

# Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species

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## Abstract

**Biodiversity studies require species level analyses for the accurate assessment of community structures. However, while specialized taxonomic knowledge is only rarely available for routine identifications, DNA taxonomy and DNA barcoding could provide the taxonomic basis for ecological inferences. In this study, we assessed the community structure of sediment dwelling, morphologically cryptic *Chironomus* larvae in the Rhine-valley plain/Germany, comparing larval type classification, cytotaxonomy, DNA taxonomy and barcoding. While larval type classification performed poorly, cytotaxonomy and DNA-based methods yielded comparable results: detrended correspondence analysis and permutation analyses indicated that the assemblages are not randomly but competitively structured. However, DNA taxonomy identified an additional species that could not be resolved by the traditional method. We argue that DNA-based identification methods such as DNA barcoding can be a valuable tool to increase accuracy, objectivity and comparability of the taxonomic assessment in biodiversity and community ecology studies.**

*Keywords:* *Chironomus*, community ecology, cytotaxonomy, DNA barcoding, DNA taxonomy

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## Introduction

Species level analyses are deemed indispensable to advance current issues in biodiversity studies, like community assembly, species richness estimators, global patterns of biodiversity or global change ecology (Gotelli 2004). However, the taxonomic identification of the usually systematically complex samples in such studies is rarely performed by respective specialists (Gaston & O'Neill 2004). Consequently, the taxonomic resolution level of large-scale routine identifications is often restricted to genera, families or even orders, at least for difficult groups. Additionally, the majority of species on this planet is lacking a formal taxonomic description and will thus not occur in published keys (May 1988). This so-called 'taxonomic impediment' is creating bias in biodiversity studies as the applied taxonomic resolution is crucial for the outcome (Bottger-Schnack *et al.* 2004; Waite

*et al.* 2004; Nahmani *et al.* 2006). DNA taxonomy (Tautz *et al.* 2003; Blaxter 2004) and DNA barcoding (Hebert *et al.* 2003) promise to be a feasible way out of this dilemma. The aim of DNA taxonomy is to find molecular defined operational taxonomic units (MOTU; Floyd *et al.* 2002; Blaxter *et al.* 2005) on the basis of sequence differences at short, orthologous marker gene sequences (Tautz *et al.* 2003; Blaxter 2004). This follows the general definition of operational taxonomic units (OTU) as groups of organisms used in a taxonomic study without designation of taxonomic rank. DNA barcoding, a system of species identification using a single gene [cytochrome oxidase I (COI)] was proposed by Hebert *et al.* (2003). Since then, the utility of DNA barcodes for species identification has been successfully demonstrated with several taxonomic groups (Hebert *et al.* 2004; Lambert *et al.* 2005; Ward *et al.* 2005; Hajibabaei *et al.* 2006), even though it received also considerable critique (e.g. Funk & Omland 2003; Meyer & Paulay 2005; Will *et al.* 2005).

In this study, we applied larval types, cytotaxonomy and DNA-taxonomy approaches to assess the regional community

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structure of a taxonomically difficult invertebrate group (Lindeberg & Wiederholm 1979), the dipteran genus *Chironomus* Meigen, a group of nonbiting midges. This species-rich genus has a worldwide distribution and is of substantial ecological relevance because of its abundant and ubiquitous occurrence in many freshwater habitats. Despite the highly similar resource use of *Chironomus* larvae and their morphological similarity, it is known that several species may co-occur at the same location (Tokeshi 1995c). As sediment dwellers, the composition of chironomid larvae communities has been considered to be among the most promising biological indicators of water and sediment quality (Lindegaard 1995). However, most chironomid larvae community studies have been hampered by the notorious difficulties of species level identification and were therefore restricted to higher taxa (Nyman *et al.* 2005) or larval types (Real & Prat 2000). Up to date, the cytotoxicological analysis of polytene chromosome banding patterns has been considered to be the only reliable method of *Chironomus* species identification because of the absence of conspicuous differentiating traits in both adults and larvae (Martin 1979). However, the method is time-consuming and requires considerable expertise, in particular for the interpretation of banding patterns. Consequently, only comparatively few ecological community studies on *Chironomus* have used this method (Butler *et al.* 2000), even though the taxonomic resolution may influence conclusions critically (Wymer & Cook 2003).

The goals of this contribution are threefold: first, we want to delineate MOTU in a regional sample of *Chironomus* larvae community using COI sequences. Second, if possible, the identified MOTU shall be linked to recognized species using cytotoxicology, phylogenetic inference and comparison to published COI sequences utilizing the DNA barcoding approach. Third, we want to compare tests for nonrandom

patterns in *Chironomus* community structure using the taxonomic resolution as suggested by larval types, cytotoxicology and DNA-based methods.

## Materials and methods

### Sampling

The sampling area lies in the middle of the upper Rhine valley in a rectangle of about 40–60 km between 49°09'–49°33'N and 8°10'–8°13'E (Fig. 1). It comprises the Rhine valley plain, in the west limited by the mountains of the Pfälzer Wald and in the East by the rising hills of the Odenwald range. The area is hydrologically characterized by the presence of many drainage ditches, slowly flowing small streams, temporary puddles, the oxbows and the main stream of the river Rhine. As *Chironomus* has active dispersal distances of several hundred metres and can be passively dispersed much farther by wind (Armitage 1995), we had no a priori reasons to assume that dispersal restrictions may have played a role in structuring the *Chironomus* assemblages on the chosen spatial scale, moreover since no obvious geographical obstacles to dispersal are present in the area.

The sampling took place from mid-September to November 2004, thus sampling the overwintering generation of *Chironomus* larvae (Tokeshi 1995a). Thirty-nine sampling sites were chosen opportunistically within the area, taking care to cover the area evenly. The different habitats present (puddles, ditches, streams and oxbows) were sampled approximately representative according to their abundance in the landscape. An area of 1 × 1 m was sampled with a 30 × 40 cm net of 0.5 mm mesh size. All instar stage 4 *Chironomus* larvae (L4) found, as identified by the presence of ventral tubuli, were brought alive into the laboratory and scored for their larval type.

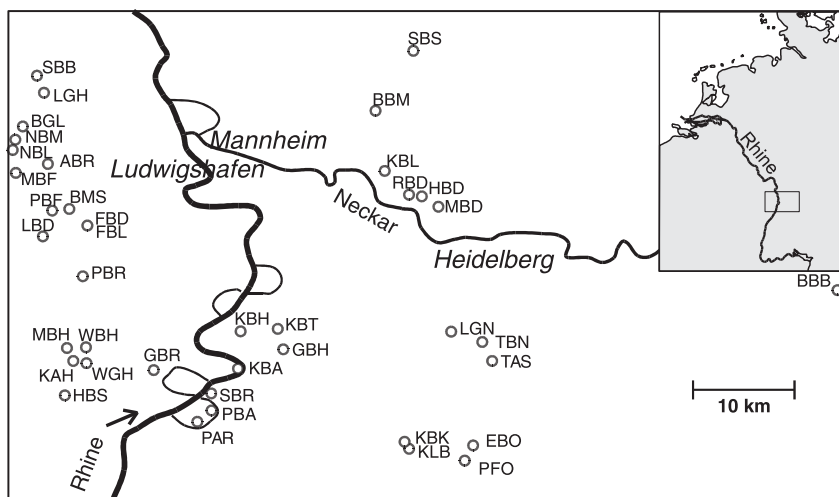


Fig. 1 Distribution of the sampling sites on the Rhine river plain in southwestern Germany.

*DNA isolation, COI and cyt b PCR and sequencing*

Larvae were kept in the laboratory for at least 5 days without feeding to remove potential (polymerase chain reaction) PCR-inhibiting substances from the gut (Carew *et al.* 2003). Head and first body segments were removed from each larva for polytene chromosome analysis and eventual museum deposition of the head capsule (voucher deposited at the Zoologische Staatssammlung, München). Remaining caudal tissue was homogenized in 700 µL standard cetyltrimethyl ammonium bromide (CTAB) buffer containing 0.1 mg/mL Proteinase K. After digestion for at least 1 h at 62 °C, chloroform/isoamyl alcohol 24:1 treatment was performed followed by 1 h precipitation at –20 °C. DNA pellets were washed twice with ethanol 70% and resolved in 30 µL water. COI fragments were amplified on a Tetrad PTC-225 Thermocycler performing initial five steps with 0.5 min 92 °C, 0.5 min 48 °C and 1 min 72 °C followed by 35 steps with 1 min 92 °C, 1 min 72 °C and 1.5 min 72 °C. Reaction mix contained 0.2 mM dNTPs, 3 mM MgCl<sub>2</sub>, 1 × reaction buffer (20 mM Tris-HCl, 50 mM KCl; Invitrogen), 0.3 µM of each *Chironomus* specific primer (forward: 5'-TCGAGCAGAA-TTAGGACGACC, reverse: 5'-AGGATCACCCACCA-GCAGG) and 1 U *Taq* DNA polymerase (Invitrogen) in a total volume of 10 µL. *Cyt b* amplification was performed in 15 µL reaction mix containing 0.13 mM dNTPs, 3.3 mM MgCl<sub>2</sub>, 1 × reaction buffer (Invitrogen), 0.4 µM of each primer (forward: 5'-TATGTTTTACCATGAGGACAAATATC, reverse: 5'-TATTTCTTTCTTATGTTTTCAAAC), 1.25 µL DMSO and 0.7 U *Taq* DNA polymerase (Invitrogen). About 40 ng of column-purified (EZNA Cycle-Pure Kit, Peqlab) DNA was used for cycle sequencing with the DTCS QuickStart sequencing kit (Beckman Coulter) following the instructions of the supplier and read automatically on a CEQ2000 capillary sequencer of the same manufacturer. Sequences were aligned using CLUSTAL W (Thompson *et al.* 1994) followed by manual corrections. Sequences were collapsed to haplotypes and deposited in GenBank (Accession nos DQ910547–DQ910729).

*Larval and cytotoxicological analysis*

The sampled L4 larvae were assigned to larval groups according to Kiknadze *et al.* (1991). The specific identity of a subsample of 100 individuals was determined using polytene chromosome analysis, following the protocol of Keyl & Keyl (1959). Briefly, salivary glands were prepared from fresh larval tissue and fixed in 50% acetic acid. Chromosomes were stained in 2% Orcein acetic acid for 15 min and fixed on glass slides for microscopical analysis. Expert cytotoxicological species determination was kindly performed by Wolfgang Wülker, Freiburg im Breisgau. Chromosome preparations were deposited together with the respective head capsules.

*MOTU delineation*

The MOTU delineation approach relied on sequence divergence. To this end, the most likely models of sequence evolution and their parameters according to the Akaike information criterion (AIC) were inferred for the COI data set using MODELTEST version 3.6 (Posada & Crandall 1998). The model was then used to compute pairwise sequence divergence estimates between all individuals. To visualize the results, an unrooted neighbour-joining (NJ) phenogram was constructed with PAUP 4.10b (Swofford 1998). Support of nodes by the data was estimated using the bootstrap (Felsenstein 1985). MOTU were then defined as least inclusive terminal groups (i.e. closest to the tips) with 90% bootstrap support or more, using 1000 bootstrap replicates. It should be noted that just as OTU in traditional taxonomy, MOTU thus do not necessarily equate to biological species, but should be treated rather as taxonomical hypotheses in need for additional evidence of their mutual reproductive isolation (Mayr 1969).

*Posterior assignment of MOTU to recognized species*

We checked whether the results of MOTU delineation and polytene chromosome analysis were congruent. To test the applicability of DNA barcoding, COI sequences of the sample specimen were assigned to sequences of cytotoxicologically identified specimen using the program *taxi* (Steinke *et al.* 2005). This program calculates sequence divergences between a query sequence (taxon to be barcoded) and each of a set of reference sequences defined by the user. Sequence divergence was calculated according to the best-fit model inferred by MODELTEST. Respectively, the first individual of each nominal species as identified by polytene chromosome analysis was arbitrarily taken as the reference sequence against which all other (query) sequences were tested. Additionally, the *Chironomus* s. str. COI sequences published in Guryev *et al.* (2001; GenBank Accession nos AF192187–AF192217) were added to the reference sequence set. The specific identity of these individuals was also established with cytotoxicology.

*Phylogenetic relations of inferred taxa*

The inferred MOTU were placed in the phylogenetic context of the genus *Chironomus*. We used the mitochondrial COI and cytochrome *b* (*cytb*) sequences of the *Chironomus* s. str. species published in Guryev *et al.* (2001; Accession nos AF192156–AF192185) to construct a phylogeny, including the respective sequences of a representative individual of each MOTU inferred here. Guryev *et al.* (2001) identified the specimen used in their study also by cytotoxicological analysis, thus ensuring the comparability of taxonomic assignments. We used a Bayesian approach of statistical

phylogeny reconstruction. To this end, the most likely models of sequence evolution and their parameters according to the AIC were inferred for each DNA data partition (596 bp COI, *cytb* 685 bp) using MODELTEST version 3.6 (Posada *et al.* 1998). The weighted average model parameters (Posada & Buckley 2004) were used as priors in the Bayesian analysis. A 99.9% credible set of phylogenetic trees was estimated with the program MRBAYES (Ronquist & Huelsenbeck 2003) by sampling the tree space using a Metropolis-coupled Markov chain Monte Carlo. Initial runs as well as a posterior inspection of the likelihoods in the final run showed that a burn-in phase of 10 000 generations was largely sufficient for the likelihood values to reach convergence. The chain was run for 10 million generations and sampled every 100th generation. A majority-consensus tree, rooted with *Archaeochlus drakensbergensis* as outgroup, was computed from the sampled trees, excluding the trees sampled in the burn-in phase.

#### Assessment of community structure

For each taxonomic technique, the species-site matrix was analysed by detrended correspondence analysis (DCA), an eigen analysis ordination technique based on reciprocal averaging (Hill & Gauch 1980). The abundance data were logarithmically transformed to obtain normally distributed variables and rare species were down-weighted. Detrending was performed by segments and nonlinear rescaling of axes. Calculations were performed in CANOCO 4.5 (Ter Braak & Smilauer 2002).

We used a permutation approach to test for nonrandom patterns of species co-occurrence in a presence/absence matrix. As test statistic, we have chosen the number of pairs of checkerboard species and the C-score. The first index is the number of mutual absences from all sites for all unique pairs of species in the assemblage (Diamond 1975). The second index, introduced by Stone & Roberts (1990), quantifies the average amount of co-occurrence among all unique pairs of species in the assemblage. In a competitively structured community, both the observed number of checkerboard pairs and the C-score should be significantly larger than expected by chance. We have tested the observed statistics against a null hypothesis of random community assembly. The associated null distribution was simulated by 9999 permutations with fixed row and fixed column sums, as recommended in the manual of the ECOSIM 7.0 software (Gotelli & Entsminger 2001).

## Results

#### Sampling results

The number of *Chironomus* larvae found at 39 sampling sites ranged from 5 to 16 individuals with a mean of  $10.1 \pm 5.5$  (mean  $\pm$  s.d., Table 1). In total, 432 *Chironomus* larvae were

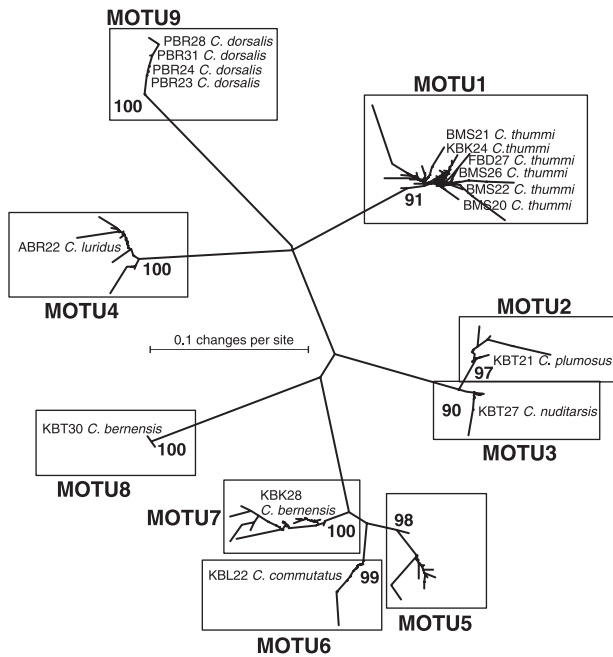
**Table 1** Sampling site abbreviations, their geographical position and the number of *Chironomus* L4 larvae found

Sampling site	Latitude	Longitude	No. of larvae found
ABR	49.4777	8.3207	12
BBB	49.3677	9.0267	12
BBM	49.5268	8.6142	13
BGL	49.5121	8.2977	13
BMS	49.4380	8.3392	12
EBO	49.2263	8.7006	6
FBD	49.4363	8.3249	10
FBL	49.4246	8.3557	8
GBH	49.3144	8.5307	10
GBR	49.2932	8.4151	5
HBD	49.4499	8.6542	8
HBS	49.2730	8.3339	8
KBA	49.2950	8.4909	5
KBH	49.3296	8.4913	26
KBK	49.2283	8.6405	7
KBL	49.4719	8.6223	9
KBT	49.3313	8.5248	19
KLB	49.2264	8.6429	7
LBD	49.4145	8.3165	11
LGH	49.5431	8.3168	23
LGN	49.3291	8.6814	7
MBD	49.4408	8.6674	11
MBF	49.4710	8.2921	13
MBH	49.3033	8.3440	9
NBL	49.4996	8.2911	13
NBM	49.4919	8.2894	9
PAR	49.2456	8.4579	10
PBA	49.2582	8.4700	5
PBF	49.4363	8.3249	12
PBR	49.3789	8.3512	14
PFO	49.2137	8.6936	11
RBD	49.4519	8.6422	13
SBB	49.5579	8.3099	15
SBR	49.2749	8.4628	12
SBS	49.5813	8.6471	21
TAS	49.3025	8.7166	6
TBN	49.3207	8.7079	10
WBH	49.3145	8.3361	6
WGH	49.3003	8.3542	11
			$\Sigma$ 432

investigated. We found two larval types, the *thummi*-form (73.2%) and the *plumosus*-form (26.8%). Seventy-eight out of 100 chromosome preparations were of sufficient quality for species identification. Cytotaxonomical analysis revealed the presence of seven chromosomal *Chironomus* species: *C. thummi*, *C. bernensis*, *C. luridus*, *C. plumosus*, *C. nudatarsis*, *C. commutatus* and *C. dorsalis*.

#### MOTU delineation with DNA taxonomy

Forward COI sequencing resulted in readable sequences of at least 416 bp length. The 436 COI sequences obtained



**Fig. 2** Neighbour-joining phenogram of ML-distances among *Chironomus* COI haplotypes. MOTU were inferred as least inclusive terminal groups with bootstrap values of 90% or more. Position of haplotypes from cytotaxonomically determined individuals are indicated.

were collapsed into 190 different haplotypes, which were used for subsequent analyses. The sequence evolution model chosen by the AIC was the general time reversible model with invariant sites and gamma distributed rates (GTI + I + G). The neighbour-joining tree of the pairwise sequence divergences showed nine terminal clades with at least 90% bootstrap support (Fig. 2). These clades were defined as MOTU1-9. The mean uncorrected sequence divergence within MOTU ranged from 0.004 (MOTU8) to 0.081 (MOTU6). The smallest among MOTU mean distance observed was 0.057 (between MOTU2 and MOTU3). Average uncorrected mean distance among two MOTU was 0.156.

### Species identification

PCA and MOTU yielded consistent results in the sense that all inferred MOTU harboured only a single polytene chromosome identified species. Chromosomal *C. thummi* individuals appeared only in MOTU1, *C. plumosus* in MOTU2, *C. nuditarsis* in MOTU3, *C. luridus* in MOTU4, *C. commutatus* in MOTU6, and *C. dorsalis* in MOTU9. One of these chromosomal species, however, occurred in two MOTU: chromosomal *C. bernensis* individuals belonged to the highly divergent MOTU 7 and 8 (Fig. 2). It was therefore possible to conclude from these inferred MOTU on the chromosomal species, but not necessarily vice versa. We did not obtain chromosome preparations for MOTU5.

### Posterior identification of taxa with barcoding methods

All haplotypes were assigned to reference sequences of nine different taxa. In all but one case, the most similar reference sequence was identical with the cytotaxonomic identification. For the exception MOTU8, the most similar reference sequence was derived from *C. heterodentatus*. MOTU5, for which no chromosomal identification was available, was identified as *C. annularius*.

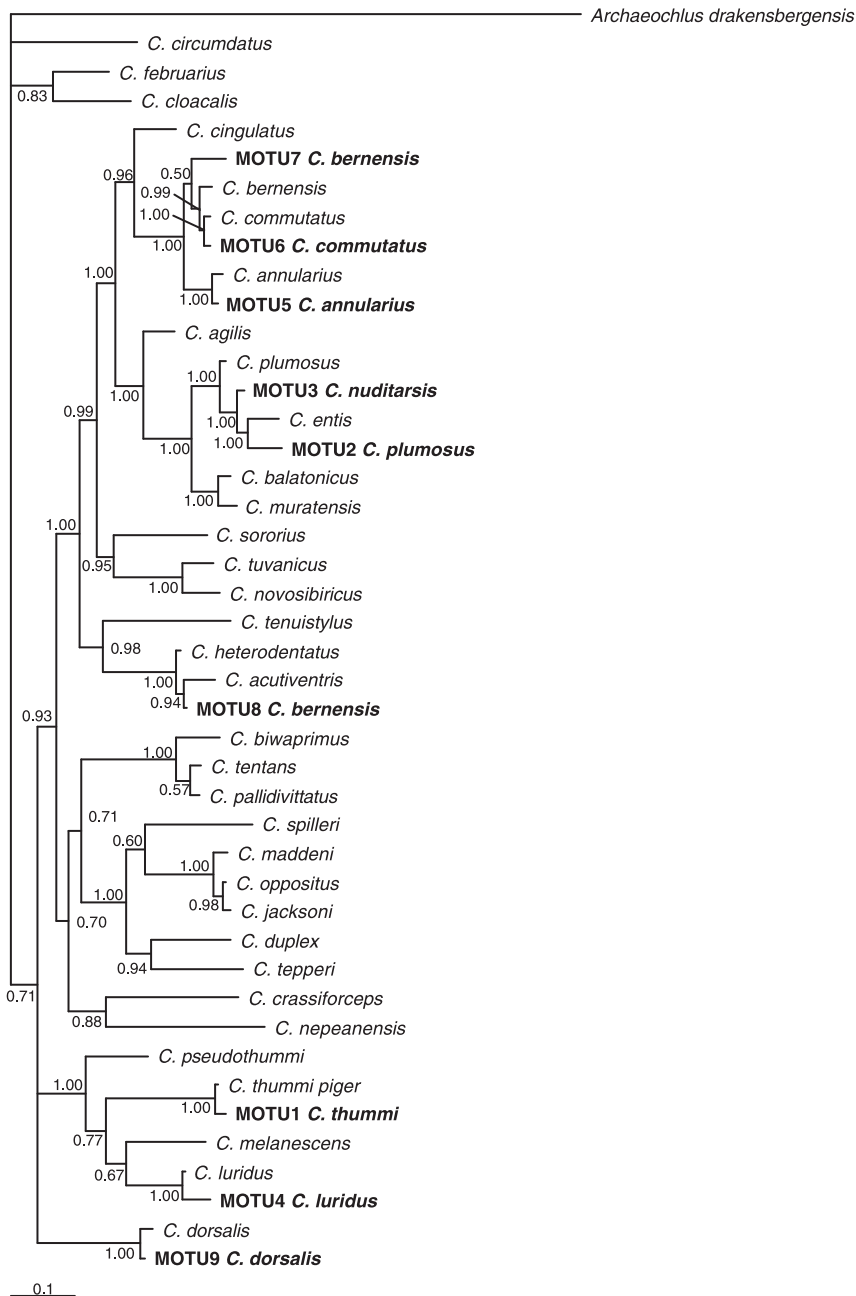
### Phylogenetic relations of inferred taxa

Four of the nine inferred MOTU grouped monophyletically with posterior probabilities of 1.00 with individuals of the same nominal species as identified by Guryev *et al.* (2001) (Fig. 3). One species identified here as *C. nuditarsis* had no nominal counterpart in the phylogeny. MOTU5 was most closely related to *C. annularius*. The remaining three MOTU revealed some taxonomic problems, however. MOTU7 *C. bernensis*, grouped with little support closely to the respective nominal species as identified by Guryev *et al.* (2001), but formed no monophyletic clade. The MOTU8, identified by polytene chromosomes also as *C. bernensis* here, grouped in a divergent clade with *C. acutiventris* to which it showed a COI sequence divergence of 0.054. MOTU2, identified as *C. plumosus*, formed a monophyletic clade with the North American species *C. entis* with a sequence divergence of 0.102. *C. plumosus* as identified by Guryev *et al.* (2001) was two nodes distinct from this clade, each node supported by high posterior probability (Fig. 3).

### Inference of community structure using different taxonomic approaches

The species/site matrix for all taxonomic approaches can be found in Table 3. As larval type identification revealed only two forms, only a single DCA axis could be extracted, representing all inertia. Sampling sites with exclusively thummi-type larvae were grouped on the left side of the axis, sites harbouring also plumosus-type larvae according to the relative abundance more to the right (Fig. 4). Sites with only plumosus-type larvae were not encountered. According to this criterion, the average species richness per site was  $1.62 \pm 0.49$ .

*C. thummi* was with more than 60% by far the dominant species in the survey. No other species exceeded a proportion of more than 8% (Table 2). Polytene chromosome taxonomy-based DCA extracted two axes with eigenvalues larger than 0.1. These axes accounted for 40.0% of total variation in species data. Axis 1 opposed sampling sites where *C. commutatus*, *C. plumosus* and *C. bernensis* were frequent against sites with more *C. dorsalis* and *C. luridus*. Axis 2 distinguished then between the latter species (Fig. 4). The number of species found per site ranged from 1 to 5 (rarefied



**Fig. 3** Majority consensus tree of Bayesian phylogeny inference of *Chironomus* species, using mitochondrial COI and *cytb* sequences, using *Archaeochlus drakensbergensis* as outgroup. Inferred MOTU are indicated in bold face. Numbers at nodes denote their posterior probability of existence, given the data and the model of sequence evolution employed.

with 500 replicates to the smallest sample size of 5–3.36) with an average of  $2.03 \pm 1.06$  (rarefied  $1.68 \pm 0.72$ ).

DNA taxonomy resulted in a very similar ordination compared to the previous approach (Fig. 4). This was mirrored in the correlation coefficients of the site scores that were higher than 0.98 among the first two axes of the two approaches, respectively. The maximum species richness also reached 5 species (rarefied 3.52), but the average was slightly increased to  $2.10 \pm 1.19$  (rarefied  $1.71 \pm 0.84$ ).

Co-occurrence analyses are not feasible for two taxa, however, a  $\chi^2$ -test revealed that the larval types were not evenly distributed ( $\chi^2$  observed = 289,  $P < 0.0001$ ). For both

polytene chromosome identification and DNA taxonomy, the number of checkerboard species was significantly larger than expected by chance (Table 3). Also the C-score was significantly high for both taxonomic approaches.

## Discussion

### *Correspondence of MOTU and traditionally recognised Chironomus species*

Cytological differences exploited by cytotaxonomy have proved to be of great value for the discrimination of

**Table 2** Taxonomic identifications of *Chironomus* larvae per sampling site according to larval type, polytene-chromosome cytotaxonomy and DNA-barcoding

Larval-type	thummi	plumosus	plumosus	plumosus	plumosus	thummi	thummi	thummi	plumosus
Cytotaxonomy	<i>C. thummi</i>	<i>C. plumosus</i>	<i>C. nuditaris</i>	<i>C. luridus</i>	<i>C. annularius</i>	<i>C. commutatus</i>	<i>C. bernensis</i>	<i>C. bernensis</i>	<i>C. dorsalis</i>
DNA-taxonomy	MOTU1	MOTU2	MOTU3	MOTU4	MOTU5	MOTU6	MOTU7	MOTU8	MOTU9
	<i>C. thummi</i>	<i>C. plumosus</i>	<i>C. nuditaris</i>	<i>C. luridus</i>	<i>C. annularius</i>	<i>C. commutatus</i>	<i>C. bernensis</i>	?	<i>C. dorsalis</i>
Sampling site									
ABR	1	—	—	3	8	—	—	—	—
BBB	11	—	—	1	—	—	—	—	—
BBM	13	—	—	—	—	—	—	—	—
BGL	13	—	—	—	—	—	—	—	—
BMS	8	—	—	4	—	—	—	—	—
EBO	2	—	—	4	—	—	—	—	—
FBD	1	—	—	—	9	—	—	—	—
FBL	8	—	—	—	—	—	—	—	—
GBH	10	—	—	—	—	—	—	—	—
GBR	4	—	—	1	—	—	—	—	—
HBD	7	—	—	1	—	—	—	—	—
HBS	8	—	—	—	—	—	—	—	—
KBA	—	1	—	—	—	—	4	—	—
KBH	24	—	1	—	—	—	1	—	—
KBK	—	—	—	—	—	—	7	—	—
KBL	—	—	—	—	—	9	—	—	—
KBT	3	2	6	—	—	—	6	2	—
KLB	2	—	1	—	—	—	2	2	—
LBD	11	—	—	—	—	—	—	—	—
LGH	23	—	—	—	—	—	—	—	—
LGN	—	—	1	—	—	—	6	—	—
MBD	11	—	—	—	—	—	—	—	—
MBF	13	—	—	—	—	—	—	—	—
MBH	1	—	2	4	2	—	—	—	—
NBL	7	2	1	—	3	—	—	—	—
NBM	5	—	—	—	2	—	2	—	—
PAR	—	8	—	—	—	1	1	—	—
PBA	—	5	—	—	—	—	—	—	—
PBF	11	—	—	—	—	—	—	—	1
PBR	10	—	—	—	—	—	—	—	4
PFO	9	—	2	—	—	—	—	—	—
RBD	13	—	—	—	—	—	—	—	—
SBB	6	—	1	—	8	—	—	—	—
SBR	1	4	4	—	2	—	1	—	—
SBS	21	—	—	—	—	—	—	—	—
TAS	2	—	—	4	—	—	—	—	—
TBN	1	—	—	9	—	—	—	—	—
WBH	—	1	1	—	—	—	3	1	—
WGH	11	—	—	—	—	—	—	—	—

**Table 3** Result of co-occurrence analyses for cytotaxonomy and DNA taxonomy. See text for details

Index	Cytotaxonomy				DNA taxonomy			
	Observed index	Mean/variance simulated indices	<i>p</i> observed > expected	<i>p</i> observed < expected	Observed index	Mean/variance simulated indices	<i>p</i> observed > expected	<i>p</i> observed < expected
Checkerboard pairs	12	7.16/2.12	0.000	1.000	18	11.84/4.25	0.002	0.999
C-score	32.39	29.79/1.22	0.015	0.985	37.31	34.32/0.67	0.001	0.999

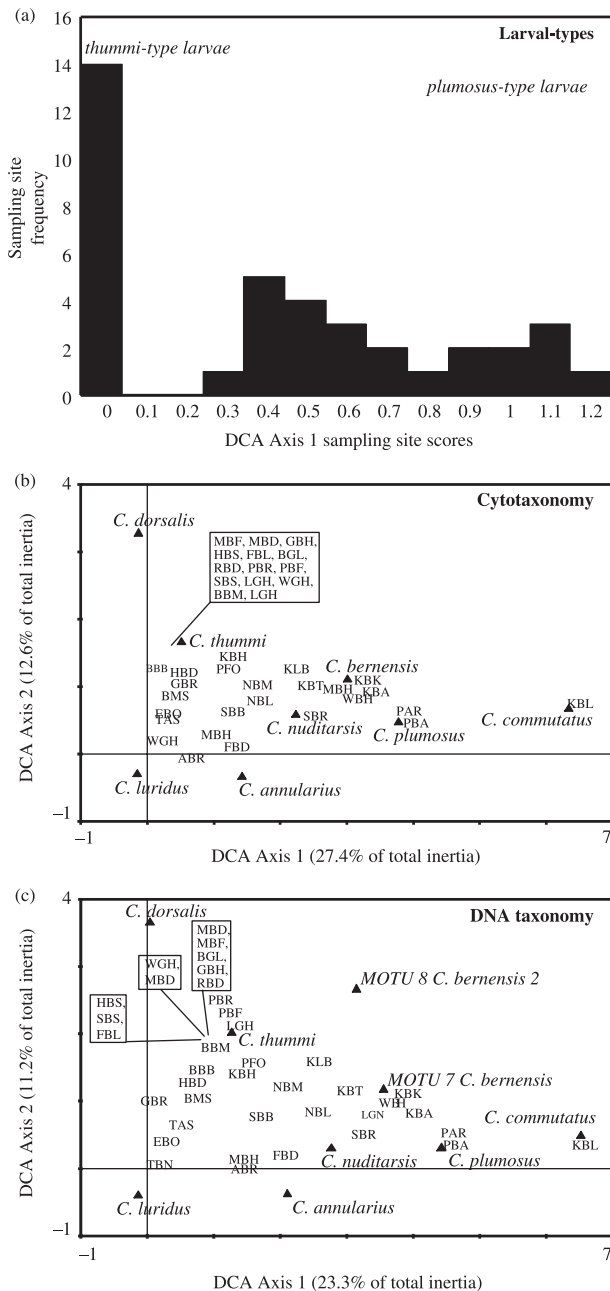


Fig. 4 Community structure of *Chironomus* larvae, inferred with detrended correspondence analysis, after (a) larval-type (b) cytotaxonomy and (c) DNA taxonomy.

morphologically similar *Chironomus* species, in particular their larvae (Martin 1979). These structural chromosomal mutations, like inversions, duplications, deletions and insertions allow a direct assessment of species status, because they confer mutual reproductive isolation and may be even the ultimate reasons for speciation (Michailova 1989). The chromosomal patterns are therefore the basis for the current taxonomy of the genus *Chironomus*. The majority of

MOTU as defined by the DNA-taxonomy approach corresponded to species as recognised by differing polytene chromosome banding patterns, thus linking DNA approaches with traditional taxonomy (Fig. 2).

Individuals with differing chromosome patterns belong to different species (Michailova 1989); the opposite, however, needs not necessarily be true: MOTU7 and 8 were both cytologically identified as *C. bernensis*, but proved highly divergent regarding their COI haplotypes. As their mutual sequence divergence (17.7%) is almost double as high as the divergence among, e.g. the well-defined *Chironomus* species *C. commutatus* and *C. annularius* (9.5%), we may assume their specific distinctness. This is supported by the phylogenetic analysis. MOTU7 formed no monophyletic group with the same nominal taxon of Guryev *et al.* (2001) and MOTU8 grouped with high support into a rather distantly related group with *Chironomus heterodontatus* and *Chironomus acutiventris*. Additional analyses would be necessary to clarify the relationship between MOTU8 and these latter taxa. *Chironomus bernensis* as defined by chromosomal banding patterns may thus constitute a cryptic species complex, also deserving additional attention.

Similar problems were encountered with *Chironomus plumosus*. The correspondingly identified individuals here and in Guryev *et al.* (2001) clustered not together in the phylogeny (Fig. 3). Polymorphisms in the cytogenetic structure of this widespread species are known (Butler *et al.* 1999; Gunderina *et al.* 1999) and may instead represent specific differences.

Our results indicate that the use of bootstrap values for the delineation of MOTU is useful, as it provides a statistical support assessment of the inferred unit by the data (Felsenstein 1985). This appeared particularly necessary in our case, because the readable sequences derived from unidirectional sequencing of the field samples were, with 416 bp, comparatively short. The unproblematic delineation has shown, however, that it is possible to obtain robust MOTU with such short sequences. A uniform sequence divergence threshold, as initially suggested by Blaxter (2004) is probably not adequate to delimit MOTU, because intraspecific population processes may differ among species, leading to highly variable coalescence times. The reciprocal monophyly criterion is thus probably better, as it avoids the problems associated with para- and polyphyletic species (Meyer & Paulay 2005).

#### DNA barcoding can re-identify the *Chironomus* species found by cytotaxonomy and DNA taxonomy

The posterior species identifications using sequence similarity to cytologically identified reference sequences yielded groups that were identical with the MOTU as defined by DNA taxonomy. The barcoding approach could therefore be used to link the COI sequences to the traditional

taxonomy. MOTU5, for which we lacked a polytene chromosome preparation here, is linked to the recognized species *C. annularius* via the chromosomal identification of the individual from which the reference COI haplotype was gained by Guryev *et al.* (2001). Even though our reference database contained only a small fraction of all described *Chironomus* species and identifications in an undersampled phylogeny without independent proof must be treated with caution (Meyer & Paulay 2005), the potential of the method for routine identification in ecological studies became obvious. The high success with which DNA barcoding correctly grouped the COI sequences is consistent with its success in identifying species in other studies on invertebrates (Hebert *et al.* 2003; Ball & Herbert 2005; Hogg & Hebert *et al.* 2004) and vertebrates (Hebert *et al.* 2004).

#### *Chironomus* larval communities showed signs of competitive structuring

Only two types of larvae were identified in the samples, making useful ecological inference impossible. This lacking taxonomic resolution was already viewed as problematic in studies of *Chironomus* community structures (Prat *et al.* 1983; Butler *et al.* 2000; Wymer & Cook 2003).

The resolution of cytotaxonomy was only slightly different from that of DNA taxonomy, yielding thus very similar results, even though the former achieved a less complete assessment of the *Chironomus* diversity present. The significant two DCA axes represented not more than 34.5% and 40% of total variation in species composition, respectively. The large residual variation could be due to stochastic variation in assemblage composition or sampling error. Sampling error might have been introduced by the comparatively low number of larvae found per sampling unit. This may be explained by our concentration on instar stage 4 larvae (L4) in order to yield comparable results with cytological taxonomic identification methods. Additionally, the sampling period was scheduled in autumn in order to avoid the large fluctuations in abundance among species throughout summer. However, the overwintering larvae will foster the next year's first generation and should thus include the majority of species present at a site (Tokeshi 1995a). Moreover, present knowledge about the dispersal biology of the Chironomidae argues for a substantial role of stochasticity in dispersal and colonization (Tokeshi 1995b; Delettre & Morvan 2000), and hence, in assemblage composition. As the amount of explained variance is in concordance with the results of comparable studies (Mousavi 2002; Nyman *et al.* 2005), the relative species abundances found here may thus be representative.

Most sites were inhabited by more than a single species (Table 2). Given the highly similar resource use of *Chironomus* larvae and the ability to quickly build up large populations, one should expect strong competition for food and space

among species within the same local habitat. Indeed, we found overall evidence for a competitive structure in the *Chironomus* assemblages surveyed (Table 3). Inspection of the pairwise C-score table revealed that this pattern is mainly due to three species pairs, MOTU1 *C. thummi* vs. MOTU2 *C. plumosus*, MOTU1 *C. thummi* vs. MOTU7 *C. bernensis* and MOTU4 *C. luridus* vs. MOTU7 *C. bernensis*. Because such a pattern could also be due to different habitat preferences, it is not possible to conclude from a mutual absence directly on competitive exclusion (Stone & Roberts 1990). Indeed, *C. thummi* was present at almost all but deep, permanent water bodies, which were preferred by *C. plumosus* and *C. bernensis*, arguing for different habitat preferences.

However, most of the species show no signs of competitive interaction. Their co-occurrence might therefore be possible due to temporal resource-use partitioning or microhabitat segregation (Giller & Malmqvist 1998). Another explanation for the coexistence of up to five congeneric species at the same sampling site may be due to chance processes in disturbance and recolonization regime or other fluctuations in the abiotic environment (Cornell & Lawton 1992). More detailed inferences about the factors shaping the structure of the *Chironomus* assemblages investigated would require the inclusion of environmental data, which is beyond the scope of this contribution.

#### Advantages of DNA taxonomy and barcoding for community ecology

To our knowledge, this is the first community ecology study that incorporates DNA taxonomy and barcoding. Several advantages of these methods became obvious in this study. Most importantly, it seems a feasible way to overcome the often bewailed taxonomic impediment to biodiversity studies (Gaston & O'Neill 2004; Gotelli 2004). Even bare of taxonomic knowledge about this notoriously difficult group, we would have drawn the same conclusions on the processes structuring larval community as a taxonomical specialist. Moreover, it seems necessary to distinguish between the processes of delineation of taxonomic units at the species level and their nominal identification. Even though it is deemed desirable in community ecology that the individuals in a sample are identified to the species level (Gotelli 2004), it is not inherently necessary for ecological process inference to assign Linnean names to the evolutionary relevant entities encountered. It would have thus not been mandatory to assign the inferred MOTU to described species to perform the ecological analyses. Therefore, the application of DNA taxonomy for ecological inference seems useful also for incompletely known taxa. A related issue in community ecology is the presumably great number of undescribed or cryptic species that consequently do not figure in determination keys (May 2004). Many other DNA-taxonomy studies have shown the

potential of the method to reveal this cryptic diversity (e.g. Pfenninger *et al.* 2003; Blaxter *et al.* 2004; Hebert *et al.* 2004; Hogg & Hebert 2004; Barber & Boyce 2006). As illustrated here with MOTU8, yet-unrecognized lineages will be inevitably identified by the objective delineation approach. The danger of assigning cryptic or not-quite fitting individuals to a species only because it is represented in the available key and thus losing relevant ecological and evolutionary resolution is greatly reduced. The possibility to recognize new taxa constitutes also an advantage over (restriction fragment length polymorphism) RFLP-based methods of identification (e.g. Carew *et al.* 2003; Sharley *et al.* 2004) for chironomids. We recognize, however, the danger that DNA taxonomy may also fail to resolve recently diverged taxa, especially if the species have ancestrally polymorphic mitochondrial haplotypes that do not sort according to subsequent speciation events (Funk & Omland 2003).

The evolutionary entities used in a study can later be unequivocally re-identified by their barcode sequence(s) (Hebert *et al.* 2002), and this regardless whether or not the inferred MOTU initially was matched to a contemporarily recognized species, belonged to a yet undescribed lineage or will be the 'victim' of a future taxonomic revision, which is in this study for example likely for MOTU2, assigned to *C. plumosus*. The need for species-level identification in biomonitoring is contentious (see Bailey *et al.* 2001; Lenat & Resh 2001), but DNA barcoding could provide the option of species-level identification when taxonomic discrimination at the species level is warranted. It could also ensure uniform quality of taxonomic results in studies where the quality of taxonomic data might be compromised by the inability to identify early instars, damaged specimens, or fragments of specimens (Stribling *et al.* 2003). Moreover, the increased taxonomic resolution delivered by DNA barcoding would provide more sensitive measures of the magnitudes and types of environmental impacts (Lenat & Resh 2001). In a way, DNA taxonomy and barcoding make thus ecological research independent of the imponderability of future taxonomical developments and keep ecological studies comparable over space and time.

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- 
- Markus Pfenninger is interested in processes structuring biodiversity on various spatial and temporal scales. The work is part of Carsten Nowak's PhD thesis on the anthropogenic impact on taxonomic and genetic variability in *Chironomus*. Christoph Kley performed his diploma thesis in this framework. Dirk Steinke works on practical and theoretical aspects at barcoding. Bruno Streit has a longstanding interest in aquatic community ecology.
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