Contributions to Zoology, 77 (2) 83-90 (2008)

Mitochondrial patterns of intra- and interspecific differentiation among endemic freshwater crabs of ancient lakes in Sulawesi

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Key words: species flock, adaptive radiation, ecotypes, morphotypes, genetic barcoding, Cox1 mtDNA

Abstract

Due to their long-term ecological stability, ancient lakes represent very interesting systems for studying differentiation and speciation processes. High degrees of endemism and specialisation are characteristic features of their fauna. The Malili lakes from the Indonesian island Sulawesi are considered an ancient limnic system, and recent research has increased the number of recognized species and the knowledge about ecological and morphological diversification within a variety of animal taxa. Here we show that the local endemic gecarcinucid freshwater crabs are more differentiated than qualitative morphological characters and the current taxonomy may indicate. The morphologically and ecologically well characterised species *Nautilothelphusa zimmeri* Balss, 1933 consists of two allopatric populations with one of the groups being more closely related to sympatric *Parathelphusa ferruginea* Chia and Ng, 2006 according to mitochondrial genetic data. One haplotype is even shared by both species. Nevertheless, in this study lack of gene flow between both species is demonstrated. To our knowledge, this is the first report of DNA sequence identity between two different genera along the same cytochrome oxidase I gene segment that is being proposed for barcoding studies.

Contents

Introduction

On the Indonesian island of Sulawesi, formerly known as Celebes, two unconnected systems of ancient lakes, i. e. Lake Poso and the Malili lake system, harbour several endemic species and species flocks that apparently evolved as a consequence of adaptive radiation (von Rintelen *et al.*, 2004; Roy *et al.*, 2004, 2007; Herder *et al.,* 2006). The Malili lake system consists of the three major lakes, Matano, Mahalona and Towuti, and two additional smaller ones. Overall, the water of these lakes is clear and oligotroph with very limited biological production (Haffner *et al.,* 2006). Lake Matano is especially remarkable by being a graben lake of approximately 590m depth (Haffner *et al.,* 2001) with an age of about 2-4 Myr (Brooks, 1959; Hamilton, 1997).

A variety of animal taxa evolved species flocks within the Malili lake system, e. g. gastropods (von Rintelen *et al.*, 2004), fish (Roy *et al*., 2004, 2007; Herder *et al.*, 2006) and shrimps (von Rintelen *et al.*, in preparation), of which the phylogenetic relationships and adaptive radiations are being studied. Within the lakes, there are also at least five endemic species of freshwater crabs (Chia and Ng, 2006) belonging to three different ecotypes (Schubart and Koller, 2006; Schubart and Ng, in press). The latter authors have shown that both Lake Poso and the Malili lake system have been colonized independently at least twice. Part of the ecological and taxonomic diversity of these crabs can thus not be the consequence of a single adaptive radiation. The previous studies also showed that two species of freshwater crabs from the Malili lakes, namely *Parathelphusa ferruginea* and *Nautilothelphusa zimmeri* (from lakes Matano and Mahalona), share identical haplotypes of the mitochondrial 16S rRNA gene. In contrast, the Lake Matano population of *N. zimmeri* forms the outgroup to this clade of sympatrically occurring populations of *N. zimmeri* and *P. ferruginea* from lakes Mahalona and Towuti, despite the fact, that the monotypic genus *Nautilothelphusa* appears morphologically and ecologically well characterised and differentiated.

The aim of the present study was to investigate the phylogenetic mitochondrial history of *Nautilothephusa zimmeri* and *Parathelphusa ferruginea* with more detail. A more variable genetic marker and a larger sample size was therefore used to help distinguish consistently between representatives of both species in Lake Mahalona and Lake Towuti and to test, whether *P. ferruginea* mtDNA maintains its intermediate phylogenetic position between two populations of *N. zimmeri*, or whether this pattern would change with a better resolved phylogeny. Compared to the previous qualitative work, the larger sample size of this study allows application of quantitative population genetic methods in order to determine whether there is ongoing gene flow among the sympatric species.

Material and Methods

Between 2000 and 2004, 71 specimens of freshwater crabs (Brachyura: Gecarcinucidae, see Klaus *et al*., 2006) were collected from 24 sampling points (Fig. 1) from the Indonesian island of Sulawesi. Most of the specimens were collected from the Malili lakes (Table 1). The specimens were preserved and stored in ethanol (75-99%). In addition, tissue was obtained from museum collections of the Raffles Museum Zoological Reference Collection Singapore (ZRC) and the Museum für Naturkunde Berlin (ZMB).

We amplified and sequenced 658bp of the cytochrome oxidase I (Cox1) gene from 30 specimens of *Nautilothelphusa zimmeri* (10 from Lake Matano, 8 from Lake Mahalona and 12 from Lake Towuti), 24 specimens of *Parathelphusa ferruginea* (6 from Lake Mahalona, 16 from Lake Towuti and 2 from tributaries to Lake Towuti), six of *Parathelphusa pantherina* (Schenkel, 1902) from Lake Matano, two of *Syntripsa matannensis* (Schenkel, 1902) from Lake Matano, and three of *Syntripsa flavichela* Chia and Ng 2006 from Lake Mahalona and Lake Towuti. As river species, we included two specimens of *Parathelphusa pallida* (Schenkel, 1902) and one specimen each of *Parathelphusa possoensis* (Roux, 1904), *Parathelphusa sarasinorum* (Schenkel, 1902), *Parathelphusa celebensis* (de Man, 1892) and *Parathelphusa lokaensis* (de Man, 1892). Among those, *P. lokaen-*

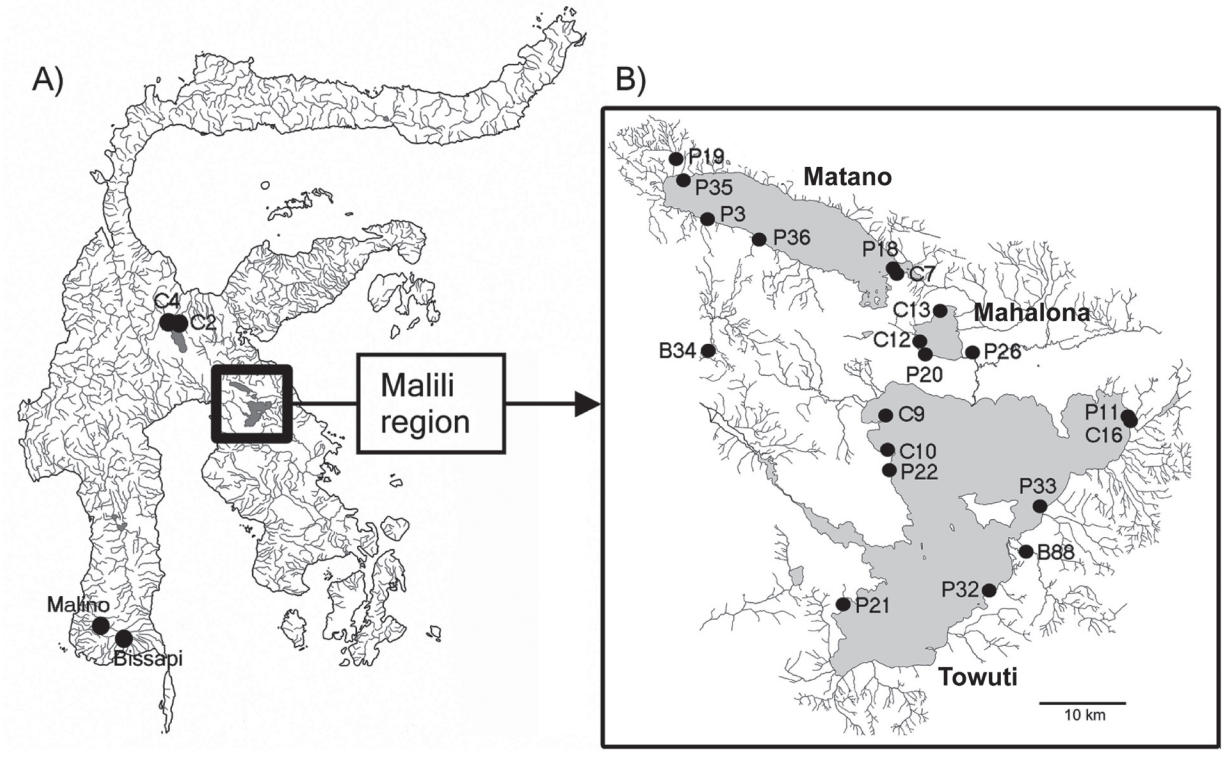

Fig. 1. Maps with the location of the freshwater-crab sampling points: A) The island of Sulawesi, B) the Malili region (modified from von Rintelen and Glaubrecht, 2005).

sis is the only Sulawesi freshwater crab species not occurring within the Malili region and was used as an outgroup, because it may share a relatively recent common ancestor with the fauna from and around the lakes.

DNA was extracted from leg muscle tissue with the Puregene method (Gentra Systems) and used for polymerase chain reactions (PCR) with the primers LCO 1490 (GGT CAA CAA ATC ATA AGA TAT TGG) (Folmer *et al.*, 1994), COL6b (ACA AAT CAT AAA GAT ATY GG) and COH6 (TAD ACT TCD GGR TGD CCA AAR AAY CA) (Schubart and Huber, 2006). PCRs were carried out with the following profile: 40 cycles with 45 sec 94°C, 1 min 48-50°C and 1 min 72°C for denaturing, annealing and extension respectively. PCR products were purified with the Quick-Clean kit (Bioline) or Microcon filters (Millipore), cycle sequenced with the Big Dye Terminator v1.1 (AB Applied Biosystems) and analysed with the automated capillary DNA sequencer ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Sequences of all haplotypes have been submitted to the European Molecular Biology Laboratories (EMBL) and are accessible under FM 177599 - FM 177643.

Alignment of the sequences was carried out with the program BioEdit (Hall, 1999). Absence of insertions/deletions and stop codons suggested lack of nuclear copies and resulted in one obvious alignment. The aligned sequences were analyzed for the best fitting model of DNA evolution with the software Modeltest 3.6 (Posada and Crandall, 1998). The resulting model of evolution GTR+I+G with the corresponding parameters (gamma = 0.871; I = 0.523) was used for Minimum Evolution (ME) and Bayesian Inference (BI) analyses. We used three different methods of phylogenetic inference for our dataset, Maximum Pasimony (MP) and Minimum Evolution (ME) using the software package PAUP* 4.0 (Swofford, 2001) and the Bayesian analysis (BI) using MrBayes v.3.0b4 (Huelsenbeck and Ronquist, 2001). MP trees were calculated with a heuristic search. Confidence values for the different groups within the trees were calculated with the bootstrap method (2,000 pseudoreplicates). Starting trees were obtained by stepwise random sequence addition with ten repetitions and five trees held per step. Branch swapping was conducted with the tree-bisection-

Table 1. List of sampling points from which freshwater crabs were collected in Sulawesi

Sampling point		Location	Species & number of specimens	Museum collection
B34	non-tributary river	2°36.53' S, 121°15.56' E	1 *P. pallida*	ZMB
B88	tributary to L. Towuti	2°48.93' S, 121°35.01' E	1 *P. ferruginea*	ZMB
C2	Tentena at L. Poso	01°45'44.4''S, 120°38'21.5''E	1 *P.sarasinorum*	ZRC
C4	tributary to L. Poso	01°45'0.9''S, 120°32'19.9''E	1 *P.possoensis*	ZRC
C7	east Matano	02°31'48.7''S, 121°27'9.9''E	1 *N. zimmeri*	ZRC
C9	northwest Towuti	02°40'37.7''S, 121°26'26.0''E	1 *N. zimmeri*	ZRC
C10	northwest Towuti	02°42'37.7''S, 121°26'26.0''E	2 *P. ferruginea*	ZRC
C12	west Mahalona	02°35'57.2''S, 121°28'19.2''E	1 *N. zimmeri*	ZRC
C13	north Mahalona	02°33'54.3''S, 121°29'44.1''E	1 *P. ferruginea*	ZRC
C16	northeast Towuti	02°40.89'S, 121°41.49'E	1 *P. ferruginea*	ZRC
P3	south Matano	02°33.566' S, 121°25.187' E	4 *N. zimmeri*, 1 *P. pantherina*, 1 *S. matannensis*	ZMB
P11	northeast Towuti	02°40.82' S, 121°41.43' E	1 *N. zimmeri*, 3 *P. ferruginea*, 1 *S. flavichela*	ZMB
P18	east Matano	02°31.492' S, 121°26.996' E	5 *N. zimmeri*, 2 *P. pantherina*, 1 *S. matannensis*	ZMB
P19	tributary to L. Matano	02°24.932' S, 121°13.594' E	1 *P. pallida*	ZMB
P20	southwest Mahalona	02°36.64' S, 121°28.54' E	7 *N. zimmeri*, 2 *S. flavichela*	ZMB
P21	southwest Towuti	02°51.732' S, 121°23.907' E	3 *N. zimmeri*, 1 *P. ferruginea*	ZMB
P22	west Towuti	02°43.696' S, 121°26.360' E	2 *N. zimmeri*	ZMB
P26	Tominanga River	02°36.638' S, 121° 31.822' E	1 *P. ferruginea*	ZMB
P32	southeast Towuti	02°51.38' S, 121°32.73' E	2 *N. zimmeri*, 5 *P. ferruginea*	ZMB
P33	east Towuti	02°46.20' S, 121°35.97' E	3 *N. zimmeri*, 4 *P. ferruginea*	ZMB
P35	northwest Matano	2°25'56.20"S, 121°14'19.91"E	2 *P. pantherina*	ZMB
P36	south Matano	2°29'38.06"S, 121°18'30.47"E	1 *P. pantherina*	ZMB
unspecified:	L. Mahalona	unknown	5 *P. ferruginea*	
Bissapi	South Sulawesi	5°26'46.96"S, 119°58'7.17"E	*P. celebensis*	ZMB
Malino	South Sulawesi	5°15'43.74"S, 119°44'56.63"E	*P. lokaensis*	ZMB

reconnection (TBR) algorithm, holding multiple trees (MulTrees). Maximum number of trees was set to 5,000 (max trees). Otherwise, the default options of PAUP* 4.0 were used. Only minimal trees were retained and zero length branches were collapsed. The Bayesian analysis was run with four MCMC chains for 2,000,000 generations, saving a tree every 500 generations. After excluding a burn-in phase of 20,000 generations with possible random and suboptimal trees, the posterior probabilities of the phylogeny were determined for the remaining trees. Consensus trees were constructed using the 'sumt' option of MrBayes. As a measure of genetic differentiation between taxa, the Φ_{ST} value was calculated with an AMOVA (Excoffier *et al.* 1992) using the software Arlequin ver. 3.0 (Excoffier *et al.*, 2005).

Results

The analyses of sequence data of 658 basepairs of the cytochrome oxidase I gene from 71 specimens supports the idea that the freshwater crab species occurring in the Malili lakes do not originate from one single common ancestor (Fig. 2).

One lineage (cluster I) contains the generalist and detritivore ecotypes of the lakes, *Parathelphusa pantherina*, *Parathelphusa ferruginea* and *Nautilothelphusa zimmeri*, and forms a well-supported clade in the analyses (BI 0.98; MP 76; ME 69). The molluscivore species *Syntripsa matannensis* and *Syntripsa flavichela* comprise the other lineage (cluster II; BI 1.0; MP 100; ME 100), which does not represent the sister clade to cluster I. Instead, the phylogenetic relationship of cluster II is either unresolved, as in the case of the MP and ME analyses (Fig. 2), or it groups together with the well-supported group (BI 0.92; MP 97; ME 95) of *Parathelphusa possoensis* and *Parathelphusa sarasinorum* from Lake Poso and tributaries in the BI (confidence of 0.85 not shown). Cluster I represents a sister taxon to *Parathelphusa pallida*, a riverine freshwater crab occurring in the Malili region (BI 0.97; MP 59; ME 65). The phylogenetic position of *Parathelphusa celebensis*, another riverine species from the south of Sulawesi, is very basal and clearly separated from the Malili lakes species.

Within the Malili lakes lineages, the phylogenetic pattern reported previously could be confirmed (Schubart and Ng, in press). The homogeneity of *P.*

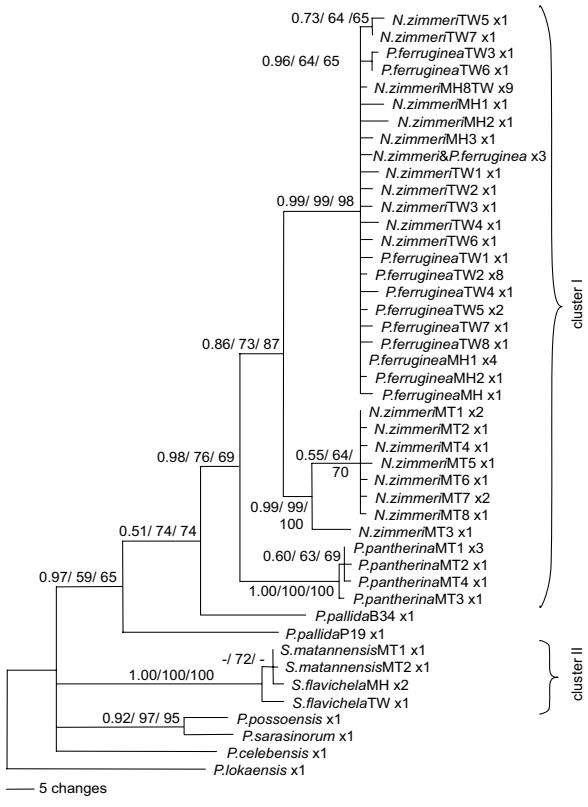

Fig. 2. Bootstrap 50% majority-rule consensus tree (MP topology) of phylogenetic relationships of the freshwater crab species from the Malili lake system (*Parathelphusa pantherina*, *P. ferruginea*, *Nautilothelphusa zimmeri*, *Syntripsa matannensis* and *S. flavichela*), also including *P. sarasinorum* from Lake Poso and the river species *P. pallida*, *P. celebensis*, *P. possoensis* and the outgroup *P. lokaensis*. Bayesian Inference, Maximum Parsimony and Minimum Evolution topologies with GTR+I+G model of evolution where applicable. Confidence values from 2,000 bootstrap replicates (MP/ME) or posterior probabilities (BI) based on 658 basepairs of the Cox1 gene (only values above 50/0.5 shown). MT: Lake Matano; MH: Lake Mahalona; TW: Lake Towuti. Letters behind locality identify haplotype names; 'x' numbers behind taxa reflect the frequency of the corresponding haplotype in the sample.

pantherina, the generalist from Lake Matano, is very well supported by our results (BI 1.0; MP 100; ME 100) and this species forms the sister taxon to the cluster of all *P. ferruginea* and *N. zimmeri* (BI 0.86; MP 73; ME 87). Within the latter cluster, the generalist *P. ferruginea* (from Lake Mahalona and Lake Towuti) groups together with the sympatric population of *N. zimmeri* (BI 0.99; MP 99; ME 98), whereas the Matano population of *N. zimmeri* is well separated from the first group (BI 0.99; MP 99;

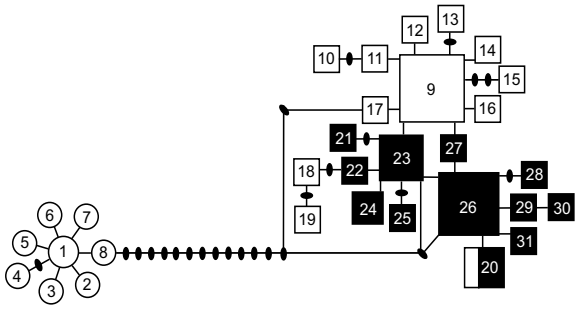

Fig. 3. Minimum parsimonious spanning network of *N. zimmeri* and *P. ferruginea* of a 658 bp fragment from the Cox1 gene. Each line represents one substitution; missing haplotypes are represented by black ovals. The size of a circle or square is representative for the frequency of the haplotype (N = 1-9). Circles correspond to Lake Matano, squares to Lake Mahalona & Lake Towuti; white circles and squares correspond to *N. zimmeri*, black squares to *P. ferruginea*; haplotype 20 was shared by one *N. zimmeri* specimen and two *P. ferruginea* specimens.

ME 100) (Fig. 2). In the minimum spanning network, *N. zimmeri* from Lake Matano is separated from the conspecific population in lakes Mahalona and Towuti and from *P. ferruginea* by at least 13 mutational steps (Fig. 3).

Within the cluster of the generalist (*P. ferruginea* morph) and the detritivore (*N. zimmeri* morph) forms from lakes Mahalona and Towuti, a weak separation of the two species can be observed, but not very distinct and consistent. Three haplotypes of *N. zimmeri* (18, 19, 20) are positioned within the *P. ferruginea* cluster, one of them is even shared by both species (Fig. 3). The pairwise Φ_{ST} value of 0.057 between the two species is low, yet highly significant ($p<0.001$), indicating a restriction of gene flow, but maybe very recent or incomplete. In comparison to that, the Φ_{ST} value between the Matano population and the Mahalona/Towuti population of *N. zimmeri* is much higher ($\Phi_{ST}= 0.462$; $p<0.001$). This finding is surprising, since morpholgically and ecologically the two populations of *N. zimmeri* are very similar and clearly derived from the bodyplan of riverine species and those of lacustrine generalists as *P. ferruginea*.

Discussion

Our genetic results render additional support to the previous finding that the extant freshwater crab species from the Malili lake system did not evolve from one single ancestor (see also Schubart and Ng, in press). There were at least two separate colonization events, resulting in two not directly related clusters, i.e. cluster I *(Parathelphusa pantherina, Parathelphusa ferruginea & Nautilothelphusa zimmeri)* and cluster II *(Syntripsa matannensis & Syntripsa flavichela)* (Fig. 2). These two groups do not only differ genetically, but are also ecologically separated, as the species of cluster I comprise the generalist (*P. pantherina*, *P. ferruginea*) and the detritvore (*N. zimmeri*) ecotypes, whereas the genus *Syntripsa* of cluster II appears to be molluscivorous judging by the chelar morphology (Chia and Ng, 2006; Schubart and Ng, in press). In terms of morphology and behaviour, the generalists are closest to the riverine forms and are therefore considered as the most underived ecotype in the lakes, with the detritivore (feeding on detritus and other small food items) and the molluscivore (specialized in feeding on hard-shelled molluscs) ecotypes being specialized to lake habitats (Chia and Ng, 2006). For the genus *Syntripsa* we could not detect a closely related sister taxon among the adjacent river populations. As no molluscivore riverine species is known, we also assume for this group a generalist ancestor, specializing on the abundant snails encountered in the lakes as a food source. The extremely strong crushing chela of these crabs and the thicker shells of the snail species of the lakes in comparison to riverine species points to a coevolution of the molluscivore crabs and the molluscs in an arms race (von Rintelen *et al*., 2004).

With the cytochrome oxidase I gene sequence data, we could confirm and expand the 16S rRNA based results of Schubart and Ng (in press). The Matano population of *N. zimmeri* forms an outgroup to the cluster of *P. ferruginea* and *N. zimmeri* from the lakes Mahalona and Towuti (Fig. 2), which would make the latter species a paraphylum according to mtDNA, indicating that either this generalist is derived from the detritivore type or that the detritivore ecotype evolved two times independently. This result is the more surprising as the populations of *N. zimmeri* are morphologically very similar with very characteristic features missing in *P. ferruginea*, e. g. the squarish carapace and the broadened propodi of the last pair of locomotory legs.

A qualitative separation of *P. ferruginea* and the sympatrically occurring *N. zimmeri* population was not possible with the analysed 658 bp long fragment of the Cox1 gene. In the minimum spanning

network (Fig. 3), some of the *N. zimmeri* haplotypes are positioned in between those from *P. ferruginea* and in one case a haplotype is even shared by two individuals of *P. ferruginea* and one individual of *N. zimmeri*. Howewer, here we show that these two groups differ significantly in their haplotype composition, which is indicated by a low, but highly significant Φ_{ST} value ($\Phi_{ST} = 0.057$, $p<0.001$). A clearer separation may be possible with a more variable genetic marker or a longer fragment of the Cox1.

Although the results of mitochondrial genetic markers and morphology are in conflict with each other and data from nuclear genetic markers are still unavailable (Koller and Schubart, in progress), the two populations of *N. zimmeri* (Matano vs. other two lakes) differ genetically to a great extent and we can already claim lack of gene flow between them ($\Phi_{ST} = 0.462$; $p<0.001$). The question remains, whether these populations can be separated by morphological features. Separating qualitative morphological characters were not found during our investigations, but morphometric analyses are being carried out to distinguish between the two allopatric populations (Koller and Schubart, in progress).

Genetic distinction between the two populations of the detritivore *N. zimmeri* fits very well the finding that other crab ecotypes from the lakes are always represented by different species in Matano versus Mahalona/Towuti: *P. pantherina*, *S. matannensis* in Lake Matano and *P. ferruginea*, *S. flavichela* in the lakes Mahalona and Towuti. A similar pattern of consistent differences in species composition of these lakes can also be found in the endemic fish (Family Telmatherinidae) (Herder *et al.*, 2006) and in atyid shrimps (genus *Caridina*) (Roy *et al.*, 2006) of the Malili region. But in the case of the fish, all endemic species of Lake Matano originated from a single colonisation event, whereas for freshwater crabs, descendants of two colonisations can be found in all three major lakes. The viviparous freshwater gastropod genus *Tylomelania* also colonised the Malili lake sytem several times independently, but only one clade spread to all three major lakes, whereas the other two clades in this area are primarily restricted to the lake they colonised first (von Rintelen *et al.*, 2004). Overall, ecological adaptions, i.e. trophic specialisation, are a general feature of speciation in the Malili lakes (see Roy *et al.*, 2007), going along with potential others like sexual selection, alloparty or coevolution in the case of molluscs and snail-eating crabs.

Our results are also of importance with respect to the emerging field of barcoding animal life. Hebert *et al.* (2003) promoted the same Cox1 gene region as used in this study as the best marker for a DNA barcoding system to render fast and accurate taxonomic identification and aiding the discovery of new species. Such a species-specific barcode would be of great advantage for establishing and managing a taxonomic system of the animal kingdom, with a vast number of not yet described species. Moritz and Cicero (2004) pointed out possible limitations of mtDNA for recognizing species boundaries. Retention of ancestral polymorphism, male-biased gene flow, selection on any part of the mitochondrial genome, introgression and paralogy due to nuclear copies of the mitochondrial marker are factors that could produce phylogenetic signals of the mtDNA, which may deviate from the actual phylogeny of the species. Especially for closely related, young species and rapidly evolving lineages, problems in these regards have to be expected (Monaghan *et al.*, 2006). In the case of *Nautilothelphusa zimmeri* and *Parathelphusa ferruginea*, species (and genus) identification by a Cox1 barcode would be impossible. Due to the genetic identity of at least one haplotype, this Cox1 region is not unique for any of the two species and therefore a genetic misidentification up to genus level would become possible. The genus *Nautilothelphusa* is closely related to *Parathelphusa ferruginea*. Thus, retention of *Nautilothelphusa* as a valid genus makes this a case of imperfect taxonomy (Meyer and Paulay, 2005) by making the genus *Parathelphusa* paraphyletic.

The here described pattern of shared haplotypes by two morphologically clearly divergent species and genera could possibly be explained by incomplete lineage sorting or introgression. Llopart *et al.* (2005) described for two West African species of *Drosophila* with a hybridization zone the genetic patterns of introgression, resulting in a situation in which the mitochondrial genome of one species was apparently completely replaced by the one from a second species. The importance of introgressive hybridization during invasion of new environments and speciation has been highlighted repeatedly (e.g. Dowling and Secor, 1997; Salzburger *et al.*, 2002; Seehausen, 2004). Nuclear genetic data (in progress) may clarify, whether a similar case of introgression and mtDNA replacement has taken place in the crabs from Sulawesi and possibly provide independent evidence for other interesting aspects of this

study, like the deep and complete genetic separation between the two populations of *N. zimmeri* from the different lakes.

Acknowledgements

We want to thank Fabian Herder (Forschungsmuseum Alexander Koenig, Bonn, Germany), Matthias Glaubrecht, Thomas and Kristina von Rintelen (Museum für Naturkunde, Berlin, Germany) for collecting specimens, organisation and support during the field trips and the exchange of ideas. The staff of INCO in Soroako, Lake Matano, kindly supported us by providing accommodation, transport and general logistics, which facilitated our field enormously. We are grateful for the continuous support by Peter K.L. Ng (National University of Singapore) and for identification of some of the river forms. Our thanks also go to Juergen Heinze (University of Regensburg) and his staff for the use of all the necessary facilities. Finally, we like to thank one anonymous reviewer for expert comments.

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Received: 19 February 2008
Accepted: 20 June 2008