European Mosquito Bulletin ***29*** *(2011), 22-37*
Journal of the European Mosquito Control Association
ISSN 1460-6127; w.w.w.e-m-b.org
First published online 18 March 2011

Distribution and frequency of *Culex pipiens* and *Culex torrentium* (Culicidae) in Europe and diagnostic allozyme markers

Thomas Weitzel, Kathrin Braun, Amandine Collado, Artur Jöst and Norbert Becker
German Mosquito Control Association (GMCA/KABS), Ludwigstr. 99, D-67165 Waldsee, Germany.
E-mail: info@kabs-gfs.de, internet: www.kabsev.de

Abstract

The morphologically similar *Culex pipiens* sensu lato and *Cx. torrentium* are sympatric in much of Central, Eastern and Northern Europe. Since morphological separation of females of these mosquitoes is extremely difficult and uncertain, diagnosis depends upon time consuming microscopic examination of male hypopygial characters. Consequently the possible, or even probable, occurrence of *Cx. torrentium* has been ignored in numerous field studies which have recorded (perhaps erroneously) presence of *Cx. pipiens* only. As a consequence, knowledge of the true geographical distribution, frequency of occurrence and ecological characteristics of these mosquitoes is deficient or confused.

In this study, the species diagnostic enzyme markers, adenylate kinase (AK) and 2-hydroxybutyrate dehydrogenase (HBDH) were shown to discriminate between *Cx. pipiens* and *Cx. torrentium* larvae as well as male and female adults. Diagnostic allele and genotype differentiations were quantified. In total, 4,040 mosquitoes of *Cx. pipiens* sensu lato and *Cx. torrentium* from Germany and other countries were studied.

Culex torrentium was shown to be frequently encountered in Central Europe. Though genetically and reproductively separated from *Cx. pipiens,* it exhibits similar ecological characteristics, and there is a high degree of association between these taxa in the breeding sites.

Of 2052 specimens collected from ovitraps in South-West Germany, 54.8% were identified as *Cx pipiens* and 45.2% as *Cx. torrentium*. In 712 samples taken from natural and artificial breeding sites, 59% were identified as *Cx. pipiens* and 41% as *Cx. torrentium*. In contrast, of adult specimens caught in CO_2-traps, only 3.9% were *Cx. torrentium,* and the other 96.1% were *Cx. pipiens,* indicating selective attraction exerted by the CO_2-traps. Both species were also found in other parts of Germany.

Neither *Cx. torrentium* nor *Cx. quinquefasciatus* were detected in samples obtained from Turkey, Cyprus, Greece, Serbia and South Eastern France, though all these contained *Cx. pipiens*. It was only in samples from Germany and Luxembourg that *Cx. torrentium* was frequent.

Key words: *Culex pipiens*, *Culex torrentium*, allozymes, genetic markers, adenylate kinase

Introduction

Culex pipiens L. is one of the most common and widespread Holarctic mosquitoes, with a distribution covering all temperate regions. Two biotypes with many behavioural differences are present in its Palaearctic distribution, the anautogenous, ornithophic *Cx. pipiens* nominate biotype, and the autogenous, strongly anthropophilic, *Cx. pipiens molestus* biotype. Formerly regarded as separate taxonomic entities, lack of diagnostic morphological characters led to the current classification. Though regarded as ornithophilic, occasional feeding on humans and other mammals has been observed by the nominate biotype (Gingrich & Casillas, 2004, Petric *et al.*, 1999). *Culex pipiens* biotype *molestus* is autogenous and, strongly anthropophilic. Formerly known as *Cx. molestus* Forskal, the absence of reliable morphological diagnostic characters led to its current taxonomic ranking (Harbach & Harrison, 1984, Becker *et al.*, 2010). The *molestus* biotype is largely an urban mosquito in the extensive northern parts of its distribution, where it is almost exclusively confined to hypogeal aquatic developmental sites, quite different from the surface or more or less elevated natural and container habitats used by the nominate biotype of *Cx. pipiens*

The morphologically similar *Culex torrentium* Martini occurs in Western Palaearctic regions where, due to its close resemblance to *Cx. pipiens*, the situation is confused. The species was not described until 1925, a date when mosquito systematics were already well advanced (Harbach *et al.*, 1985). The variable numbers and lengths of larval setae and observed differences in egg rafts morphology are not sufficiently diagnostic (Dahl, 1988). Becker *et al.* (2010) describe differences in the number of larval setae with diagnostic relevance, but well preserved samples are needed. Almost all morphological characters of males are variable and overlapping (Dahl, 1988). Mohrig (1969) mentioned differences in wing vein shape, but such characters can only be used by specialists. Morphological differences in the dorsal arms of the aedeagus of adult males are the only reliable diagnostic characters separating *Cx. torrentium* from *Cx. pipiens* (Mohrig, 1969, Becker *et al.*, 2010).

Field studies are difficult when morphological identification of species is tedious and unsure. Thus knowledge about the quantitative, spatial and temporal distribution of both species is incomplete and they may have been confused in former studies (Vinogradova *et al.*, 2007). These species are not separated in many publications and are sometimes handled as bundles of "*Cx. pipiens/torrentium"* (e.g. Schäfer *et al.*, 2004), or the presumptive existence of *Cx. torrentium* seems to have been neglected (e.g. Rydzdanicz & Lonc, 2003), or collectors were overwhelmed by high densities of *Cx. pipiens,* particularly when females from CO_2-trap collections were studied (Petric *et al.*, 1999). Therefore it is essential to consider genetic markers.

Vector surveys and control programmes require separation of the clearly ornithophilic *Cx. torrentium* and the partially anthropophilic *Culex pipiens*. Since West Nile virus outbreaks became epidemic in the USA and in parts of Europe, the zoogeography, taxonomy and identification of species of *Cx pipiens s.l.* and related taxa have received high attention. Sindbis virus has been detected recently in a *Cx. torrentium* population in Germany (Jöst *et al.*, 2010). Until it was established experimentally that *Cx. torrentium* was susceptible to Sindbis virus in Sweden, Norway and Russia, no differentiation of *Cx. torrentium* females from CO_2-trap catches was made (Lundström, 1994). Ornithophilic mosquito species may act as vectors between bird populations and species. Hence the potential of arbovirus transmission depends directly on the abundance of enzootic vectors. In addition to *Cx. torrentium,* the role of the nominate biotype of *Cx. pipiens,* has to be taken into consideration, since it has been found to be the most ornithophilic of eight common mosquito species, yet

also feeds on mammals (Gingrich & Williams, 2005). Other mosquito species with catholic host preferences, including the *molestus* biotype of *Cx pipiens*, may serve as bridge vectors between birds and man (Medlock *et al.*, 2005, Becker *et al.*, 2010).

Molecular techniques have recently been used to discriminate between these *Culex* mosquitoes. Miller *et al.* (1996) showed by ITS-sequences of rDNA, that *Cx. torrentium* is phylogenetically closest to the Culex Pipiens Complex. This was confirmed by Weitzel *et al.* (2009), but considerable genetic distances were measured. Microsatellite-sequences were analysed by Smith & Fonseca (2004) and variable restriction sites of the mitochondrial COI region were found to be valid for distinction between *Cx. pipiens* and *Cx. torrentium* (Shaikevich, 2007, Hesson *et al.*, 2010).

Enzyme electrophoretic methods have also been used to discriminate between morphologically similar *Culex* species, e.g. the *Culex sitiens* group (Chapman *et al.*, 2000), four *Culex* species from Florida (Knight & Nayar, 2004), *Cx. pipiens* and *Cx. quinquefasciatus* (Pryor & Daly, 1991, Urbanelli *et al.*, 1995, Cui *et al.*, 2007, Weitzel *et al.*, 2009) and between the nominate and *molestus* biotypes of *Cx. pipiens* (Lopatin, 1993, Chevillon *et al.*, 1995, Byrne & Nichols, 1999, Weitzel *et al.*, 2009).

Urbanelli *et al.* (1981) found diagnostic enzyme markers (AK-78, AK-100) to distinguish both species in Italy. Dahl (1988) mentioned allozymic differentiation at three loci between Swedish populations of *Cx. pipiens* and *Cx. torrentium*. Recently, Weitzel *et al.* (2009) published a comprehensive set of allozyme markers to distinguish *Cx. torrentium* from members of the *Culex pipiens s.l.* and other *Culex* species. Some of these are used in this study.

The basis of this study was the establishment of easily applicable genetic markers for diagnosis of *Cx. pipiens* and *Cx. torrentium,* as used by Weitzel *et al.* (2009). Allele and genotype distribution at both AK- and HBDH-loci will be described and quantified in geographical populations. Thousands of specimens of both sexes and of all developmental stage from different sources were screened for their species membership and correlated with their geographic distribution. Additional samples of presumptive *Cx. pipiens* (nominate biotype) / *Cx. torrentium* populations from Turkey, Cyprus, Greece, Serbia, France and Luxembourg were assayed. As control taxa for evaluation of the genetic markers employed, the anthropophilic *molestus* biotype of *Cx. pipiens* from underground pits and *Cx. quinquefasciatus* from different tropical and subtropical regions were employed.

The aim of the study was to estimate the local occurrence and the geographic distribution of both *Cx torrentium* and the nominate biotype of *Cx. pipiens*. Furthermore, the capability of CO_2-traps for representative recording of these two mosquitoes was analysed.

Materials and Methods

Mosquito collection

Most *Cx. pipiens* and *Cx. torrentium* samples were collected from localities in the Upper Rhine Valley, Germany. Three sampling methods were performed and the samples were stored in liquid nitrogen until used for enzyme electrophoresis.

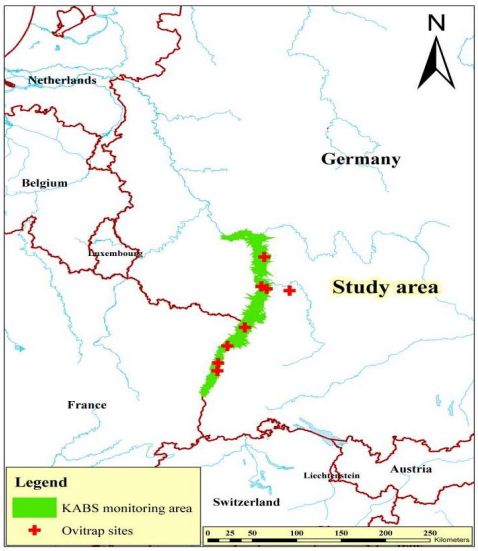

Fig 1. Area of sampling by ovitraps, dipping and CO_2-traps in South-West Germany (samples taken from other German and European regions not shown)

Fig 2. Ovitraps exposed in yards, gardens and natural habitats, either containing clear water or hay infusion**.**

Ovitraps: 96 buckets with black-painted bottoms were used as ovitraps scattered in eight communities over the study area in the manner of a transect (Fig 1). Each ovitrap contained 2 litres of either clear rain water or eutrophic hay infusion, (Fig 2). Larvae and eggs were collected from ovitraps every other week, i.e. nine times from May to September 2004, and reared to 4th instar larvae or adulthood. Four buckets were placed in the urban areas, four at the periphery of settlements where garden barrels were frequent, and another four in meadows and forests. During the two weeks in the ovitrap the larvae developed undisturbed by external influences. After removal of the larvae and eggs, ovitraps were refilled. Altogether, larvae were collected from 864 ovitraps during the season.

Natural and artificial breeding sites in Germany and other European countries: In total, samples from 46 different sites in Germany, Western Turkey, Cyprus, Greece, Serbia, South-eastern France and Luxembourg were collected by dipping in any type of natural and artificial breeding sites and sometimes by CO_2-traps. In urban areas, larvae were collected from barrels and open sewage reservoirs. Rural populations came from flood plains, ditches and ground pools in forests and meadows.

Anthropophilic *Cx. pipiens* biotype *molestus* served as control and was collected from four enclosed underground habitats (cesspits) in different localities in Germany. They were tested for autogeny by rearing them without a blood meal through two generations (Weitzel *et al.*, 2009). *Cx. quinquefasciatus* formed another control group and were collected in sewage systems in Luang Prabang, Laos and Los Angeles, USA. Additional *Cx. quinquefasciatus* samples were received from three breeding stocks of Bayer CropScience, Monheim, originating from Indonesia and Malaysia. They were identified by MDH (NADP) allozyme electrophoresis (Urbanelli *et al.*, 1997, Weitzel *et al.*, 2009) as well as by examination of the anatomy of the adult male hypopygia.

CO_2-traps: Presumptive *Cx. pipiens* (nominate biotype) / *Cx. torrentium* adult females were obtained from 189 CO_2-trap-collections (Bioquip) for four years from May to September throughout the study area in Germany (Fig. 1). In 2001 the whole GMCA-area along the Rhine River has been sampled and between 2002 and 2004 identical operations were carried

out in the Southern part along the French border. The traps were supplied with 1 kg dry ice and exposed without light from afternoon until the next morning. In total, 621 *Cx. pipiens / Cx. torrentium* individuals were taken from the traps and transported on dry ice to the laboratory.

Species identification and enzyme electrophoresis

A representative selection of 62 *Cx. pipiens* and 61 *Cx. torrentium* adult males from above ground breeding sites were identified by hypopygia anatomy (Mohrig, 1969, Dahl, 1988, Becker *et al.*, 2010) on a cooling plate (-10°C). Heads and thoraces were removed and used for allozyme analysis to elaborate the genetic enzyme markers, so ensuring their reliability for the study (Weitzel *et al.* 2009).

Adenylate kinase (AK, E.C. 2.7.4.3.) and 2-hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30) allozymes were used in this study to discriminate between *Cx. torrentium* and the *Cx pipiens* biotypes.

Sample preparation: Mosquitoes were homogenized by ultrasound (Bandelin sonopuls GM 70) in grinding buffer (10 mM Tris / citric acid, pH 7.5, 10 mM 2-mercaptoethanol) and centrifuged for 1 min at 16,000 rpm at 4°C in a Minifuge (eppendorf 5415R). Between 2 and 4 µl of supernatant per sample were used.

Electrophoresis: Horizontal thin 1% agarose gels (LEEO, Schreiber & Weitzel, 1995) were run at 500Vh and +4°C in Multiphor II electrophoresis units (LKB Pharmacia).
AK: Electrophoresis buffer was prepared with 0.155 M Tris, 0.05 M citric acid, pH 7.0 (250 ml for each buffer tank). The corresponding gels were prepared with 90 ml of 15.5 mM Tris, 5 mM citric acid; pH 7.0.

AK is a monomeric enzyme that exhibits a main band with a nongenetic (physiological) sub-band after electrophoresis. Thus, homozygous banding patterns consist of two bands (Fig 3) and heterozygous patterns of four bands.

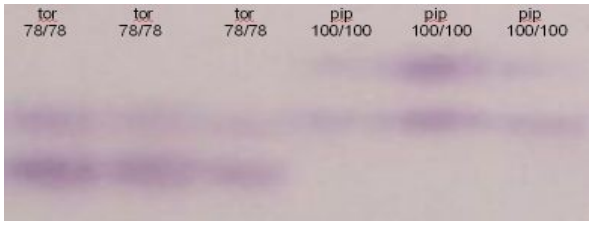

Fig 3. AK banding pattern

HBDH: 0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA and 0.01 M $MgCl_2$, pH 8.3 (250 ml for each buffer tank) and 90 ml of 10 mM Tris, 10 mM maleic acid, 1 mM EDTA and 1 mM $MgCl_2$, pH 8.3 for the agarose gel.
HBDH is an enzyme with a dimeric molecule structure, producing single bands when its underlying gene locus was inherited homozygous. Three bands are produced when it is heterozygous, with the intermediate band having a double staining intensity (Fig 4).

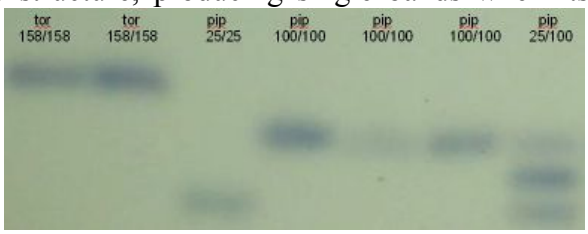

Fig 4. HBDH banding pattern

Staining recipes for AK followed Harris and Hopkinson (1976) protocol and Murphy *et al.* (1996) for HBDH.

Nomenclature of allozymes: Enzyme bands were labelled by their relative electrophoretic mobility. The most common allozyme in a *Cx. pipiens* biotype *molestus* reference population was named "100" in relation to its real migration distance. Other allozymes were named with numbers depending on their electrophoretic mobility relative to the most common allozyme.

e.g. the observed allozymes of AK were called AK-53, AK-78, AK-87, AK-100, AK-124 and AK-154, where AK-100 was the most frequent band in the *molestus* reference population. Genotypic combinations were indicated by the numbers of their respective allele compositions, e.g. AK-100-100 (homozygous) or AK 100-124 (heterozygous).

Results

Detection, application and reliability of genetic enzyme markers

Species diagnostic banding patterns of AK and HBDH were found to discriminate between individuals of *Cx. torrentium* and of *Cx. pipiens s.l.* The number of identified specimens, the genotype distributions and allele frequencies are presented in the appendix, together with calculations of expected genotype frequencies, variances and confidence intervals. Each enzyme system used separately provided sufficient diagnostic value, above 99% and up to 99.99% when used in combination. In some cases, HBDH staining reactions were too weak to be reliably read, mostly when larvae samples were used instead of adults, which explains why the number of examined specimens with HBDH is lower than with AK. Nevertheless, 4038 out of 4040 specimens (99.95%) could reliably be diagnosed and only 2 (0.05%) specimens remained unidentified.

AK genotype combinations specific for *Cx. pipiens s.l.* were found in 99.6% of the specimens of *Cx. pipiens.* The species specific genotype combination "100-100" was by far the most frequent (98.4%). The most common allele "100" was sometimes combined with the rare *pipiens*-alleles, "100-154" (0.28%) and "100-124" (0.66%). Other rare combinations were "124-124" (0.24%) and "78-124" (0.05%). All these combinations were found to be species specific for *Cx. pipiens*. The combination "78-100" (0.42%) was found rarely in *Cx. torrentium* (Fig 5, Table 4).

AK genotypes specific for *Cx. torrentium* were found in 98.9% of the specimens. The genotype combination "78-78" (88.0%) was the most common. Also the combination "53-78" (10.8%) was frequently found and typical for *Cx. torrentium*. The genotypes "78-87" (0.08%) and "78-100" (1.14%) were rare.

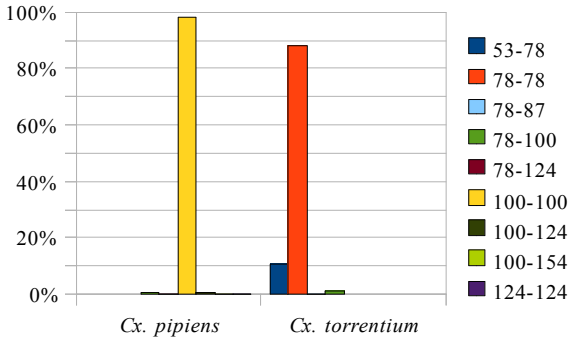

Fig 5. AK genotype distribution

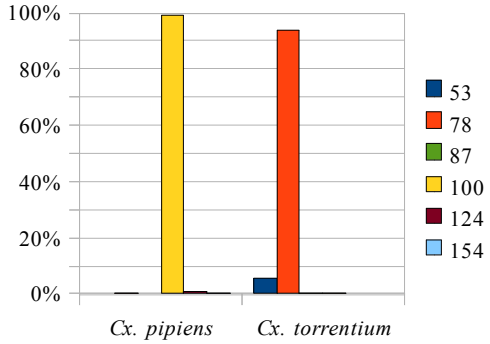

Fig 6. AK allele frequencies

Calculations of expected genotype frequencies, variances and confidence intervals of allele frequencies (Table 4, Table 5) demonstrate the high degree of reliability of the measured values based on the huge sample size and the optimized methodology. Deviations at rare variants are negligible for the purpose of species diagnosis. AK-100 was the most frequent allozyme in *Cx. pipiens* (in both the nominate and *molestus* biotypes) and in *Cx.*

quinquefasciatus, whereas AK-78 and, at a lower frequency AK-53, were typical in populations of *Cx. torrentium* (Fig 6, Table 5).

Every genotype combination provided 100% confidence in discriminating between *Cx. pipiens* and *Cx. torrentium* except "78-100". However, this genotype was rarely observed (with frequencies of 0.33% and 0.6% respectively in *Cx. pipiens* and *Cx. torrentium*: see appendix). Analysis with the HBDH marker system confirmed this species diagnosis. If both enzyme marker systems failed in identifying an individual it was removed from the analysis.

HBDH showed a high degree of species specific allele and genotype distribution and was found to differentiate between *Cx pipiens* and *Cx. torrentium* (Table 6, Table 7). Results from HBDH served as control of the AK-marker. The genotype distribution was as in AK, but with a high degree of intraspecific polymorphism and heterozygosity of individual alleles in *Cx. pipiens*. More than 99% of the results of HBDH reliably distinguished the species. The genotypic and allelic differentiation of the both species is shown in Fig 7 and Fig 8.

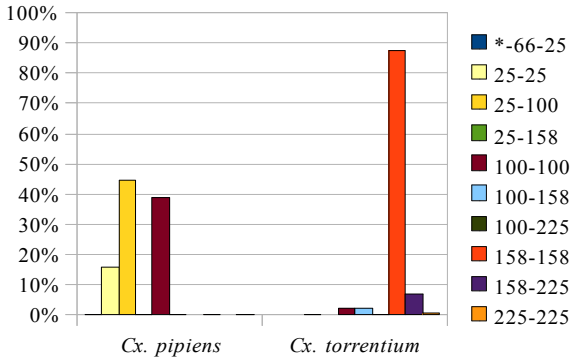

Fig 7. HBDH genotype distribution

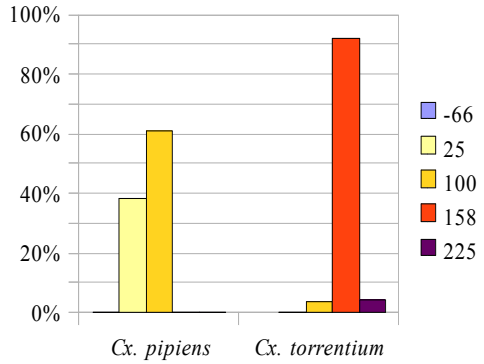

Fig 8. HBDH allele frequencies

Abundance of *Cx. pipiens* and *Cx. torrentium*

The 96 ovitraps were employed from mid-May to September. *Culex* sp. larvae were recorded in 442 of 864 ovitrap collections in the Upper Rhine Valley (Germany). After rearing the larvae from 333 ovitraps successfully, 2052 specimens were analysed using allozyme electrophoresis. This random selection produced 1124 specimens (54.8%) of *Cx. pipiens* (and given the open nature of their aquatic sources almost certainly of the nominate biotype) and 928 (45.2%) of *Cx. torrentium* (Table 1.).

The species proportion found in 33 artificial and natural breeding sites in the study area was similar: Of 420 specimens, (59.0%) were *Cx. pipiens* and 292 (41.0%) were *Cx. torrentium*.

In contrast to these results from larval populations, CO_2-traps yielded 597 specimens of *Cx. pipiens* (96.1% of total catch), but only 24 specimens (3.9%) of *Cx. torrentium* (Table 1.). Altogether, 621 specimens of both species were caught in 189 traps during four seasons in the study area.

Table 1. Species proportions in 3 sample categories in the Upper Rhine Valley, Germany

	Total	*Cx. pipiens*	proportion	*Cx. torrentium*	proportion
Ovitraps	2052	1124	54.8%	928	45.2%
Natural/artificial breeding sites	712	420	59.0%	292	41.0%
CO2-traps	621	597	96.1%	24	3.9%

Geographic distribution

There was an obvious geographic difference in abundance of the two *Culex* species in both ovitraps and natural breeding sites (Fig 9), with *Cx. torrentium* equally or more frequent in the northern part of the study area and *Cx. pipiens* predominant in the South. This was not surprising, but is remarkable when the limited extent of the sample area (between the latitudes of 48° and 50°) is taken into account.

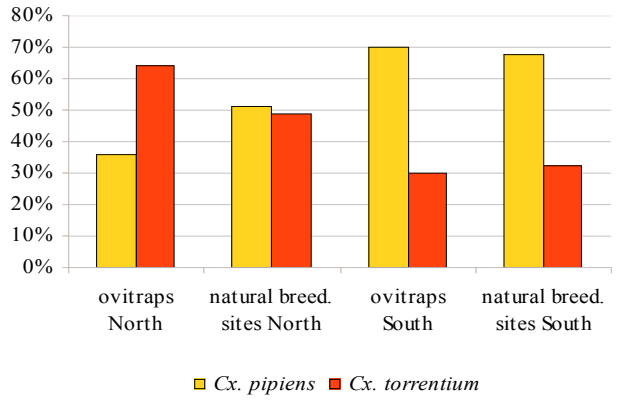

Fig 9. Species proportions in Northern and Southern part of the study area

Because assessment of seasonal abundance strongly depends on the seasonality of the species being studied, as well as the location of the traps, the ovitraps were utilised from May to September and were placed in different type of habitats in rural, suburban and urban areas of each community. In Table 2 the species proportions in 8 different community locations, each with 12 ovitraps, are presented.

Table 2. Results of collections from ovitraps in the close vicinity of different communities in south-western Germany

	geo-coordinates		*Cx. pipiens*		*Cx. torrentium*	
location	**North**	**East**	**number**	**proportion**	**number**	**proportion**
Gernsheim	49°45'1.40"N	8°29'8.95"E	80	35.4%	146	64.6%
Waldsee	49°23'45.81"N	8°26'29.39"E	101	48.1%	109	51.9%
Ketsch	49°22'0.46"N	8°32'0.80"E	112	35.9%	200	64.1%
Spechbach	49°20'44.11"N	8°53'5.95"E	125	38.9%	196	61.1%
Steinmauern	48°54'1.64"N	8°11'49.31"E	151	75.1%	50	24.9%
Freistett	48°40'0.16"N	7°56'29.34"E	143	62.2%	87	37.8%
Altenheim	48°27'53.26"N	7°48'34.08"E	263	80.9%	62	19.1%
Schwanau	48°23'12.41"N	7°45'19.76"E	149	65.6%	78	34.4%
Total			1124	54.8%	928	45.2%

Both species were also found in other parts of Germany, and in Luxembourg. *Culex torrentium* was found only at latitude higher than 48°N. *Culex pipiens* from southern France, Serbia, Greece, Turkey and Cyprus possessed the same alleles of AK and HBDH as those from Central Europe, with only slightly different frequencies. (Table 3) MDH (NADP) allozyme analysis did not detect the presence of *Cx. quinquefasciatus.*

Table 3. Species records in Mediterranean and Central European countries

Country	Location	North	East	source	*Cx. pipiens*	*Cx. torrentium*
Cyprus	Nikosia	35°9'38.25"N	33°21'31.61"E	sewage plant	7	
Turkey	Izmir	38°25'16.08"N	27°7'47.84"E	ovitrap	17	
Greece	Marathon	38°16'2.06"N	24°9'38.30"E	container	6	
Serbia	Hajdukovo	46°6'43.97"N	19°49'20.68"E	cellar, diapause	21	
Serbia	Novi Sad	45°14'29.87"N	19°50'42.00"E	puddle	1	
France	Cressin	45°47'17.20"N	5°46'6.96"E	sewage plant	38	
France	St. Désirat	45°15'18.23"N	4°47'7.87"E	container	1	
Luxembourg	4 diff. sites	49°36'N	6°6'E	CO_2 trap	7	
Luxembourg	Dondelange	49°41'15.82"N	6°1'47.69"E	container	2	10
Luxembourg	Bous	49°33'43.50"N	6°19'80.90"E	CO_2 trap	11	1
Germany	Weimar	50°58'44.99"N	11°19'27.41"E	container	29	
Germany	Fürth	49°28'24.83"N	10°59'27.53"E	container		10
Germany	Munich	48° 8'20.85"N	11°34'48.67"E	container	7	3

Discussion

The screening of 4040 specimens belonging to the *Culex pipiens s.l.* and *Cx. torrentium* by the genetic enzyme markers AK and HBDH gave a comprehensive insight into the species abundance and geographic distribution, and also the specific composition of CO_2 trap collections in Central Europe.

Most remarkably they showed *Cx. torrentium* to be nearly as frequent and widespread as *Cx. pipiens* in the open ground level larval habitats in Germany. Therefore, *Cx. torrentium* should also be considered as one of the most abundant mosquito species in Central Europe. Moreover, due to the lack of migration barriers, *Cx. torrentium* may be as common as *Cx. pipiens* in other parts of Europe with a temperate climate, from northern France to Belgium, The Netherlands, Poland, Ukraine, Baltic States, Russia and other areas.

Species records depend first of all on the choice of the correct sampling sites and on the seasonal activity of the species. Both aspects were taken into account by sampling mosquitoes in various types of breeding sites and by regularly collecting eggs and larvae from ovitraps throughout the summer (from May to September). Adjacently located ovitraps containing either rainwater or hay infusion possibly boosted their efficacy by offering alternatives to gravid mosquitoes.

The number of *Cx. torrentium* individuals caught in 189 CO_2 traps (24) was strikingly low when compared to the number *Cx. pipiens* caught (597 individuals) and the species was obviously under-represented. A possible explanation may lie in the standardised height of one metre above ground (Becker *et al.* 2010) being too low for strictly ornithophilic mosquitoes like *Cx. torrentium,* which probably spend their time around nesting zones (Petric *et al.* 1999). Similar results were obtained by Küpper *et al.* (2006) who observed an under-representation of the number of adults of both species when using a different trapping system.

Until the 1970s, many authors classified *Cx. torrentium* as rare (e.g. Mohrig, 1969), breeding in clear water in natural breeding sites. Moreover, this species was probably often neglected or mistakenly classified as *Cx. pipiens*. Later, Struppe (1989) investigated the species composition of larval populations from ovitraps and natural breeding sites in different urban zones of West-Berlin (North-Eastern Germany). Based solely on the observation of adult male hypopygia, Struppe's study revealed a proportion of 52.65% *Cx. torrentium* and 47.35% *Cx. pipiens* out of 3833 male specimens analysed. 68.1% of *Cx. pipiens* and 31.9% *Cx. torrentium* came from natural breeding sites whereas 25.2% *Cx. pipiens* and 74.8% *Cx. torrentium* came from ovitraps. It remains unclear why such differences are observed, and it is uncertain whether or not one can extrapolate Struppe's results from Berlin to the rest of Germany or other parts of Europe.

Küpper *et al.* (2006) recorded twice as many *Cx. torrentium* than *Cx. pipiens* from a larval survey in western-Germany. Similar studies in England, (Jupp, 1979) found that 62.1% of 206 samples were *Cx. torrentium*. Also, from collected egg rafts in southern England, Gillies & Gubbins (1982) observed proportions of 79.7% and 83.6% of *Cx. torrentium* from one year to another. In contrast, only 32.6% and 24.7%, respectively, of male larvae were identified as *Cx. torrentium.* However, the British Islands may not be representative of temperate continental Europe, due to their special postglacial zoogeographic history. It is well known, that *Cx. torrentium* is ubiquitous in northern Europe (Utrio, 1976, Dahl, 1988, Hesson *et al.*, 2010). In France, *Culex torrentium* has been reported in the Pyrenees around 1500m altitude in swampy fields (Sicart, 1954). Moreover, in 1960, Doby & Rault found this species "extremely usual/common" as it was easily sampled in 'Haute-Savoie' from a few hundred meters of altitude. More recently, Schaffner (1998) reported the species as being present in France and Wegner (2009) found *Cx. torrentium* larvae in central Poland. Identifications of 460 *Cx. pipiens* and 321 *Cx. torrentium* were made in a study covering a wide area of European Russia (Vinogradova *et al.*, 2007). Both species were detected even at latitudes above 60°N. However, *Cx. torrentium* was found to be frequent and widespread at latitudes above 50° suggesting that it is ubiquitous at northern rather than southern latitudes.

In the present study, investigations of mosquito samples from other European countries found no *Cx. torrentium* were at latitudes below 48°N, where *Cx. pipiens* predominated. Nevertheless *Cx. torrentium* has already been described in several Mediterranean countries such as in Italy (Urbanelli *et al.*, 1981) and Spain (Aranda *et al.*, 1999). In Turkey, Parrish (1959) found *this species* only in coastal regions whereas *Cx. pipiens* was widespread. Nicolescu (1998) found both species in the south of Romania. In contrast, in the species chart of Snow & Ramsdale (1999) *Cx. torrentium* is missing in Bulgaria, Greece, Turkey and Mediterranean islands. Thus it was not likely to be detected by a few hundreds of samples collected at low altitudes in the present study. Nevertheless the allozyme marker system would be valid for discrimination of Mediterranean *Cx. torrentium* from *Cx. pipiens s.l.*

Culex quinquefasciatus has not been collected in Greece, Turkey and Cyprus (Snow & Ramsdale, 1999; Simsek, 2003). However, Violaris *et al.* (2009) stated that *Cx. torrentium*

and *Cx. quinquefasciatus* are absent from Turkey and Cyprus, but reported *Cx. pipiens* to be abundant.

Success and practicability of allozyme technique for species diagnosis

Species diagnostic allele distribution was successfully used for species identification on a large scale. In this study, AK and HBDH allozymes were used in combination to increase the confidence of species identification up to 99.99%. However, the genotype-combination AK-78/100 is the exception as it does not discriminate between *Cx. pipiens* and *Cx. torrentium;* but this would only occur at an extremely low percentage (below 1%, see appendix). Because the probability of finding this genotype in a sampled population is very low, the allozyme technique is adequate for *Culex* species identification. As a matter of fact, two specimens out of 4040 (0.05%) possessed the rare genotype AK-78/100 in combination with a weak HBDH staining reaction, so that only two samples remained unidentified.

In addition to their high reliability, both marker systems were shown by Weitzel *et al.* (2009) to be diagnostic also for *Cx. torrentium* versus *Cx. pipiens* biotype *molestus, Cx. quinquefasciatus, Cx. modestus, Cx. territans* and *Cx. stigmatosoma* and *vice versa.*

Generally, the allozyme technique can be applied to most kinds of organisms. The reliability of marker systems is obvious by their banding pattern, and is guided by the use of reference samples. The inheritance of cytoplasmic enzyme-genes follows Mendelian rules. The allele combinations (genotypes) are usually readable in the banding pattern. Accordingly, the Hardy-Weinberg equilibrium gives a guideline for the data structure. Thousands of base pairs of encoding DNA are represented by only a dozen enzyme loci. In case of doubt, a sample can be checked by other differentiated enzyme loci as was demonstrated in this study. The technique is cheap, easy to apply and the chemicals are easily available. No toxic substances are needed, no toxic waste is produced and no radiation is emitted. The material costs for determination of one sample is about 0.30 €, far lower than for a PCR sample. In one day, 88 samples were identified using both AK and HBDH marker systems with 44 samples per gel. A higher efficiency is possible, especially when only AK is used and in consequence a 1% portion of junk is acceptable. This procedure allows huge sample sizes. For each run in thin agarose gels, about 10% of the body of a mosquito is needed, thus in vector studies the samples can be divided and one part of the mosquitoes used for electrophoresis, the other part for further processing. The preparation process and conduction of the protein electrophoresis involves common laboratory practice easily performed by a technician or a student. In the last decades the technique has been optimized to a higher degree of efficiency and the data evaluation computerized. A large amount of data is available for comparison.

Acknowledgements

Thanks are due to the German Mosquito Control Association (GMCA/KABS) and Gesellschaft zur Förderung der Stechmückenbekämpfung (GFS) for financial support. We are very grateful to Matthias Beck (Germany, USA), Wolf-Peter Pfitzner, Achim Kaiser and Andreas Arnold (Germany), Marija Zgomba and Duşan Petrić, University of Novi Sad, Serbia, and Delphine Rey and Rémi Foussadier, EID Rhône Alpes, France for the collection of mosquito samples. Specimens of *Cx quinquefasciatus* from Malaysia and Indonesia were provided by Bayer CropScience, Monheim, Germany. We thank Klaus Hoffmann and Kerstin Pietsch for technical assistance.

References

Aranda, C., Eritja, R., Schaffner, F. & Escosa, R. (2000) *Culex (Culex) torrentium* Martini (Diptera, Culicidae) a new species from Spain. *European Mosquito Bulletin* **8**, 7-9.

Byrne, K. & Nichols, R.A. (1999) *Culex pipiens* in London Underground tunnels: differentiation between surface and subterranean populations *Heredity* **82**, 7-15.

Becker, N., Petrić, D., Zgomba, M., Boase, C., Dahl, C., Lane, J. & Kaiser, A. (2003, 2010) *Mosquitoes and their control*. 1st and 2nd ed. Kluwer Academic / Plenum Publishers, New York.

Chapman, H.F., Kay, B.H., Ritchie, S.A., van den Hurk, A.F. & Hughes, J.M. (2000) Definition of Species in the *Culex sitiens* Subgroup (Diptera: Culicidae) from Papua New Guinea and Australia. *Journal of Medical Entomology* **37**, 736-742.

Chevillon, C., Eritja, R., Pasteur, N. & Raymond, M. (1995) Commensalism, adaption and gene flow: mosquitoes from the *Culex pipiens* complex in different habitats. *Genetical Research* **66**, 147-157.

Cui F., Qiao, C.-L., Shen, B.-C., Marquine, M., Weil M. & Raymond M. (2007) Genetic differentiation of *Culex pipiens* (Diptera: Culicidae) in China. *Bulletin of Entomological Research* **97**, 291–297.

Dahl, C. (1988) Taxonomic studies on *Culex pipiens* and *Cx. torrentium*. Pp 149-175 in: Service, M.W. (ed.), *Biosystematics of Haematophagous Insects*. Clarendon Press, Oxford.

Doby, J.M. & Rault, B. (1960) Complément à l'étude des caractères morphologiques différentiels de *Culex pipiens* Linné, 1758 et de *Culex torrentium* Martini, 1924 (Diptera Culicidae). *Cahiers des Naturalistes, nouvelle série* **16**, 113-122.

Gillies, M.T. & Gubbins, S.J. (1982) *Culex (Culex) torrentium* Martini and *Cx. pipiens* (L.) in a Southern English County, 1974-1975. *Mosquito Systematics* **14**, 127-130.

Gingrich, J.B. & Casillas, L.J. (2004) Selected mosquito vectors of West Nile virus: comparison of their ecological dynamics in four woodland and marsh habitats in Delaware. *Journal of the American Mosquito Control Association* **20**, 138-145.

Gingrich, J.B. & Williams, G.M. (2005) Host-feeding patterns of suspected West Nile virus mosquito vectors in Delaware, 2001-2002. *Journal of the American Mosquito Control Association* **21,** 194-200.

Harbach, R.E. & Harrison, A.M. (1984) *Culex* (*Culex*) *molestus* Forskål (Diptera: Culicidae): Neotype designation, description, variation, and taxonomic status. *Proceedings of the Entomological Society of Washington* **86**, 521-542.

Harbach, R.E., Dahl, C. & White, G. (1985) *Culex* (*Culex*) *pipiens* Linnaeus (Diptera: Culicidae): concepts, type designations, and description. *Proceedings of the Entomological Society of Washington* **87**, 1-24.

Harris, H. & Hopkinson, D.A. (1976) *Handbook of enzyme electrophoresis in human genetics*. North Holland Publishers Company, Amsterdam, Oxford.

Hesson, J., Lundström, J., Halvarsson, P., Erixon, P. & Collado, A. (2010) A sensitive and reliable restriction enzyme method to distinguish between *Culex torrentium* and *Culex pipiens* (Diptera: Culicidae). *Medical Veterinary Entomology* **24**, 142-149.

Jöst, H., Bialonski A., Storch V., Günther S., Becker N. & Schmidt-Chanasit J. (2010) Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. *Journal of Clinical Microbiology* **48**, 1900-3.

Jupp, P.G. (1979) *Culex (Culex) pipiens* and *Cx. torrentium* (Diptera: Culicidae) in England: Notes on their Taxonomy and Biology. *Mosquito Systematics* **11**, 121.

Knight, J.W. & Nayar, J.K. (2004). Identification of four commom *Culex* (*Culex*) (Diptera:Culicidae) species from Florida with isoenzyme analysis. *Florida Entomologist* **87**, 1-5.

Küpper, S., Schulze, S., Maier, W.A. & Kampen, H. (2006) Beitrag zum Vorkommen und zur Verbreitung von Stechmücken (Diptera: Culicidae) in Nordrheinwestfalen mit besonderer Berücksichtigung des Großraums Bonn. *Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie* **15**, 337-344.

Lopatin, O.E. (1993) On the genetic structure of anthropophilous populations of *Culex pipiens*. *Krovososushchiye dvukrylyye*, 82-85.

Lundström, J.O. (1994) Vector competence of Western European mosquitoes for arboviruses: a review of field and experimental studies. *Bulletin of the Society of Vector Ecology* **19**, 23-36.

Medlock, J.M., Snow K.R. & Leach, S. (2005) Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors. *Medical and Veterinary Entomology* **19**, 2-21.

Miller, B.R., Crabtree, M.B. & Savage H.M. (1996) Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. *Insect Molecular Biology* **5**, 93-107.

Mohrig, W. (1969) *Die Culiciden Deutschlands*. Parasitologische Schriftenreihe **18**, 260 pp.

Murphy, R.W., Sites, J.W., Buth, D.G. & Haufler, C.H. (1996) Proteins: Isozyme Electrophoresis. In: *Molecular Systematics* 2nd ed. (Hillis, D.M., Moritz, C. & Mable, B.K.). Sinauer Associates, Sunderland, Massachusettes, USA, 51-120.

Nicolescu, G. (1998) A general characterisation of the mosquito fauna (Diptera: Culicidae) in the epidemic area for West Nile virus in the south of Romania. *European Mosquito Bulletin* **2**, 13-18.

Parrish, D.W. (1959) The Mosquitoes of Turkey. *Mosquito News* **19**, 264-266.

Petrić, D., Zgomba, M. Bellini, R., Veronesi, R., Kaiser, A. & Becker, N. (1999) Validation of CO_2 Trap Data in three European Regions. *Proceedings of the 3rd International Conference on Urban Pests*, 437-445.

Pryor, S.C. & Daly, J. (1991) Temporal Variation in morphological and genetic characteristics within a hybrid population of *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology* **28**, 481-486.

Rydzdanicz, K. & Lonc, E. (2003) Species composition and seasonal dynamics of mosquito larvae in the Wroclaw, Poland area. *Journal of Vector Ecology*. **28**, 255-266.

Schreiber, A. & Weitzel, T. (1995) Biochemical Systematics of Sea Eagles (Genus *Haliaeetus* Savigny 1809), with a Note on Allozyme Differentiation Between Black and Red Kites (Genus *Milvus* L. 1758). *Biochemical Systematics and Ecology* **23**, 235-244.

Schäfer, M.L., Lundström, J.O., Pfeffer, M., Lundkvist, E. & Ladin, J. (2004) Biological diversity versus risk for mosquito nuisance and disease transmission in constructed wetlands in southern Sweden. *Medical and Veterinary Entomology* **18**, 256-267.

Schaffner, F. (1998) A revised checklist of the French Culicidae. *European Mosquito Bulletin* **2**, 1-9.

Shaikevich, E.V. (2007) PCR-RFLP of the COI gene reliably differentiates *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *Cx. torrentium* of the Pipiens Complex. *European Mosquito Bulletin* **23**, 25-30.

Sicart, M. (1954) Présence de *Culex torrentium* dans les Pyrénées et comparaisons avec *Culex pipiens* du même gite. *Societé d'Histoire Naturelle de Toulouse Bulletin* **89**, 228-230.

Simsek, F.M. (2003) Seasonal Population Dynamics and Breeding Habitat Diversity of *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae) in Gölbasi District, Ankara, Turkey. *Journal of the Society for Entomological Research* **5**, 51-62.

Smith, J.L. & Fonseca, D.M. (2004) Rapid assay for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *American Journal for Tropical and Medical Hygiene* **70**, 339-345.

Snow, K. and Ramsdale, C. (1999) Distribution chart for European mosquitoes. *European Mosquito Bulletin* **3**, 14-31.

Struppe, Th. (1989) Biologie und Ökologie von *Culex torrentium* (Martini) unter besonderer Berücksichtigung seiner Beziehungen im menschlichen Siedlungsbereich. *Angewandte Zoologie* **17**, 257-86.

Urbanelli, S., Cianchi, R., Petrarca, V., Sabatinelli, M., Colluzzi, M. & Bullini, L. (1981) Adattamento all'ambiente erbano nella zanzara *Culex pipiens* (Diptera: Culicidae). In: Moroni, A., Ravera, O., Anelli, A. (eds.) *Ecologia, Atti XI Congressi nazionali.* Societa Italiana E. Zara, Parma. 305-316.

Urbanelli, S., Silvestrini, F., Sabatinelli, G., Ravelorifera, F., Petrarca, V. & Bullini, L. (1995) Characterization of the *Culex pipiens* Complex (Diptera: Culicidae) in Madagascar. *Journal of Medical Entomology* **32**, 778-786.

Urbanelli, S., Silvestrini, F., Reisen, W. K., De Vito, E. & Bullini, L. (1997) Californian hybrid zone between *Culex pipiens pip*iens and *Cx. p. quinquefasciatus* revisited (Diptera: Culicidae). *Journal of Medical Entomology* **34**, 116-127.

Utrio, P. (1976) Identification Key to Finnish Mosquito Larvae (Diptera, Culicidae). Annales Agriculturar Fenniae **15**, 128 -136.

Vinogradova, E.B., Shaikevich, E.V. & Ivanitsky, A.V. (2007) A study of the distribution of the *Culex pipiens* complex (Insecta: Diptera: Culicidae) mosquitoes in the European part of Russia by molecular methods of identification. *Comparative Cytogenetics* **1**, 129-138.

Violaris, M., Vasquez, M.I., Samanidou, A., Wirth, M.C. & Hadjivassilis, A. (2009) The mosquito fauna of the republic of Cyprus: a revised list. *Journal of the American Mosquito Control Association* **25**, 199-202.

Wegner, E. (2009) A study of mosquito fauna (Diptera: Culicidae) and the phenology of the species recorded in Wilanów (Warsaw, Poland). *European Mosquito Bulletin* **27**, 23-32.

Weitzel, T., Collado, A., Jöst, A., Pietsch, K., Storch, V. & Becker, N. (2009) Genetic differentiation of populations within the Culex Pipiens Complex (Diptera: Culicidae) and phylogeny of related species. *Journal of the American Mosquito Control Association* **25**, 6-17.

Appendix

Details of genotype and allele frequencies of adenylate kinase and 2-hydroxybutyrate dehydrogenase and the respective number of mosquito specimens are presented. Calculations of expected genotype frequencies following Hardy-Weinberg equilibrium, and the variances and confidence intervals of allele frequencies give an idea of the reliability of the data.

Table 4. AK genotype distribution in German *Cx. pipiens*, *Cx. torrentium* and control taxa

			AK genotypes								
Species		**Total**	**53-78**	**78-78**	**78-87**	**78-100**	**78-124**	**100-100**	**100-124**	**100-154**	**124-124**
Cx. pipiens	number	2133	0	0	0	7	1	2101	13	6	5
	frequency		0.0000	0.0000	0.0000	0.0033	0.0005	0.9850	0.0061	0.0028	0.0023
	frequ. expected		0.0000	0.0000	0.0000	0.0037	0.0000	0.9823	0.0112	0.0028	0.0000
Cx. torrentium	number	1236	142	1085	1	8	0	0	0	0	0
	frequency		0.1149	0.8778	0.0008	0.0065	0.0000	0.0000	0.0000	0.0000	0.0000
	frequ. expected		0.1079	0.8816	0.0008	0.0061	0.0000	0.0000	0.0000	0.0000	0.0000
Cx. pipiens **biot. *molestus***	number	180	0	0	0	0	0	179	0	1	0
	frequency		0.0000	0.0000	0.0000	0.0000	0.0000	0.9944	0.0000	0.0056	0.0000
	frequ. expected		0.0000	0.0000	0.0000	0.0000	0.0000	0.9945	0.0000	0.0055	0.0000
Cx. quinque-fasciatus	number	181	0	0	0	0	0	178	3	0	0
	frequency		0.0000	0.0000	0.0000	0.0000	0.0000	0.9834	0.0166	0.0000	0.0000
	frequ. expected		0.0000	0.0000	0.0000	0.0000	0.0000	0.9835	0.0164	0.0000	0.0001

Table 5. AK allele distribution in *Cx. pipiens*, *Cx. torrentium* and control taxa

			AK alleles					
Species		**Total**	**53**	**78**	**87**	**100**	**124**	**154**
Cx. pipiens	number (2n)	4266	0	8	0	4228	24	6
	frequency		0.0000	0.0019	0.0000	0.9911	0.0056	0.0014
	variance		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	confidence (+/-)		0.0000	0.0013	0.0000	0.0029	0.0023	0.0011
Cx. torrentium	number (2n)	2472	142	2321	1	8	0	0
	frequency		0.0574	0.9389	0.0004	0.0032	0.0000	0.0000
	variance		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	confidence (+/-)		0.0094	0.0096	0.0008	0.0023	0.0000	0.0000
Cx. pipiens	number (2n)	360	0	0	0	359	0	1
biotype. *molestus*	frequency		0.0000	0.0000	0.0000	0.9972	0.0000	0.0028
	variance		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	confidence (+/-)		0.0000	0.0000	0.0000	0.0055	0.0000	0.0055
Cx. quinquefasciatus	number (2n)	362	0	0	0	359	3	0
	frequency		0.0000	0.0000	0.0000	0.9917	0.0083	0.0000
	variance		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	confidence (+/-)		0.0000	0.0000	0.0000	0.0095	0.0095	0.0000

Table 6. HBDH genotype distribution in German *Cx. pipiens*, *Cx. torrentium* and control taxa

			HBDH genotypes										
Species		**Total**	**-66--66**	**-66-25**	**25-25**	**25-100**	**25-158**	**100-100**	**100-158**	**100-225**	**158-158**	**158-225**	**225-225**
Cx. pipiens	number	1647	0	5	264	733	1	641	0	0	2	0	1
	frequency		0.0000	0.0030	0.1603	0.4451	0.0006	0.3892	0.0000	0.0000	0.0012	0.0000	0.0006
	frequ. exp.		0.0000	0.0012	0.1479	0.4706	0.0012	0.3742	0.0019	0.0007	0.0000	0.0000	0.0000
Cx. torrentium	number	957	0	0	0	1	0	21	23	1	838	67	6
	frequency		0.0000	0.0000	0.0000	0.0010	0.0000	0.0219	0.0240	0.0010	0.8757	0.0700	0.0063
	frequ. exp.		0.0000	0.0000	0.0000	0.0000	0.0010	0.0012	0.0646	0.0029	0.8513	0.0771	0.0017
Cx. pipiens **biotype** ***molestus***	number	68	0	0	0	20	0	48	0	0	0	0	0
	frequency		0.0000	0.0000	0.0000	0.2941	0.0000	0.7059	0.0000	0.0000	0.0000	0.0000	0.0000
	frequ. exp.		0.0000	0.0000	0.0216	0.2509	0.0000	0.7275	0.0000	0.0000	0.0000	0.0000	0.0000
Cx. quinque-	number	102	1	13	87	0	0	0	0	0	1	0	0
fasciatus	frequency		0.0098	0.1275	0.8529	0.0000	0.0000	0.0000	0.0000	0.0000	0.0098	0.0000	0.0000
	frequ. exp.		0.0054	0.1348	0.8403	0.0000	0.0180	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000

Table 7. HBDH allele frequencies. variances and confidence intervals

			HBDH alleles				
Species		**Total**	**-66**	**25**	**100**	**158**	**225**
Cx. pipiens	number (2n)	3328	5	1282	2033	6	2
	frequency		0.0015	0.3852	0.6109	0.0018	0.0006
	variance		0.0000	0.0001	0.0001	0.0000	0.0000
	confidence (+/-)		0.0013	0.0169	0.0169	0.0015	0.0008
Cx. torrentium	number (2n)	1968	0	1	68	1816	83
	frequency		0.0000	0.0005	0.0346	0.9228	0.0422
	variance		0.0000	0.0000	0.0000	0.0000	0.0000
	confidence (+/-)		0.0000	0.0010	0.0082	0.0120	0.0091
Cx. pipiens **biotype**	number (2n)	244	0	33	211	0	0
molestus	frequency		0.0000	0.1352	0.8648	0.0000	0.0000
	variance		0.0000	0.0005	0.0005	0.0000	0.0000
	confidence (+/-)		0.0000	0.0438	0.0438	0.0000	0.0000
Cx. quinquefasciatus	number (2n)	238	15	221	0	2	0
	frequency		0.0630	0.9286	0.0000	0.0084	0.0000
	variance		0.0002	0.0003	0.0000	0.0000	0.0000
	confidence (+/-)		0.0315	0.0334	0.0000	0.0118	0.0000