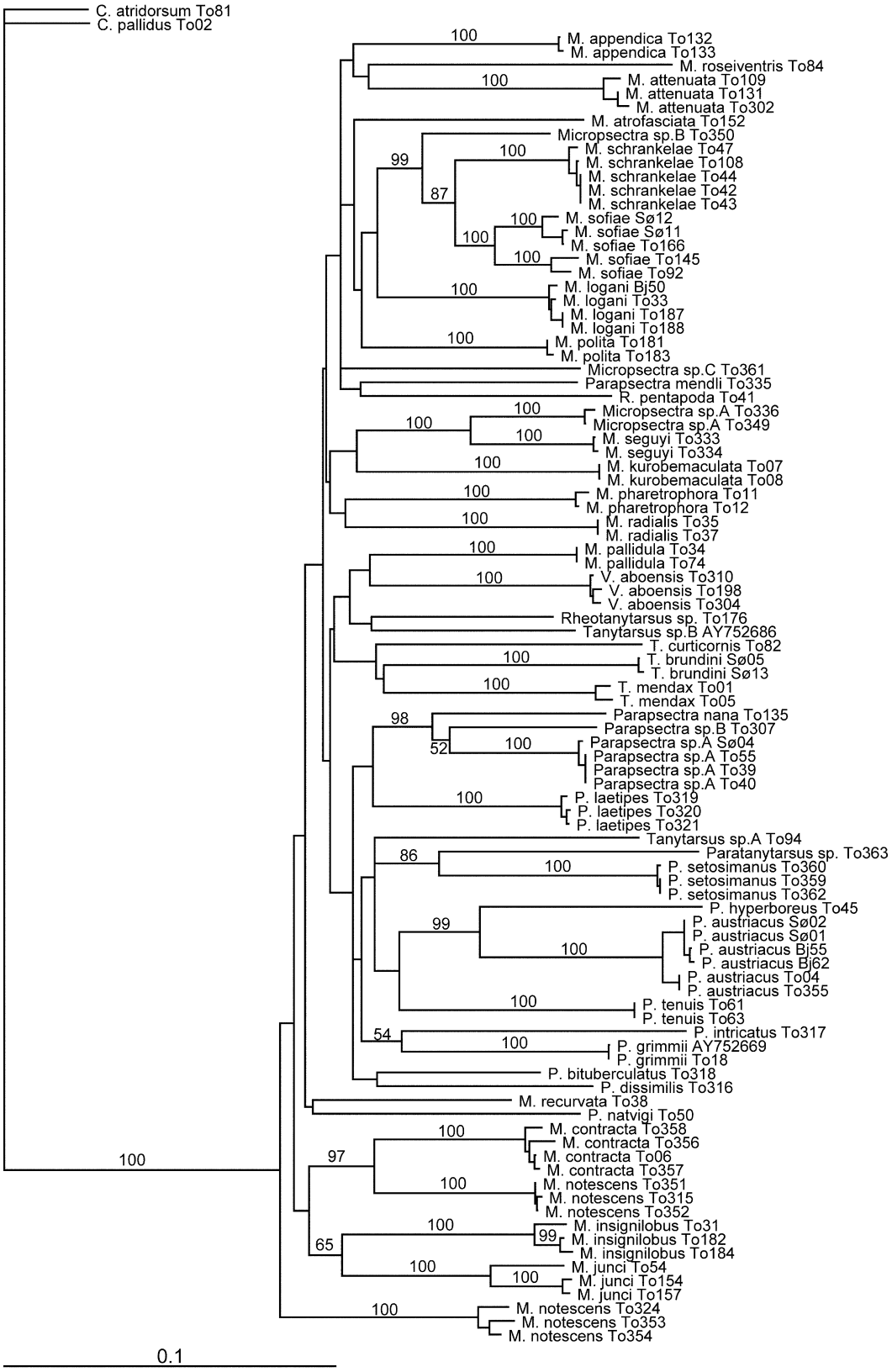

Fig. 1. Neighbour joining tree of partial cox1 sequences. Bootstrap values on branches. Scale = K2P-distance.

required to obtain sequences from various taxa. We have amplified partial cox1 gene sequences from 47 species of Chironomidae in this study, and only discovered one species from which cox1 was impossible to amplify with the standard barcode primers. The reason for failed amplification of our only template of *Micropsectra calcifontis* has yet

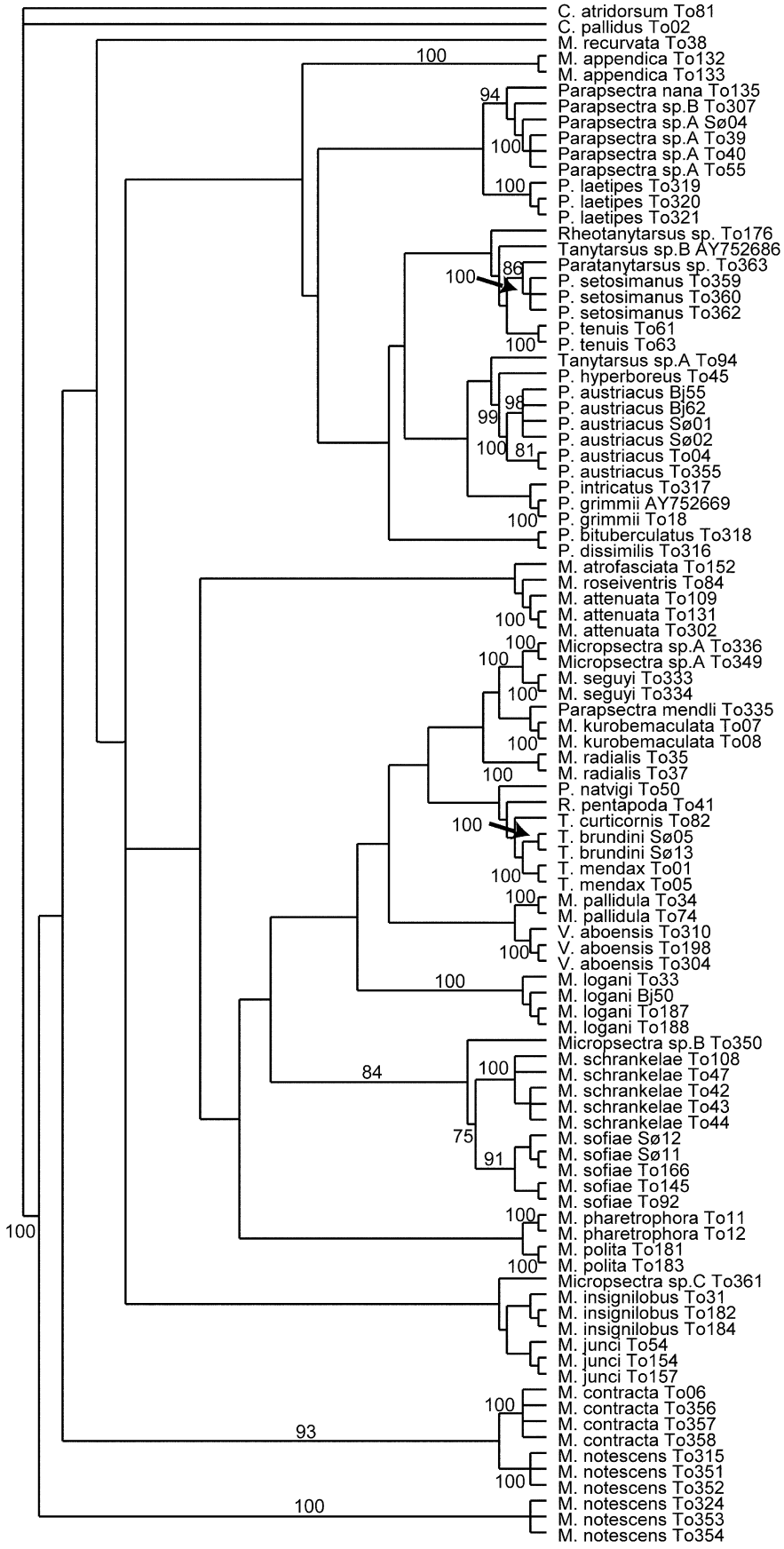

Fig. 2. Strict consensus tree from maximum parsimony analyses based on partial cox1 sequences. Bootstrap values on branches.

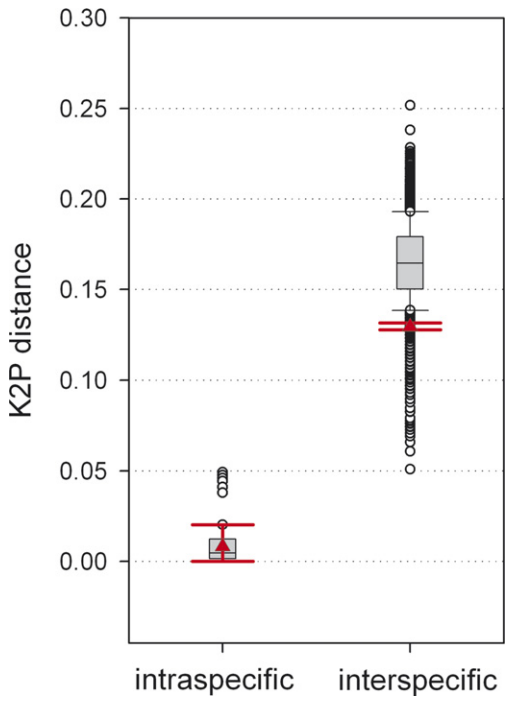

Fig. 3. Pairwise cox1 sequence differences within and between species based on the Kimura-2-parameter substitution model. Grey boxes are 75th percentiles, whiskers are 90th percentiles and dots are outliers. Broad whiskers represent the two paraphyletic 'mitotypes' of *Micropsectra notescens*.

to be evaluated, but it is probable that there is primer site incompatibility in this species since we have amplified the genes cox2 and 16s from the same DNA template. We have also tested these primers on chironomid species from other subfamilies (Diamesinae, Orthocladiinae and Tanypodinae), and the amplification success rate exceeds the desirable 95% limit for large scale DNA-barcoding (Hajibabaei et al., 2005). Importantly, no differences were detected in amplification success of different life stages. We have, however, mixed experience with PCR on various bulk fixed material and slight modification of standard sampling protocols for ecological studies might be necessary if barcode identification is going to be a useful direct tool for freshwater biologists.

4.2. The promise of perfectly matching sequences

When evaluating cox1 as a species identification tool, the case of *Paratanytarsus grimmii* warrants special comments because it may be seen as an ideal example of successfully implemented barcode philosophy. The fact that we found a perfect match between the cox1 sequence of a pre-identified specimen from Norway and an Australian sequence already filed in GenBank under the same species name (Carew et al., 2005) is clearly a success for the current state of biotechnology and a glossy demonstration of the potentials of identification via barcoding. It is worth mentioning that an indispensable component of conventional identification skills contributed to the revelation of this particular achievement. *P. grimmii* is known as a frequent pest in freshwater supplies (Langton et al., 1988) and more detailed studies should indicate whether the observed lack of genetic differences is caused by a recent origin of the wide distribution of this species.

We also observed no intraspecific divergence when comparing several other species (Figs. 1 and 3). This is more trivial when the sequences derive from the same population, but nonetheless important for the potential utility of barcoding in the identification of chironomids. Our preliminary results hence imply very promising prospects for the use of DNA barcodes as a means to identify species, at least in a local geographical setting. However, it is also required that barcodes robustly discriminate between inclusion and exclusion of group membership even when there is sequence variability within the group. For the purpose of identification of known species we would clearly prefer barcodes that are unique and non-overlapping for each species on a broad spatial scale. Disregarding *M. notescens*, we found that both NJ and MP analyses resulted in monophyletic species. We therefore predict that if a species is represented in a DNA sequence library, there is a very good chance for correct identification. However, due to low sample sizes intraspecific variation is probably underestimated for most of the taxa analysed in our study, and interspecific variation might be overestimated through undersampled true sister species pairs (Meyer and Paulay, 2005). Thus, broader sampling of most taxa in our study group may potentially result in narrower gaps, or even partial overlap, between intra- and interspecific variation (see Meyer and Paulay, 2005; Fig. 2).

4.3. Cryptic species and gene trees vs species trees

Although it has been emphasized that DNA barcoding is just a limited facet of taxonomy and systematics (Besansky et al., 2003), the difference between identification and classification is sometimes blurred in the barcode literature and cox1 sequencing has been suggested as a powerful tool for discovery of new species (Hebert et al., 2004a,b; Smith et al., 2006). Using this approach, candidates of cryptic species were detected among the specimens identified to *M. notescens* on the basis of morphology (Figs. 1 and 2). We were unable to find any morphological differences in the adult males, except that the Hainich specimens were about 5% smaller. Size is usually not considered to be a good diagnostic character for species of Chironomidae since size variation clearly is temperature and nutrient dependent (see Willassen, 1985; Kobayashi, 1998; McKie and Cranston, 2005). It remains to be seen if pupal characters provide support for morphological delineation of the two '*notescens* species'.

Studies of size related phenotypic plasticity may have contributed scepticism towards the taxonomic value of sophisticated morphometric analyses. Our findings of mitochondrial divergence in *Micropsectra* perhaps speak for

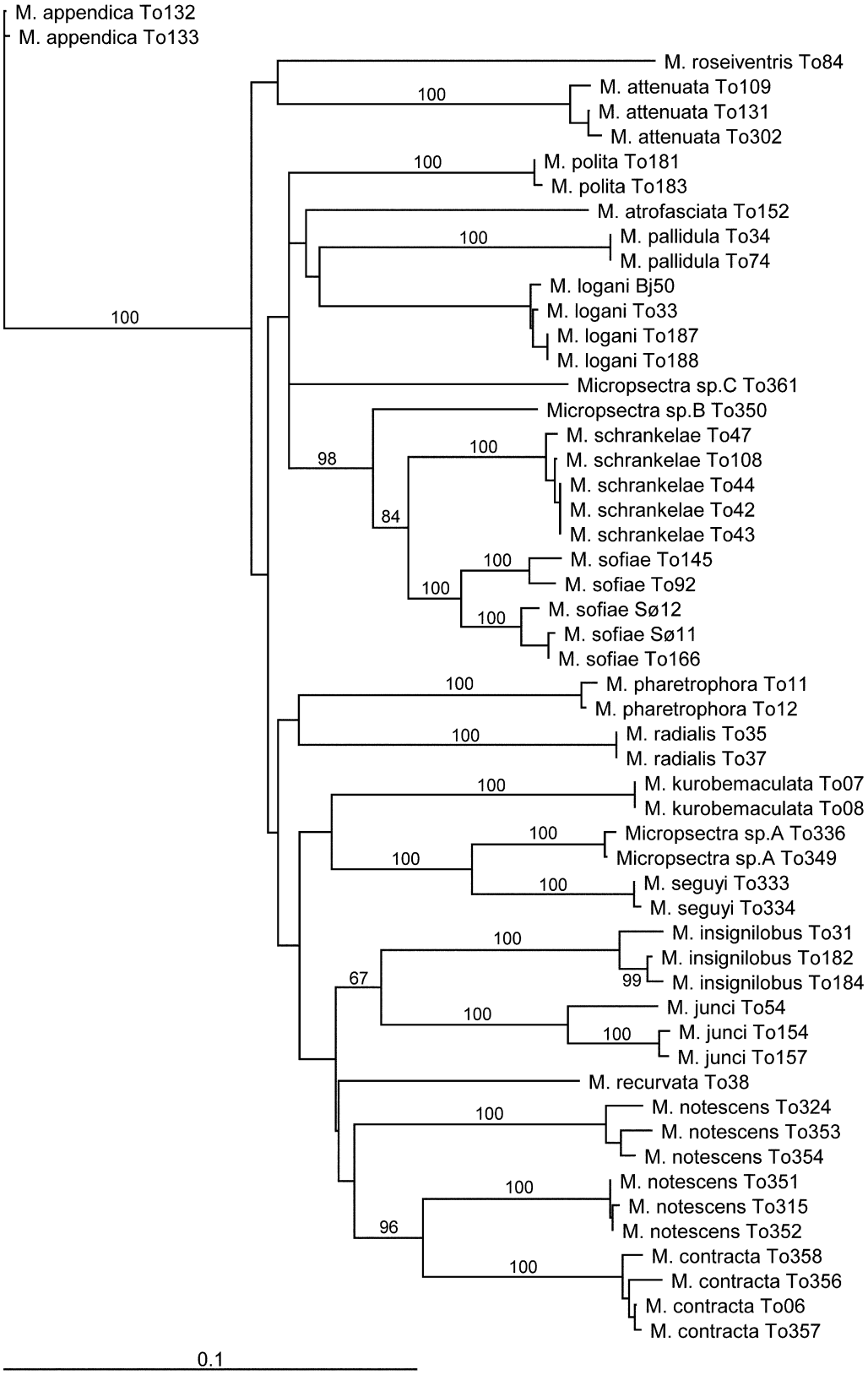

Fig. 4. Neighbour joining tree of partial cox1 sequences from *Micropsectra*. Scale = K2P-distance.

fine-scaled morphometrics and more detailed field studies including behavioural observations (e.g. Lindeberg, 1967). A considerable number of cryptic species have also been discovered by karyotype studies in the closely related tribe Chironomini, and similarity in cox1 sequences seem to almost perfectly reflect karyological species concepts in

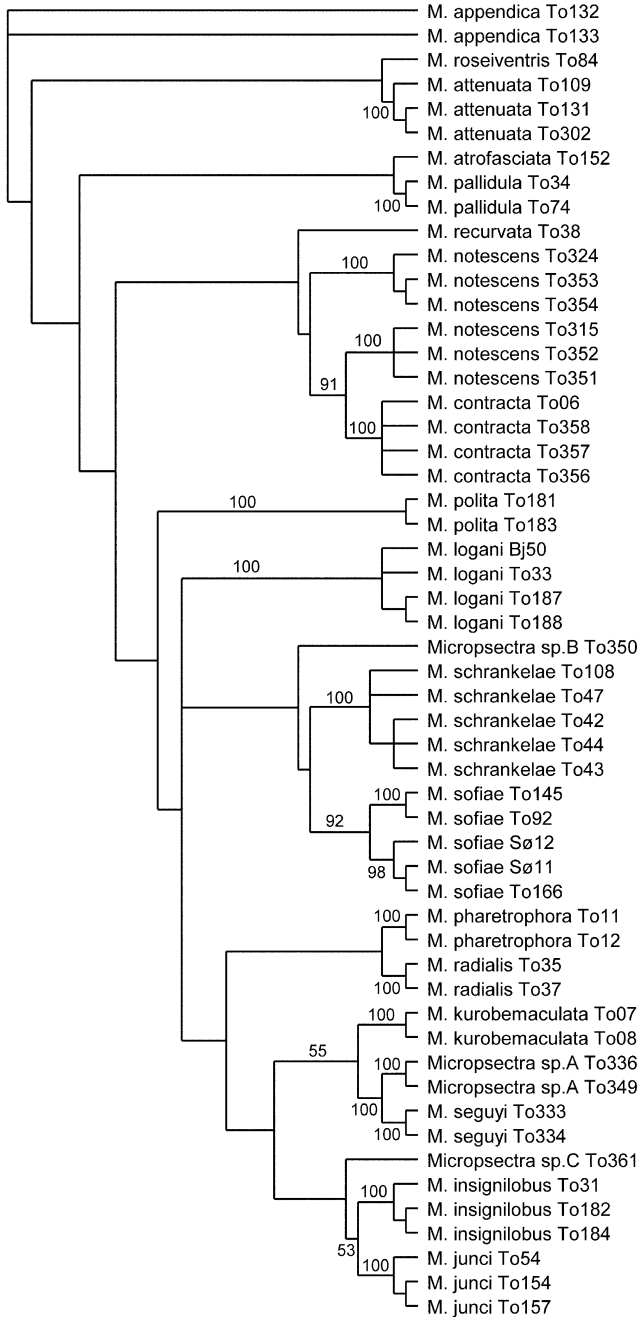

Fig. 5. Strict consensus tree from maximum parsimony analyses of partial cox1 sequences from *Micropsectra*. Bootstrap values on branches.

the morphologically challenging genus *Chironomus* (Pfenninger, in press). However, partial cox1 sequences do not necessarily reveal the whole truth of unknown species diversity. It may be argued that discovery of cryptic species based on non-monophyletic grouping in a NJ tree is resting on an underlying phylogenetic species concept. We see that *M. notescens* haplotypes become paraphyletic instead of polyphyletic when the analysis is confined to con-generic taxa only (Figs. 4 and 5). We ascribe the misleading tree pattern in Figs. 1 and 2 in part to saturation of phylogenetic signal and recognise that *M. notescens* might well be just a single species that has been subject to incomplete lineage sorting of an ancient polymorphism (Funk and Omland, 2003). Needless to say, caution should be exercised before such observations (Figs. 1 and 2) are interpreted in terms of cryptic species. There is a fair possibility that other genes will produce other tree topologies for *M. notescens* and a well-corroborated phylogeny including nuclear markers should clearly be preferred before taxonomic action is taken in terms of erecting new species based on a phylogenetic species concept. In fact, discordances between mitochondrial and nuclear genes are known from many taxonomic groups, and concordance of the two types of markers would certainly strengthen hypotheses on separate status of particular lineages. Nevertheless, barcoding surveys such as this seem effective in identifying evolutionary interesting 'problem taxa' that clearly deserve more attention with regard to the species genetics and ecology. Such discoveries are in our opinion scientifically intriguing and contribute to drive taxonomic research ahead.

4.4. The problem of unknowns

Many of the central issues in antagonist debates about the "promise and perils" of DNA barcoding (Besansky et al., 2003; Blaxter, 2004; Hebert and Gregory, 2005; Lorenz et al., 2005; Moritz and Cicero, 2004; Wheeler, 2005; Will and Rubinoff, 2004; Will et al., 2005) are essentially rooted in phylogenetics. It is the ambition of modern systematics to make classification systems that reflect the patterns of descent of taxa. However, cox1 gene trees may not be congruent with species trees in terms of monophyletic groups (e.g. Kizirian and Donnelly, 2004) and this is why available cox1 sequences may have poor predictive power in identification of unknowns. We observed that trees constructed on partial cox1 gene sequences did not reflect the taxonomical hierarchy of the study group. *Micropsectra*, *Parapsectra* and *Paratanytarsus* did not appear monophyletic, neither with NJ nor with character based maximum parsimony. Thus, the prospects of identifying a sequence to the correct genus or species group are not good unless a near perfect match is already represented in the DNA sequence library. This conclusion was incidentally confirmed by the results of the identification requests we made via the BOLD site (http://www.barcodinglife.org) in April 2006 on selected sequences produced in the present work: for *M. notescens* three haplotypes were identified to the genus *Tanytarsus* with 100% probability, whereas the other haplotypes came out as *Belvosia* (Diptera: Tachinidae) or *Tortricidia* (Lepidoptera: Limacodidae) with 100% probability. Provided that *M. notescens* is a single species, our results show that the problem also extends to the species category. The BOLD identification engine has been improved since our initial searches in April 2006 and the DNA sequence library on Chironomidae species has expanded considerably. Thus, the prospects of obtaining a correct identification of some chironomids by searching public DNA sequence databases are much better today. Our findings do, however, illustrate the more general point

also observed by Vences et al. (2005b): a comprehensive DNA sequence library is essential for correct identification to species, genus, family or even order level. Since cox1 at least in some cases seems to be a relatively poor predictor of placement in higher level taxa, we recommend that identifications by the BOLD facility must be cautiously evaluated as the system at present may return high probabilities of placements that obviously are erroneous. Is it, however, fair to expect correct identifications to be retrieved from a system that does not yet hold that information? We think not. After all, a traditional identification key to the birds of Europe may fail to identify windblown endemics from the Nearctic. Nevertheless, it is perhaps time to think about disclaimers on DNA based identifications or at least downscale the probabilities of correct species identifications from less comprehensive DNA libraries.

5. Conclusions

The partial cox1 gene sequences of the species included in this study clearly have sufficient variation for species discrimination (Figs. 1 and 2) and no species had identical sequences. Thus, the taxa examined to this point can be identified by character based genetic differences (DeSalle et al., 2005). The toll for the gain in discrimination power between species may be a loss of phylogenetic signal. Combined with incomplete lineage sorting of cox1 this may obstruct the placement of unknowns in the correct higher taxa. We see no other remedy to this difficulty than to provide unknowns with barcodes. As with commercial product barcodes, DNA barcodes have to be *defined* to represent known 'items', be it species, populations within species or cryptic taxa with unresolved taxonomic status. Despite the problems discussed, we are of the opinion that the establishment of a cox1 library for chironomids will be very useful for species identification. Effective and high throughput identification is essential for the success of large freshwater bio-monitoring projects, and DNA-barcoding is a powerful tool to do exactly this.

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References

Aagaard, K., Solem, J.O., Bongard, T., Hanssen, O., 2004. Studies of aquatic insects in the Atna River 1987–2002. Hydrobiologia 521, 87–105.

Asari, H., Kasuya, S., Kobayashi, T., Kondo, S., Nagano, I., Wu, Z.L., 2004. Identification of closely related *Hydrobaenus* species (Diptera: Chironomidae) using the second internal transcribed spacer (ITS2) region of ribosomal DNA. Aquat. Insects 26, 207–213.

Barrett, R.D.H., Hebert, P.D.N., 2005. Identifying spiders through DNA barcodes. Can. J. Zool. 83, 481–491.

Besansky, N.J., Severson, D.W., Ferdig, M.T., 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. Trends Parasitol. 19, 545–546.

Blaxter, M.L., 2004. The promise of a DNA taxonomy. Phil. Trans. R. Soc. Lond. B 359, 669–679.

Brodersen, K.P., Lindegaard, C., 1999. Classification, assessment and trophic reconstruction of Danish lakes using chironomids. Freshw. Biol. 42, 143–157.

Carew, M.E., Pettigrove, V., Hoffmann, A.A., 2003. Identifying chironomids (Diptera: Chironomidae) for biological monitoring with PCR-RFLP. Bull. Entomol. Res. 93, 483–490.

Carew, M.E., Pettigrove, V., Hoffmann, A.A., 2005. The utility of DNA markers in classical taxonomy: using cytochrome oxidase I markers to differentiate Australian *Cladopelma* (Diptera: Chironomidae) midges. Ann. Entomol. Soc. Am. 98, 587–594.

DeSalle, R., Egan, M.G., Siddall, M., 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Phil. Trans. R. Soc. B 360, 1905–1916.

Ekrem, T., Stur, E., in press. Description of *Tanytarsus hjulorum* new species, with notes and DNA barcodes of some South African *Tanytarsus* (Diptera: Chironomidae). In: Andersen, T. (Ed.) Contributions to the Systematics and Ecology of Aquatic Diptera—A Festschrift Honoring Ole A. Sæther, The Caddis Press, Ohio.

Ekrem, T., Willassen, E., 2004. Exploring Tanytarsini relationships (Diptera: Chironomidae) using mitochondrial COII gene sequences. Insect Syst. Evol. 35, 263–276.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Funk, D.J., Omland, K.E., 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 34, 397–423.

Gibbs, R.A., Cockerill, M., 1995. Sequencher. Gene Codes Corporation, Ann Arbor.

Hajibabaei, M., DeWaard, J.R., Ivanova, N.V., Ratnasingham, S., Dooh, R.T., Kirk, S.L., Mackie, P.M., Hebert, P.D.N., 2005. Critical factors for assembling a high volume of DNA barcodes. Phil. Trans. R. Soc. B 360, 1959–1967.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B 270, 313–321.

Hebert, P.D.N., Ratnasingham, S., deWaard, J.R., 2003b. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B (Suppl. 27), S96–S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S., Francis, C.M., 2004a. Identification of birds through DNA barcodes. PLoS Biol. 2, 1657–1663.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H., Hallwachs, W., 2004b. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. PNAS 101, 14812–14817.

Hebert, P.D.N., Gregory, T.R., 2005. The promise of DNA barcoding for taxonomy. Syst. Biol. 54, 852–859.

Kizirian, D., Donnelly, M.A., 2004. The criterion of reciprocal monophyly and classification of nested diversity at the species level. Mol. Phylogenet. Evol. 32, 1072–1076.

Kobayashi, T., 1998. Seasonal changes in body size and male genital structures of *Procladius choreus* (Diptera: Chironomidae: Tanypodinae). Aquat. Insects 20, 165–172.

Langton, P.H., Cranston, P.S., Armitage, P.D., 1988. The parthenogenetic midge of water supply systems *Paratanytarsus grimmii* Schneider (Diptera: Chironomidae). Bull. Entomol. Res. 78, 317–328.

Lindeberg, B., 1967. Sibling species delimitation in the *Tanytarsus lestagei* aggregate (Diptera, Chironomidae). Ann. Zool. Fenn. 4, 45–86.

Lorenz, J.G., Jackson, W.E., Beck, J.C., Hanner, R., 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. Phil. Trans. R. Soc. B 360, 1869–1877.

McKie, B.G., Cranston, P.S., 2005. Size matters: systematic and ecological implications of allometry in the responses of chironomid midge morphological ratios to experimental temperature manipulations. Can. J. Zool. 83, 553–568.

Meyer, C.P., Paulay, G., 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 3, 2229–2238.

Miller, K.B., Alarie, Y., Wolfe, G.W., Whiting, M.F., 2005. Association of insect life stages using DNA sequences: the larvae of *Philodytes umbrinus* (Motschulsky) (Coleoptera: Dytiscidae). Syst. Ent. 30, 499–509.

Moritz, C., Cicero, C., 2004. DNA barcoding: promise and pitfalls. PLoS Biol. 2, 1529–1531.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Paquin, P., Hedin, M., 2004. The power and perils of 'molecular taxonomy': a case study of eyeless and endangered *Cicurina* (Araneae: Dictynidae) from Texas caves. Mol. Ecol. 13, 3239–3255.

Pfenninger, M., Nowak, C., Kley, C., Steinke, D., Streit, B., in press. Utility of DNA-taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. Mol. Ecol.

Reiss, F., Säwedal, L., 1981. Keys to males and pupae of the Palaearctic (excl. Japan) *Paratanytarsus* Thienemann & Bause, 1913, n. comb., with descriptions of three new species (Diptera: Chironomidae). Ent. Scand. Suppl. 15, 73–104.

Sæther, O.A., Spies, M., 2004. Fauna Europaea: Chironomidae. In: de Jong, H. (Ed.), Fauna Europaea: Diptera: Nematocera. Fauna Europaea version 1.1, www.faunaeur.org.

Säwedal, L., 1976. Revision of the *notescens*- group of the genus *Micropsectra* Kieffer, 1909 (Diptera: Chironomidae). Ent. scand. 7, 109–144.

Sharley, D.J., Pettigrove, V., Parsons, Y.M., 2004. Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems. Austr. J. Entomol. 43, 359–365.

Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W., Hebert, P.D.N., 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). PNAS 103, 3657–3662.

Stoeckle, M., 2003. Taxonomy, DNA, and the bar code of life. Biosciences 53, 796–797.

Stur, E., Ekrem, T., 2006. A revision of West Palaearctic species of the *Micropsectra atrofasciata* species group (Diptera: Chironomidae). Zool. J. Linn. Soc. 146, 165–225.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Sunderland, Massachusetts.

Thomas, M., Raharivololoniaina, L., Glaw, F., Vences, M., Vieites, D.R., 2005. Montane tadpoles in Madagascar: molecular identification and description of the larval stages of *Mantidactylus elegans, Mantidactylus medacassus*, and *Boophis laurenti* from the Andringitra Massif. Copeia, 174–183.

Velle, G., Brooks, S.J., Birks, H.J.B., Willassen, E., 2005. Chironomids as a tool for inferring Holocene climate: an assessment based on six sites in southern Scandinavia. Q. Sci. Rev. 24, 1429–1462.

Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y., Vieites, D.R., 2005a. Performance of 16S RRNA in DNA barcoding of amphibians. Front. Zool. 2, article 5.

Vences, M., Thomas, M., Bonett, R.M., Vieites, D.R., 2005b. Deciphering amphibian diversity through DNA barcoding: chances and challenges. Phil. Trans. R. Soc. B 360, 1859–1868.

Verneaux, V., Verneaux, J., 2002. Assessing lake functioning using the macrobenthic community with special reference to Chironomidae (Diptera). A subalpine lake (Lake Annecy) as an example. Arch. Hydrobiol. 154, 61–78.

Wheeler, Q.D., 2005. Losing the plot: DNA ''barcodes'' and taxonomy. Cladistics 21, 405–407.

Will, K.W., Rubinoff, D., 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20, 47–55.

Will, K.W., Mishler, B.D., Wheeler, Q.D., 2005. The perils of DNA barcoding and the need for integrative taxonomy. Syst. Biol. 54, 844–851.

Willassen, E., 1985. (distributed 1986). A review of *Diamesa davisi* Edwards and the *davisi* group (Diptera,Chironomidae). Spixiana Suppl. 11, 109–137.

Willassen, E., 2005. New species of *Diamesa* (Diptera: Chironomidae) from Tibet: conspecific males and females associated with mitochondrial DNA. Zootaxa, 19–32.