

## Cryptic invasion and dispersal of an American *Daphnia* in East Africa

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### Abstract

We document the cryptic invasion of a North American genotype of *Daphnia pulex* into Kenya. During a survey of zooplankton samples and dormant egg banks of 41 natural lakes, ponds, and man-made reservoirs throughout central and southern Kenya, *D. pulex* was found at seven localities in the Rift Valley region. We used DNA sequencing (12S rDNA and cytochrome *c* oxidase subunit 1 gene [COI]) and microsatellite analyses (10 loci) to characterize each population genetically. A single haplotype was found for both 12S and COI sequences. Comparison with DNA sequences of the *D. pulex* complex from Europe and America reveals that the Kenyan *D. pulex* is not closely related to European *D. pulex* but clusters tightly with the American–Panarctic clade of *D. pulex* sensu stricto. Microsatellite data further reveal that all seven known Kenyan populations are genetically nearly identical and are dominated by a single clone. All populations except that in Lake Naivasha contained only one multilocus genotype, a fixed heterozygote for 3 of the 10 studied loci. Our data suggest that an obligately parthenogenetic clone of American *D. pulex* recently immigrated into Kenya and has subsequently dispersed over distances of several hundreds of kilometers. Most likely it was co-introduced accidentally during one of numerous stockings of North American fish or crayfish in Kenya's Rift Valley lakes since the mid-1920s.

Introductions and natural invasions of exotic species are increasingly posing threats to biodiversity worldwide and currently happen at dramatically increased rates as a result of human-mediated intercontinental transfers, linking regions that had been isolated for millions of years. Among cladoceran zooplankton there are several examples, including recent invasions from Africa to America (*Daphnia lumholtzi*; Havel and Hebert 1993), from America to Europe (e.g., *Daphnia parvula*; reviewed in Maier 1996), and from Europe to America (e.g., *Bosmina coregoni*, *Cercopagis pengoi*, and *Bythotrephes cederstroemi*; Mills et al. 1993;

De Melo and Hebert 1994; MacIsaac et al. 1999). Documentation of these human-mediated intercontinental transfers can be facilitated by genetic analyses, showing that many cladoceran taxa with natural transcontinental distributions and apparently uniform morphology are in fact two or more genetically distinct species (e.g., Adamowicz et al. 2004). These modern genetic studies often confirm detailed morphological analyses that already earlier questioned the traditional paradigm of panmixis and cosmopolitanism among cladocerans (Frey 1987). Even within a single continent, traditional species are often found to consist of a complex of cryptic species (reviewed in Adamowicz et al. 2004). *Daphnia pulex* Leydig belongs to one of these cosmopolitan species complexes, members of which are widely distributed over North and South America, Eurasia, and parts of Africa. Hybridization between members of this complex, allopolyploidization, and loss of sexual reproduction strongly complicate correct identification of its member taxa. Colbourne et al. (1998) showed that *D. pulex* is a polyphyletic species, with a distinct European clade at the base of the phylogenetic tree, and American–Panarctic *D. pulex*, Panarctic *Daphnia pulicaria*, and European *D. pulicaria*, among others, form inner branches of the tree. Using genetic tools, we wanted to analyze the genetic structure of Kenyan populations of *D. pulex*. We used sequencing of mitochondrial DNA, which is best suited for phylogeography and species assignment, as well as microsatellite variation analysis, which is better suited for assessing genetic diversity and differentiation between populations because of the higher mutation rate of microsatellites. Given ancient biogeographic links between continents and the dominant North–South migration routes of

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birds, we expected members of the *D. pulex* species complex encountered in East African lakes to be more closely related to the European clade than to the American–Panarctic clade. Because gene flow between populations in *Daphnia* is much lower than expected from their dispersal capacity (dispersal–gene flow paradox; De Meester et al. 2002), we expected clear differentiation between populations at the level of microsatellites.

## Materials and methods

**Sampling**—Field surveys in central and southern Kenya during August 2001 and January 2003 yielded collections of the active zooplankton community and dormant egg bank (viable dormant eggs recently deposited in surface sediments) from 41 standing waters (Mergeay et al. in press). These sample localities include the large majority of all natural freshwater lakes and a sizable portion of man-made reservoirs in the study region. Live zooplankton was collected with a conical tow net (25 cm diameter) of 150- $\mu$ m mesh, washed in the net, and fixed in 100% ethanol. Offshore surface sediments (~500 ml) containing cladoceran dormant eggs were sampled from a boat, with a weighted Wildco Fieldmaster<sup>®</sup> horizontal water sampler, or manually in shallow waters.

*Daphnia* in the zooplankton samples were identified with a compound stereomicroscope, with reference to Kofínek (1999). Ehippia (the chitinous capsules encasing cladoceran dormant eggs) were identified to species with the use of ehippium-bearing females in associated zooplankton samples or in reference collections at Ghent University and the Katholieke Universiteit Leuven. At the level of the species complex, *D. pulex* ehippia are easily identified and distinguished from all other *Daphnia* species known to occur in East Africa (Mergeay et al. in press).

**Genetic analyses**—Single dormant eggs were picked out of the ehippium and transferred to ultraviolet (UV)-sterilized 100- $\mu$ l microcentrifuge tubes in 30  $\mu$ l of Proteinase K-buffer (16 mmol L<sup>-1</sup> [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>, 67 mmol L<sup>-1</sup> Tris-HCl pH 8.8, 0.01% Tween-20, 10% dithiothreitol (DTT), and 0.5 mmol L<sup>-1</sup> Proteinase K). Adult *Daphnia* were treated similarly, in 100  $\mu$ l Proteinase K-buffer. Samples were incubated for 1 h at 56°C followed by 10 min at 96°C and 2 min centrifugation (13,000 rpm, 20 cm diameter), after which the supernatant was transferred to a new UV-sterilized microcentrifuge tube. Samples were stored at -20°C.

Sequencing involved amplification of a section of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) with primers LCOI490 and HCO2198 (Folmer et al. 1994) and a section of the mitochondrial 12S rRNA gene (12S) (primers: 5'-ATGCACTTTCCAGTACATCTAC-3' and 5'-AAATCGTGCCAGCCGTCGC-3'; Taylor et al. 1996). The total reaction volume (50  $\mu$ l) consisted of 1 $\times$  PCR buffer (Silverstar<sup>™</sup>, Eurogentec), 1.5 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 200  $\mu$ mol L<sup>-1</sup> of each desoxynucleotide triphosphate (dNTP), 0.2  $\mu$ mol L<sup>-1</sup> of each primer, 1  $\mu$ l of template DNA, 1–2 U of *Taq* polymerase, and UV-sterilized double distilled-H<sub>2</sub>O. PCR amplifications involved a denaturing step of 5 min at 95°C, followed by 30–40 cycles of 45 s at 95°C, 45 s at 53°C, 45

s at 72°C, and a final elongation of 7 min at 72°C. When possible, three individuals per population were sequenced with both primer sets. Amplification reactions were sequenced with forward primers. Sequencing reaction products were electrophoresed with an ABI PRISM 3700 capillary DNA sequencer (Applied Biosystems). Sequences were aligned in Clustal X (Thompson et al. 1997) with the default options, and electropherograms were checked visually for ambiguities. Phenetic analyses on unique COI and 12S haplotypes were performed in MEGA 2.1 (Kumar et al. 2001), including other sequences available from GenBank (<http://www.ncbi.nlm.nih.gov/>) and sequences used in Colbourne and Hebert (1996). Pairwise genetic distances were calculated by the Kimura two-parameter model (Kimura 1980), with pairwise deletion of missing sites. These genetic distances were used to construct phenograms with the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) and bootstrap values from 1,000 replicates. We used sequences of *Daphnia obtusa* as the outgroup to root the trees.

Ten microsatellite markers were used to assess genetic variation within and between populations. The following loci were used: Dpu7, Dpu40, Dpu45, Dpu46, Dpu12/2, Dp183, Dp525alt, Dp464, Dp502, and Dp496 (GenBank AF233359–AF233363, AY619196, AY619551, AY619487, AY619527, and AY619521; Colbourne et al. 2004). Forward primers were end-labeled with a fluorescent dye. The total reaction volume (10  $\mu$ l in singleplex, 25  $\mu$ l in multiplex) consisted of 1 $\times$  PCR buffer (Silverstar<sup>™</sup>, Eurogentec), 1.5 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 200  $\mu$ mol L<sup>-1</sup> of each dNTP, 0.2  $\mu$ mol L<sup>-1</sup> of each primer, 1  $\mu$ l of template DNA, 0.25–0.5 U of *Taq* polymerase, and UV-sterilized mQ-H<sub>2</sub>O. Loci Dp183, Dp502/Dp525alt, Dp464, and Dp496 were amplified in multiplex. PCR amplifications involved a denaturing step of 5 min at 95°C, followed by 30–40 cycles of 17 s at 95°C, 17 s at 52–55°C, 17 s at 72°C, and a final elongation of 7 s at 72°C. When the amount of material allowed, 30 individuals, dormant eggs, or both were analyzed. PCR products were separated and visualized on a LiCor 4200 system with a 6% polyacrylamide gel (Sequagel XR, National Diagnostics).

## Results

**Sequencing**—*D. pulex* was found in the active community, the dormant egg bank, or both in 7 of the 41 sampled waters (Table 1; Fig. 1). In Lake Naivasha and its satellite basin of Crescent Island Crater, *D. pulex* was found both in the active community and the dormant egg bank. In five other lakes, it was only found in the dormant egg bank. Sequencing of a total of 19 individuals from seven populations yielded only a single mitochondrial haplotype for both the COI and 12S sequences (GenBank AY745244–5). Comparison with available sequences from European and North American members of the *D. pulex* complex revealed a perfect match for 12S with a North American isolate of *D. pulex* from Ontario, Canada (Fig. 2a). Phenetic reconstruction corroborates the nesting of our Kenyan genotype within the American–Panarctic clade of *D. pulex* (Fig. 2a; Colbourne et al. 1998). Sequence divergence at 12S between the American–Panarctic clade of *D. pulex* (including the Kenyan genotype) and

Table 1. Kenyan waters inhabited by *D. pulex*. Numbers refer to sampling locations in Fig. 1. Active/Dormant, numbers of individuals from live zooplankton samples and dormant eggs from the surface sediment, respectively, subjected to microsatellite analysis.

No.	Name	Geographical position	Altitude (m asl)*	Maximum depth (m)	Area (km <sup>2</sup> )	Active	Dormant
14	Nairobi National Park Narogoman dam	01°21.0'S, 36°47.9'E	1,707	3.8	0.15	—	5
21	Lake Limuru 2	01°06.3'S, 36°37.8'E	2,294	2.5	0.25	—	30
23	Lake Naivasha	00°46.3'S, 36°21.7'E	1,897	4.5	150	30	30
24	Crescent Island Crater	00°45.8'S, 36°24.5'E	1,897	14.0	1.95	30	30
26	Sigawet dam	00°35.1'N, 35°13.1'E	2,014	1.5	0.05	—	1
37	Lake Ol Bolossat	00°09.9'S, 36°26.0'E	2,358	2.0	20	—	6
40	Lake Baringo	00°39.1'N, 36°03.6'E	967	4.0	108	—	9

\* asl, above sea level.

the European clade averages 7.2%. A phenetic NJ tree with COI sequences (Fig. 2b) similarly shows close affinity of the Kenyan genotype with North American *D. pulex*.

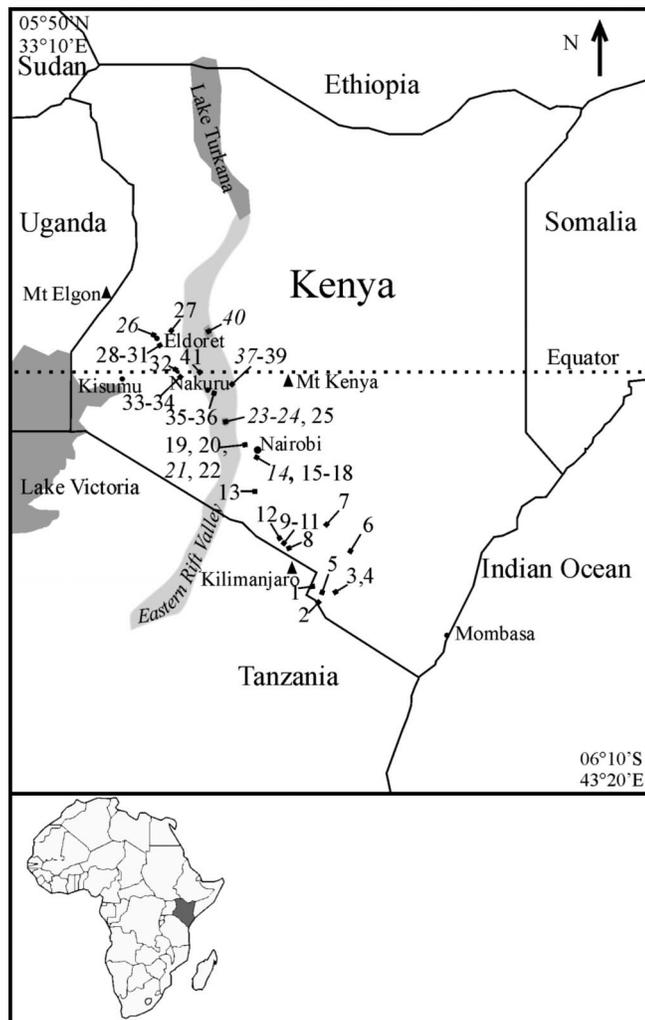


Fig. 1. Map of Kenya showing locations of sampled lakes and reservoirs. Numbers refer to locality names in Mergeay et al. (in press); numbers in italics represent waters inhabited by *D. pulex* (Table 1).

*Microsatellite analysis*—In total, 60 individuals from the active communities of Lake Naivasha and Crescent Island Crater and 111 dormant eggs from these and five other populations (Table 2) were subjected to microsatellite variation analysis. All specimens except two from Lake Naivasha shared the same multilocus genotype (ML1 in Table 2). Hence, the genetic composition of most sampled populations can be considered identical. Because this dominant multilocus genotype (MLG) is heterozygous at three loci (Dpu7, Dp12/2, and Dp496), all populations except Lake Naivasha are fixed heterozygous at these loci (Lake Naivasha not for Dp496; Table 2). The two distinct MLGs of the atypical Lake Naivasha individuals differed each at one locus only (Table 2). As a result, all sampled populations are in strong Hardy–Weinberg disequilibrium.

## Discussion

*A cryptic American invader in Africa*—Until recently, *D. pulex* was often thought not to occur in tropical and subtropical regions. Our survey and earlier reports (Lowndes 1936; Green 1995) showed that members of the *D. pulex* complex do occur in tropical Africa, although they are mostly restricted to highland regions in Uganda and Kenya where a warm-temperate climate prevails. Our genetic analyses revealed that all known Kenyan populations belong to the American clade of the *D. pulex* species complex and are not closely related to the European clade (7.2% sequence divergence at 12S). The resulting 12S phenetic tree confirms the monophyletic status of the European clade, which is distinct from the monophyletic clade of American–Panarctic *D. pulex* within the *D. pulex* complex, as well as the sister relationship between European *D. pulicaria* and *Daphnia tenebrosa*, both reported earlier by Colbourne et al. (1998). Because of a low number of informative sites among members of this clade (Colbourne and Hebert 1996), 12S rDNA sequence analysis does not resolve more detailed relationships within the American group of *D. pulex*. The main difference between our data and the more detailed phenetic tree of Colbourne et al. (1998) is that the position of the European *D. pulex* clade is switched with the position of the clade containing European *D. pulicaria* and *D. tenebrosa*. COI sequences confirm strong nesting of the Kenyan *D. pulex* within the American–Panarctic clade. This consistent pattern for

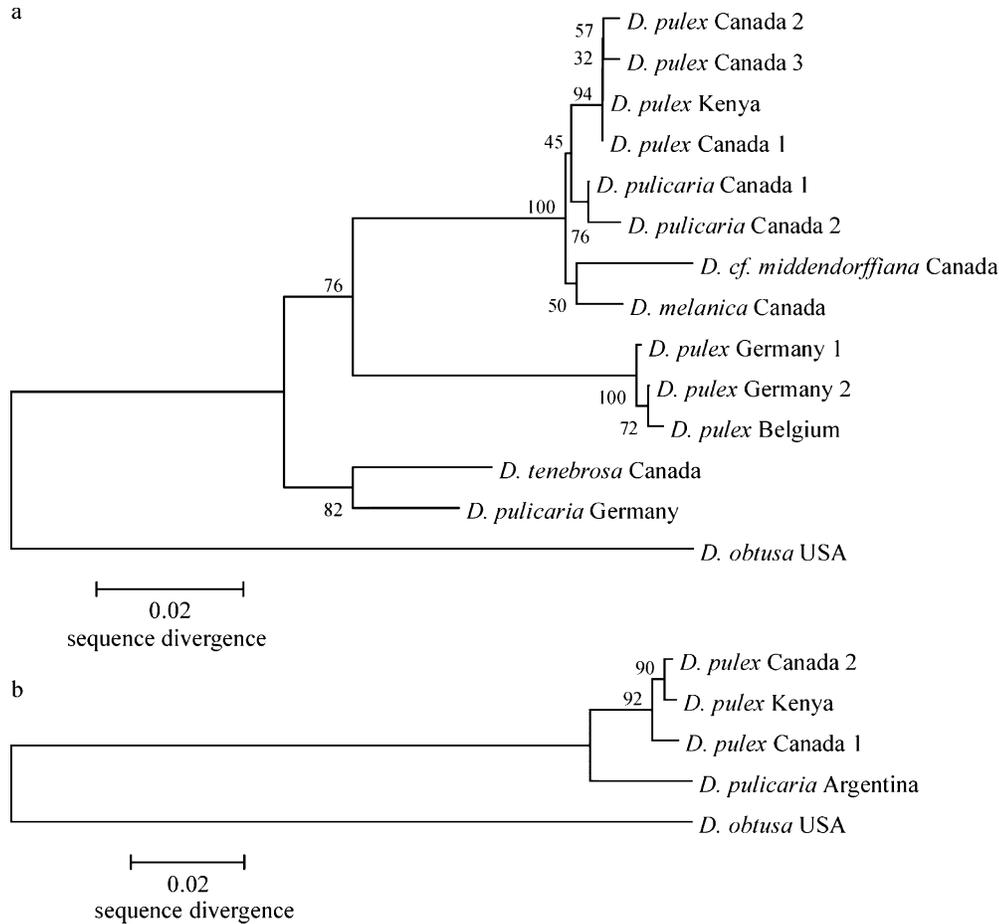


Fig. 2. (a) Neighbor-joining tree of 12S sequence variation in members of the *D. pulex* species complex (excl. *D. obtusa*). All seven Kenyan populations share the same haplotype. (b) Neighbor-joining tree of COI sequence variation of American members of the *D. pulex* complex. All Kenyan populations share the same haplotype. Scale bars indicate Kimura two-parameter genetic distance. Bootstrap values are based on 1,000 pseudoreplicates. Sequence origins with GenBank numbers, if available. 12S: *D. pulex* Canada 1, AF117817; *D. pulex* Canada 3, AY626352; *D. pulicaria* Canada 1, AY626354; *D. pulex* Canada 2, *D. pulicaria* Canada 2, *D. cf. middendorffiana*, *D. melanica*, *D. tenebrosa*, J. K. Colbourne (Colbourne and Hebert 1996); *D. pulex* Germany 1–2, AY626356–7; *D. obtusa* USA, AY626366; *D. pulex* Belgium, AY745245. COI: *D. pulex* Canada 1, AF117817; *D. pulex* Canada 2, AY380449; *D. pulicaria* Argentina, AY489525; *D. obtusa* USA, AY380446

both genes clearly points to an American origin of the Kenyan lineage. Lack of significant sequence divergence with the American clade of *D. pulex* sensu stricto suggests that the Kenyan genotype is not indigenous to Africa. Other traditional cladoceran taxa distributed over different continents almost invariably reveal cryptic endemism at the continental or subcontinental scale (e.g., Colbourne et al. 1998; Taylor et al. 1998; Cox and Hebert 2001; Adamowicz et al. 2004; Penton et al. 2004). Had this also been the case with the Kenyan *D. pulex*, we should have found a separate lineage, distinct from the American–Panarctic and the European clades.

*A single recent colonizer?*—A test of the 10 microsatellite markers used in this study on four European *D. pulex* populations revealed on average eight alleles per locus (J. Mergey unpubl.). Almost complete lack of genetic differentia-

tion on microsatellites between all Kenyan populations therefore indicates that all known Kenyan populations are derived from a single individual. Only the Lake Naivasha population showed some MLG variation, but the two distinct MLGs found at low frequency in Lake Naivasha are just one mutational step away from the dominant genotype found in all other lakes (Table 2) and have probably arisen locally as a result of slippage mutation of microsatellite repeats. North American asexual lineages of *D. pulex* (including hybrids), in contrast, usually show an extraordinary amount of clonal diversity (Innes and Hebert 1988). This reinforces our hypothesis that all seven Kenyan populations originated from a single, relatively recent colonization event followed by rapid range expansion. Although dispersal capabilities of cladocerans were first assumed to be very high (Talling 1951; Mayr 1963), they were questioned later on (Frey 1982, 1987). This study once more stresses the high potential of

Table 2. Microsatellite multilocus genotypes of *D. pulex* found in the seven Kenyan waters. Upper row is microsatellite loci screened for variation. Numbers indicate size of alleles (base pairs).

Genotype*	Dpu7	Dpu40	Dpu45	Dpu46	Dpu12/2	Dp183	Dp525alt	Dp464	Dp502	Dp496
ML1	112-114	119-119	134-134	121-121	136-142	107-107	107-107	147-147	149-149	196-202
ML2	112-114	119-121	134-134	121-121	136-142	107-107	107-107	147-147	149-149	196-202
ML3	112-114	119-119	134-134	121-121	136-142	107-107	107-107	147-147	149-149	193-202

\* ML1, all seven populations; ML2 and ML3, one individual each from Lake Naivasha.

dispersal of cladocerans. Even though the exotic genotype probably did not colonize Africa naturally, it was able to disperse successfully over distances of several hundreds of kilometers following its initial colonization. The spread of American *D. pulex* in Kenya reported here shows similarities with the rapid and successful spread of African *D. lumholtzi* in North America following co-introduction with Nile perch (Havel and Hebert 1993; Havel et al. 2000a). The invasion of American *D. pulex* into Africa would however have remained undetected without genetic analyses. Cryptic invasions like the present one probably often remain undetected, even though their effect on indigenous species and local ecosystems can be equally strong. With routine application of genetic tools for species characterization and identification, possibly many more cryptic invasions will be revealed worldwide in the near future.

*A case of co-introduction with nonnative fish?*—American *D. pulex* (or one of its hybrids) could have immigrated into Kenya in various ways and from various sources. One important possibility is an accidental introduction during one of many historical fish stockings in Lake Naivasha, one of two large freshwater lakes in Kenya's Rift Valley region. Lake Naivasha has a central location with respect to the currently known distribution of *D. pulex* in Kenya (Fig. 1), and the species is especially successful there (Mergeay et al. 2004). As an early and important center of British colonial settlement in Kenya, Lake Naivasha has been the scene of many introductions of exotic species, among which are the North American Largemouth bass (*Micropterus salmoides*), Rainbow trout (*Oncorhynchus mykiss*), and Louisiana red swamp crayfish (*Procambarus clarkii*; Siddiqui 1979). Largemouth bass was introduced repeatedly between 1927 and 1960, Rainbow trout was introduced in the early 1960s in the inflowing Malewa River, and Louisiana red swamp crayfish was introduced in 1970 (Siddiqui 1979). We surmise that American *D. pulex* was accidentally co-introduced on one of these occasions. Still, because exotic fish species have been introduced to almost every large African lake (Ogutu-Ohwayo and Hecky 1991; Muchiri et al. 1995), colonization of Kenya's Rift Valley region by this particular *D. pulex* lineage might also be a case of secondary introduction from an as yet unidentified other African location.

Kenyan *D. pulex* were found to coexist with six other species of *Daphnia* in various types of habitats, from macrophyte-rich and relatively cool to very turbid and warm lakes (Mergeay et al. in press), which suggests that this invader has a large ecological amplitude and a high potential for further dispersal and colonization of other lakes. It presently already dominates the cladoceran community in three

of the seven waters in which it was found (J. Mergeay unpubl.). Most probably, further sampling in East Africa will reveal this asexual exotic zooplankton's presence in many more lakes.

Finally, this and other studies (May 1986; Havel et al. 2000b; Vandekerckhove et al. 2005; Mergeay et al. in press) emphasize that the inclusion of samples from dormant egg bank communities gives a far better assessment of biodiversity than the use of zooplankton samples alone. Without the use of dormant egg bank samples, *D. pulex* would only have been found in one lake and its satellite basin, and the cryptic invasion of this species would, in spite of advanced genetic tools, have gone unnoticed in the majority of the waters surveyed.

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