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Direction of karyotype evolution in the bug family Nabidae (Heteroptera): New evidence from 18S rDNA analysis

CHRISTINA NOKKALA[1], VALENTINA KUZNETSOVA[2], SNEJANA GROZEVA[3] and SEPPO NOKKALA[1]

[1]Laboratory of Genetics, Department of Biology, University of Turku, FI 20014 Turku, Finland; e-mail: chrinok@utu.fi, sepnok@utu.fi

[2]Department of Karyosystematics, Zoological Institute, Russian Academy of Sciences, 199034 St. Petersburg, Russia; e-mail: karyo@zin.ru

[3]Institute of Zoology, Bulgarian Academy of Sciences, Tsar Osvoboditel 1, 1000 Sofia, Bulgaria; e-mail: sgrozeva@yahoo.com

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Abstract. The bug family Nabidae (Heteroptera) includes taxa showing either a low chromosome number 2n = 16 + XY or high chromosome numbers 2n = 26 or 32 + XY. In order to reveal the direction of karyotype evolution in the family, a molecular phylogeny of the family was created to reveal the taxon closest to the ancestral type and hence the ancestral karyotype. The phylogeny was based on a partial sequence of the 18S rDNA gene of both high and low chromosome number species belonging to the subfamilies Prostemmatinae and Nabinae. Phylogeny created by the Neighbour Joining method separated the subfamilies, Prostemmatinae and Nabinae, which form sister groups at the base of this phylogenetic tree, as well as within the Nabinae, tribes Nabini and Arachnocorini. Combining karyosystematic data with the phylogeny of the family indicated that the ancestral karyotype was a high chromosome number, consisting of 2n = 32 + XY. During the course of evolution changes have occurred both in meiotic behaviour of the sex chromosomes and in the number of autosomes. The direction of karyotype evolution was from a high to low autosome number. Abrupt decreases in the number of autosomes have occurred twice; firstly when the tribe Arachnocorini differentiated from the main stem in the subfamily Nabinae and secondly within the tribe Nabini, when within the genus *Nabis* 2n = 16 + XY species diverged from the 2n = 32 + XY species. A scheme of the sequence of events in karyotype evolution during the evolution of the Nabidae is presented.

INTRODUCTION

Nabidae is a relatively small bug family with 20 genera and approximately 400 species. The representatives of this family are distributed throughout the world. The family is one of the most primitive in the large infraorder Cimicomorpha and hence it is of major importance for the classification and phylogeny of this infraorder. According to Kerzhner (1981, 1996), the family Nabidae consists of four subfamilies: Velocipedinae, Medocostinae, Prostemmatinae (two tribes), and Nabinae (four tribes). The Nabinae and Prostemmatinae are sister groups. In contrast to Kerzhner, Schuh & Štys (1991) consider the first two subfamilies to be separate families. They based this concept on labial and membrane venation characters and the presence of Ekblom's organ in nabids.

The diploid chromosome numbers reported for 27 nabid species vary between 2n = 18 (16+XY) and 2n = 34 (32 + XY), with one species with 2n = 38 (36 + XY) (for a review see Kuznetsova et al., 2004) and the first mentioned number clearly predominates. The abundance of the karyotype 2n = 16 + XY has led to the hypothesis that it is the ancestral karyotype for the family and higher chromosome numbers represent derived characters (Leston, 1957; Ueshima, 1979; Thomas, 1996; Kuznetsova & Maryańska-Nadachowska, 2000). To account for the evolution of karyotypes, it is suggested that polyploidy (Thomas, 1996) or autosomal polyploidy (Kuznetsova & Maryańska-Nadachowska, 2000) has played a crucial role in the family Nabidae, resulting in the doubling of autosome number from 2n = 16 + XY to 2n = 32 + XY. However, the existence of polyploidy in Heteroptera is questioned by Jacobs (2002). Recently, an alternative hypothesis was put forward by Kuznetsova et al. (2004). They noted that a high chromosome number species, *Prostemma guttula,* belonging to the subfamily Prostemmatinae displays meiotic behaviour of the X and Y sex chromosomes different from that of the subfamily Nabinae, but identical to that found in the closely allied outgroup families, Anthocoridae, Miridae and Cimicidae. This finding suggests that the subfamily Prostemmatinae displayes the ancestral type of sex chromosome behaviour and hence indicates that a high autosome number is the ancestral character in the family and not a derived one. Kuznetsova et al. (2004) suggested that karyotypes in Nabidae have evolved from 2n = 34 (32 + XY), with a decrease or increase of autosome number in different taxa of the family brought about by a series of fusion or fragmentation events.

In order to distinguish between these two hypotheses, chromosome banding techniques were used to analysze karyotypes within Nabidae (Grozeva & Nokkala, 2003; Grozeva et al., 2004). However, the results obtained were inconclusive. In the present study a partial sequencing of the 18S rDNA gene, in both low and high chromosome number species in the family, is used to construct a

molecular phylogeny of the family in order to reveal the ancestral taxon and hence the ancestral karyotype, and propose the sequence of events resulting in the evolution of nabid karyotypes.

MATERIAL AND METHODS

Insects

The specimens of *Nabis* (s. str.) *brevis* Scholtz 1847, *N.* (s. str.) *ferus* (Linnaeus, 1758), *N.* (*Dolichonabis*) *limbatus* Dahlbom, 1851, *N.* (*Nabicula*) *flavomarginatus* Scholtz, 1847 were collected in the vicinity of Turku, Finland, *N.* (*Australonabis*) *biformis* Bergroth, 1927 in New Zealand, and the specimens of *Himacerus* (*Himacerus*) *apterus* Fabricius, 1798, *H.* (*Aptus*) *mirmicoides* (O. Costa, 1834) and *Prostemma guttula* (Fabricius, 1787) in the Rhineland, Germany. Specimens of *N.* (*Halonabis*) *sareptanus* Dohrn, 1862 originated from the Black Sea coast, Bulgaria and specimens of *Arachnocoris trinitatus* Bergroth from Arena Forest Reserve, Trinidad. *Himacerus* (*Stalia*) *boops* (Schiødte, 1870) originated from Dragsfjärd, situated in the southwest archipelago of Finland and *Himacerus* (*Anaptus*) *major* (A. Costa, 1848) from Vlieland, The Netherlands. *Liocoris tripustulatus* (Fabricius, 1781), Miridae, collected from the vicinity of Turku, Finland served as an outgroup. Live insects were put in 96% alcohol and stored at +4°C or –20°C.

Taxonomy

P. guttula belongs to the subfamily Prostemmatinae and all other species belong to the subfamily Nabinae. Within the Nabinae the tribe Arachnocorini is represented by the species *A. trinitatus* and the tribe Nabini by species of *Nabis* and *Himacerus* (Kerzhner, 1981, 1996).

Chromosome numbers

P. guttula, 2n = 26 + XY, *H. apterus*, 2n = 32–34 +XY, *H. mirmicoides*, 2n = 32 + XY, and *N. (Halonabis) sareptanus,* 2n = 32 + XY have high chromosome numbers, whereas *A. trinitatus* 2n = 10 + XY and *N. brevis, N. ferus*, *N. limbatus*, *N. flavomarginatus*, 2n = 16 + XY, have low chromosome numbers (for reviews see Kuznetsova & Maryańska-Nadachowska, 2000; Kuznetsova et al., 2004, 2007). The chromosome numbers of *H.* (*Anaptus*) *major* and *Nabis* (*Australonabis*) *biformis* are unknown. The chromosome number of the outgroup species *L. tripustulatus* is 2n = 32 + XY (Leston, 1957; Nokkala & Nokkala, unpubl.).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from individual fresh or alcohol-preserved specimens. Single insects were homogenized in liquid nitrogen and DNA was extracted with DNeasy™ Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was finally eluted with 100 or 200 µl of distilled water depending on the size of the specimen.

Primers used to amplify a segment of about 550 bp of the 18S ribosomal RNA gene were 5'-CTG GTT GAT CCT GCC AGT AG-3' (forward) (Sorensen et al., 1995) and 5'-CCG CGG CTGCTG GCA CCA GA-3' (reverse) (von Dohlen & Moran, 1995). The PCR reaction was carried out in a 50 µl volume containing 1× buffer for DyNAzyme supplemented with 1.5 mM $MgCl_2$, 200µM of each dATP, dCTP, dGTP and dTTP, 1 unit of DyNAzyme II DNA polymerase (all from Finnzymes, Espoo, Finland), 0.4 µM of each primer and 50 mM of template DNA. PCR amplification conditions were as follows: 5 min at 95°C, followed by 30 cycles of 40 s at 95°C, 40 s at 54°C, 45 s at 72°C and a single final extension step at 72°C for 10 min. After checking the PCR products on a 1% agarose gel containing 0.5% ethidium bromide and sized against a 100 bp DNA ladder (New England BioLabs, Ipswich, MA, USA), PCR products were purified for direct sequencing with QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions and eluted finally with 30 µl of distilled water. Both strands of PCR products were sequenced with the original primers using an ABI Prism 377® automatic sequencer.

Sequence editing and phylogenetic analysis

Sequences were edited with the aid of BioEdit program (Hall, 1999). The 18S rDNA sequence of Nabidae contains distinct conserved stretches, which allowed the manual alignment of sequences. Phylogenetic analyses were carried out using the programs in the PHYLIP package (version 3.6) by Felsenstein (2005). All sites were weighted equally. We constructed maximum likelihood, parsimony and neighbour-joining trees using the Kimura 2-parameter distances. Support for particular nodes was assessed by 1000 bootstrap replicates.

RESULTS

Nucleotide sequences

After sequence editing, sequences ranging from 483 bp (*Liocoris tripustulatus*) through 524 bp (*Nabis flavomarginatus*, *N. ferus*, *N. brevis*, *N. limbatus*) and 525 bp (*N. biformis*, *Arachnocoris trinitatus*, *Himacerus apterus*, *H. myrmicoides*, *H. boops*, *H. major*, *Prostemma guttula*) to 526 bp (*N.* (*Halonabis*) *sareptanus*) were recovered. The A+T content of all sequences is similar, ranging from 51.81% in *H. boops* to 53.71% in *A. trinitatus* (data not shown). The sequences are deposited in GenBank, accession numbers DQ199623–DQ199631, DQ199633, DQ906002–DQ906003, and EU005234.

The alignment yielded a 526 bp stretch. Among the species of Nabidae, there were only two indels; at site 14, *N.* (*Halonabis*) *sareptanus* alone showed a C, whereas at site 138 *Nabis* species other than *N. biformis* lacked a base**.** Of the 526 sites, 51 were variable and 40 phylogenetically informative. The first nucleotide in the present alignment corresponds to 18S rDNA site 50 in 21 Reduviid and one Mirid species and to site 52 in a Lygaeid species, whose complete 18S rDNA sequences are available in GenBank.

Phylogenetic analyses

The phylogenetic tree created by the neighbour joining (NJ) method and rooted by the outgroup species *Liocoris tripustulatus* (Miridae) (Fig. 1) indicates that the subfamilies Prostemmatinae and Nabinae are sister groups, supported by a bootstrap value of 68%. Within the subfamily Nabinae the tribes Arachnocorini and Nabini are seen as sister groups with 71% bootstrap support. Within the tribe Nabini, the genus *Himacerus* forms a sister group with the genus *Nabis* (98% bootstrap support). Within the genus *Nabis* the subgenus *Halonabis* forms a sister group with the remaining *Nabis* species, supported by a bootstrap value of 61%.

Topologies of trees obtained using parsimony and maximum likelihood methods were virtually identical with the one created by the NJ method. The only difference was that nodes were not supported with significant bootstrap values in as many cases as with the NJ method. The position of the subgenus *Halonabis* as a sister group

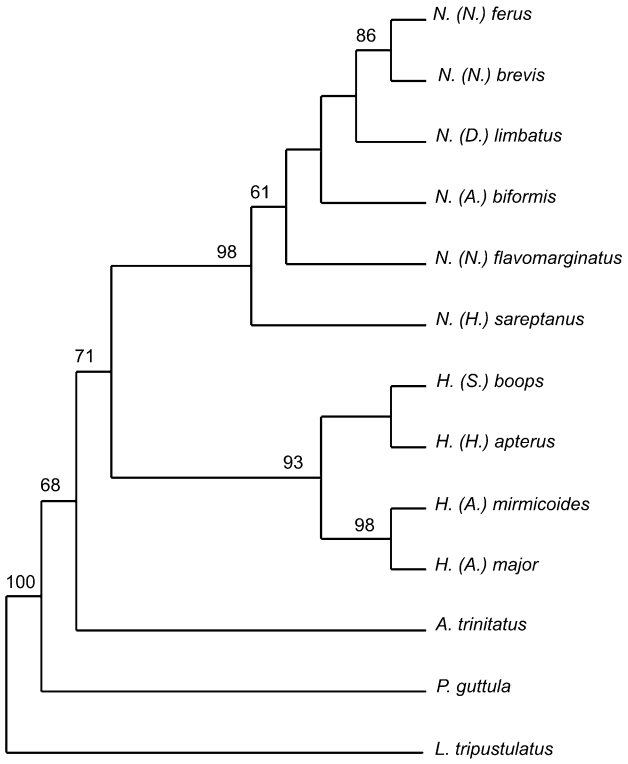

Fig. 1. Neighbour joining tree of the Nabidae resulting from an analysis of 18S rDNA data. Bootstrap values greater than 50% are shown.

of other *Nabis* species was, however, significantly supported by both parsimony and maximum likelihood phylogenies (trees not shown).

DISCUSSION

Molecular phylogeny of the family and taxonomic relationships.

The molecular phylogeny is in good accordance with the systematic division of the family Nabidae by Kerzhner (1981, 1996). The sister group relationship of the subfamilies Prostemmatinae and Nabinae is well supported. Within the subfamily Nabinae the sister group relationship of the tribes Arachnocorini and Nabini is supported by the neighbour joining phylogeny. Hence, the idea that the tribe Arachnocorini diverged early on from the main stem of Nabini (Kerzhner, pers. commun.) is supported by the molecular phylogeny. Within the tribe Nabini the position of the genus *Himacerus* as a sister group to the genus *Nabis* is unambiguous as well as the position of the subgenus *Halonabis* separate from the rest of the *Nabis* species. On the basis of chromosome number and the molecular phylogeny, *Halonabis* could even be considered a separate genus, as previously suggested by Kerzhner (1981).

Evolution of karyotypes

Kuznetsova et al. (2004) studied the karyotypes of *Prostemma guttula* and *Pagasa fusca*, and found their chromosome numbers to be high, 2n = 26 + XY. They also found that the sex chromosomes in *P. guttula* showed "touch and go" pairing at metaphase II in male meiosis. This behaviour is common to the sex chromosomes in the outgroup families Anthocoridae, Cimicidae, and Miridae within the Cimicomorpha and all other heteropteran families showing post-reduction of the sex chromosomes. However, the sex chromosomes in the subfamily Nabinae show "distance" pairing at the second metaphase. The "distance" pairing of sex chromosomes in the second meiotic division is not found elsewhere in Heteroptera and was first considered to be a unique character of the Nabidae as a whole (Nokkala & Nokkala, 1984; Kuznetsova & Maryańska-Nadachowska, 2000), but after finding "touch and go" pairing in Prostemmatinae "distance" pairing appeared to be unique only for the subfamily Nabinae (Kuznetsova et al., 2004). Based on the outgroup type of sex chromosome behaviour in Prostemmatinae, Kuznetsova et al. (2004) suggested that Prostemmatinae represents a taxon closest to the ancestral form and hence for the family a high chromosome number would be ancestral, not a low one. They also suggested that the ancestral karyotype consisted of 2n = 32 + XY and that the sex chromosomes in this taxon showed "touch and go" pairing in the second meiotic division. The idea is strongly supported by the present molecular phylogenetic analysis, which places the subfamily Prostemmatinae at the base of the tree and indicated that both subfamilies, Nabinae and Prostemmatinae, include branches made up of high chromosome number taxa, the genus *Himacerus* and the subgenus *Halonabis* in the former and the genus *Prostemma* in the latter. The outgroup family Miridae also has a high chromosome number, and their modal chromosome number is 2n = 32 + XY (Ueshima, 1979) and is represented by *L. tirpustulatus* in the present study. It is clear from the phylogeny that the chromosome number 2n = 16 appeared late in evolution after the most advanced subgenera of *Nabis* had evolved. Hence, any suggestion that a low chromosome number (2n = 16 + XY) is a plesiomorphic character in this family, as required by the polyploidy hypothesis of Thomas (1996) or the autosomal polyploidy hypothesis of Kuznetsova & Maryańska-Nadachowska (2000), can be excluded.

Combining current karyosystematic knowledge (for a review see Kuznetsova et al., 2004, 2007) with a molecular phylogeny of the family indicates the following trend in the evolution of the karyotype in the family Nabidae. The ancestral karyotype was 2n = 32 + XY and the sex chromosomes underwent "touch and go" pairing at the second meiotic division. This karyotype was selected to be ancestral, as it or something similar is characteristic of the closely related families within the Cimicomorpha, Miridae, Athocoridae, and Cimicidae, and also within the family Nabidae (Kuznetsova et al., 2004). Fusions of chromosomes resulted in the present day karyotype 2n = 26 + XY, found in *P. guttula* and *P. fusca*. The emergence of the subfamily Nabinae is accompanied by the preservation of the ancestral chromosome number 2n = 32 + XY and a change in the behaviour of the sex chromosomes, resulting in "distance" pairing

instead of "touch and go" pairing at the second metaphase. Soon after the emergence of the Nabinae, the tribe Arachnocorini diverged from the main stem. This divergence was accompanied by a marked decrease in chromosome number by chromosome fusions, resulting in the karyotype 2n = 10 + XY found in *Arachnocoris trinitatus*. In the main stem, represented by the tribe Nabini in the present study, the karyotype is 2n = 32 + XY or slightly modified by fragmentations, as in the genus *Himacerus*. On the other hand, the karyotype is preserved in its original form in subgenus *Halonabis*. This karyotype is also found in the closely related subgenus *Aspilaspis* (Kuznetsova et al., 2004). Finally, decrease in the number of autosomes by chromosome fusions within the tribe Nabini resulted in the karyotype 2n = 16 + XY, found in most of the present day *Nabis* species, as in *N. ferus*, *N. brevis*, *N. limbatus*, *N. flavomarginatus*, and twelve other *Nabis* species (Kuznetsova et al., 2004). Apparently, further divergence occurred in the tribe Nabini without changes in chromosome number as, in addition to the genus *Nabis*, the karyotype 2n = 16 + XY is also found in the genera *Lasiomerus* (Montgomery, 1901) and *Hoplistocelis* (Kuznetsova & Maryańska-Nadachowska, 2000).

In conclusion, the evolution of karyotypes in the family Nabidae has been accompanied by changes in both the number of autosomes and the meiotic behaviour of the sex chromosomes. The direction of the change is clearly from high to low number of autosomes. A marked decrease in the number of autosomes by chromosome fusions has occurred not once, but at least on two occasions during the course of evolution in this family, firstly when Arachnocorini diverged from the main stem of Nabini and secondly when the subgenera of *Nabis* diverged from the subgenus *Halonabis*.

The evolution of karyotypes in the family Nabidae involves properties common to groups with holokinetic or holocentric chromosomes. Firstly, substantial divergence can occur without any change in the number of autosomes, as shown, e.g., by the species with either low or high chromosome number within the tribe Nabini, which all share the karyotype 2n = 16 + XY or 2n = 32 + XY, respectively. The phenomenon is even more pronounced in the family Tingidae, where all the species share the same autosome number (for a review see Grozeva & Nokkala, 2001). Secondly, the emergence of some taxa was preceded by an abrupt and prominent change in the number of autosomes. As in the tribe Nabini species with either a high or low chromosome number exist, but not a single species with an intermediate number of autosomes. Thirdly, decrease in the number of autosomes by chromosome fusions is far more common than the increase in the number of autosomes by chromosome fragmentations. This difference might be due to the fact that a chromosome, be it monocentric or holocentric, has to have two functional telomeres if it is to survive a mitotic cycle. In the case of chromosome fusions, the fusion chromosome always carries functional telomeres that originated from the parent chromosomes, whereas a fragmented chromosome has to be able to develop a functional telomere.

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