RESEARCH ARTICLE



Chromosomal and DNA barcode analysis of the Polyommatus (Agrodiaetus) damone (Eversmann, 1841) species complex (Lepidoptera, Lycaenidae)

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Abstract

The *Polyommatus (Agrodiaetus) damone* (Eversmann, 1841) species complex comprises from 5 to 8 species distributed in southeastern Europe and southern Siberia. Here we used chromosomal and DNA-barcode markers in order to test the taxonomic hypotheses previously suggested for this complex. We revealed that all taxa within this group demonstrate chromosomal stasis and share the same or very similar haploid chromosome number (n = 66 or n = 67). This finding is unexpected since the karyotypes are known to be very diverse and species-specific within the other taxa of the subgenus *Agrodiaetus* Hübner, 1822. Analysis of the mitochondrial gene *COI* revealed six diverged clusters of individuals within the complex. Each cluster has a specific geographic distribution and is characterized by distinct morphological features in the wing pattern. The clusters mostly (but not always) correlate with traditionally recognized species. As a result of our study, we describe a new subspecies *P* (*A.*) *iphigenides zarmitanus* **subsp. nov**. from Uzbekistan and Tajikistan and show that the taxon originally described as *Lycaena kindermanni* var. *melania* Staudinger, 1886 represents a subspecies *P* (*A.*) *iphigenides melanius* (Staudinger, 1886). *Polyommatus (A.) samusi* Korb, 2017 (**syn. nov**.) and *P* (*A.*) *melanius komarovi* Korb, 2017 (**syn. nov**.) are considered here as junior subjective synonyms of *P* (*A.*) *iphigenides iphigenides* (Staudinger, 1886).

Keywords

Agrodiaetus, chromosomal stasis, chromosome, COI, DNA barcoding, karyosystematics, taxonomy

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Introduction

The *Polyommatus (Agrodiaetus) damone* (Eversmann, 1841) species complex is a monophyletic group (Vershinina and Lukhtanov 2017) that comprises from 5 to 8 species distributed in SE Europe, Central Asia and S Siberia (Eckweiler and Bozano 2016). The taxa of the complex were previously revised by Staudinger (1899), Forster (1956, 1960), Dantchenko and Lukhtanov (1993) and Dantchenko (1997). There are also limited molecular (Wiemers 2003; Kandul et al. 2004, 2007; Lukhtanov et al. 2005, 2009; Vodolazhsky et al. 2011; Vodolazhsky and Stradomsky 2012) and chromosomal (Lukhtanov 1989; Kandul 1997; Lukhtanov et al. 1997; Lukhtanov and Dantchenko 2002a; Lukhtanov et al. 2005) data for a few taxa of the complex. However, the complex has never been systematically studied by using chromosomal and molecular markers, although such an approach is considered as an essential requirement for revealing taxonomic structure in the subgenus *Agrodiaetus* (Lukhtanov and Dantchenko 2002b; Kandul et al. 2004).

Here we analyzed karyotypes and mitochondrial DNA-barcodes of all species of the P. (A.) *damone* complex in order to test the taxonomic hypotheses previously suggested for this group (see the references above).

The taxa P. (A.) damone walteri Dantchenko et Lukhtanov, 1993, P. (A.) damone fabiani Bálint, 1997 and P. (A.) damone bogdoolensis Dantchenko et Lukhtanov, 1997 are not considered in this paper since neither chromosomal nor molecular data are available. This also applies to P. (A.) carmon altaiensis (Forster, 1956), recently treated by Eckweiler and Bozano (2016) as a separate species. All these taxa represent the most eastern populations of the P. (A.) damone complex distributed in Mongolia, Altai and southwestern Siberia. Morphologically they are close to other populations of P damone or to P. mediator Dantchenko et Churkin, 2003. Their study will become possible in the future as soon as the material suitable for molecular and chromosomal analyses becomes available.

Material and methods

Molecular methods and DNA barcode analysis

Standard *COI* barcodes (658-bp 5' segment of mitochondrial *cytochrome oxidase subunit I*) were studied. *COI* sequences were obtained from 44 specimens representing the *P. damone* species group and from two samples [*P. damon* (Denis et Schiffermüller, 1775) and *P. icarus* (Rottemburg, 1975)] which were selected as outgroup (Table 1). Legs were sampled from these specimens, and sequence data from the DNA barcode region of *COI* were obtained at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using protocols described in Hajibabaei et al. (2005), Ivanova et al. (2006) and deWaard et al. (2008). Specimens examined are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia and in the McGuire Center for Lepidoptera and Biodiversity

| Species and subspecies | Sequence code | Field code | GenBank number | Country | Locality | Reference |
|----------------------------|---|----------------|---------------------|--------------------------|-------------------------------------|------------------------------------|
| P. damon | FJ663230 | n/a | FJ663230 | Kazakhstan | Altai | Lukhtanov et al. 2009 |
| P. damone altaicus | FJ663229 | LOWA298-06 | FJ663229 | Kazakhstan | Saur-Tarbagatai Mts | Lukhtanov et al. 2009 |
| P. damone altaicus | FJ663228 | LOWA299-06 | FJ663228 | Kazakhstan | Saur-Tarbagatai Mts | Lukhtanov et al. 2009 |
| P. damone altaicus | BPAL3395-16 | CCDB-25452_F10 | MW186990 | Russia | Altai, Jarbalyk | This study |
| P. damone altaicus | BPAL838-11 | CCDB-05724_G06 | MW186700 | Kazakhstan | Saur Mts, Saikan | This study |
| P. damone altaicus | BPAL839-11 | CCDB-05724_G07 | MW186701 | Kazakhstan | Saur Mts, Saikan | This study |
| P. damone altaicus | AY496734 | n/a | AY496734 | Russia | Altai, Aktash | Kandul et al. 2004 |
| P. damone altaicus | BPAL3394-16 | CCDB-25452_F09 | MW186989 | Russia | Altai, Chemal | This study |
| P. damone | BPAL836-11 | CCDB-05724_G04 | MW186988 | Russia | Volga, Volsk | This study |
| P. damone | BPAL837-11 | CCDB-05724_G05 | MW186992 | Russia | Volga, Volsk | This study |
| P. damone | BPAL835-11 | CCDB-05724_G03 | MW186999 | Russia | Volga, Akulovka | This study |
| P. damone | AY496735 | n/a | AY496735 | Russia | South Urals, Guberli Mts, Adaevo | Kandul et al. 2004 |
| P. damone irinae | BPAL833-11 | CCDB-05724_G01 | MW186997 | Russia | Volgograd Region, Olkhovka | This study |
| P. damone irinae | BPAL834-11 | CCDB-05724_G02 | MW186998 | Russia | Volgograd Region, Olkhovka | This study |
| P. damone irinae | AY496736 | n/a | AY496736 | Russia | Volgograd Region, Olkhovka | Kandul et al. 2004 |
| P. damone pljushtchi | AY496774 | n/a | AY496774 | Russia | Crimea, Ai Petri | Kandul et al. 2004 |
| P. damone ssp. | BPAL524-11 | n/a | MW186991 | Kazakhstan | Karaganda Region, Akchatau | This study |
| P. damone tanais | BPAL825-11 | CCDB-05724_F05 | MW186993 | Ukraine | Amvrosievka | This study |
| P. damone tanais | BPAL826-11 | CCDB-05724_F06 | MW186994 | Ukraine | Amvrosievka | This study |
| P. damone tanais | BPAL827-11 | CCDB-05724_F07 | MW186995 | Ukraine | Amvrosievka | This study |
| P. damone tanais | BPAL828-11 | CCDB-05724_F08 | MW186996 | Ukraine | Amvrosievka | This study |
| P. damone tanais | KC692328 | n/a | KC692328 | Russia | Rostov Region, Belaya Kalitva | Vodolazhsky and Stradomsky 2012 |
| P. icarus | HM913968 | n/a | HM913968 | Italy | 39.9919°N, 15.7931°E | GenBank |
| P. iphigenides | n/a | LOWA422-06 | FJ663238 | Kyrgyzstan | Transalai Mts, Nura | Lukhtanov et al. |
| P. iphigenides | | | | , 0, | | 2009 |
| P. iphigenides iphigenides | iphigenides n/a LOWA423-06 FJ663237 Kyrgyzstan Transa | | Transalai Mts, Nura | Lukhtanov et al. 2009 | | |
| P. iphigenides iphigenides | n/a | LOWA424-06 | FJ663236 | Kyrgyzstan | Transalai Mts, Nura | Lukhtanov et al. 2009 |
| P. iphigenides iphigenides | n/a | LOWA514-06 | FJ663235 | Kyrgyzstan | Alai, Tengizbai Pass | Lukhtanov et al. 2009 |
| P. iphigenides iphigenides | n/a | LOWA515-06 | FJ663234 | Kyrgyzstan | Alai, Tengizbai Pass | Lukhtanov et al. 2009 |
| P. iphigenides iphigenides | BPAL1586-12 | CCDB-03032_F06 | MW194007 | Tajikistan | Iskanderkul | This study |
| P. iphigenides iphigenides | BPAL1587-12 | CCDB-03032_F07 | MW194008 | Tajikistan | Iskanderkul | This study |
| P. iphigenides iphigenides | AY496758 | n/a | AY496758 | Kazakhstan | Shymkent Region, Ugamski Mts | Kandul et al. 2004 |
| P. iphigenides iphigenides | AY557155 | WE98001 | AY557155 | Kyrgyzstan | 25 km S Song Kul Lake | Wiemers 2003 |
| P. iphigenides melanius | BPALB479-18 | CCDB-23848_A04 | MW186954 | Tajikistan | Alai Mts, Jirgatol | This study |
| iphigenides melanius | BPALB480-18 | CCDB-23848_A05 | MW186955 | Tajikistan | Alai Mts, Jirgatol | This study |
| P. iphigenides melanius | BPALB481-18 | CCDB-23848_A06 | MW186956 | Tajikistan | Alai Mts, Jirgatol | This study |
| P. iphigenides melanius | BPALB482-18 | CCDB-23848_A07 | MW186957 | Tajikistan | Alai Mts, Jirgatol | This study |
| P. iphigenides melanius | BPALB483-18 | CCDB-23848_A08 | MW186958 | Tajikistan | Alai Mts, Jirgatol | This study |
| P. iphigenides melanius | BPALB484-18 | CCDB-23848_A09 | MW186959 | Tajikistan | Alai Mts, Jirgatol | This study |
| P. iphigenides melanius | BPALB556-18 | CCDB-23848_G09 | MW186960 | Tajikistan | Peter I Mts, Khorakul Lake | This study |
| P. iphigenides melanius | BPALB558-18 | CCDB-23848_G11 | MW186961 | Tajikistan | Peter I Mts, Mingbulak | This study |
| P. iphigenides melanius | BPALB559-18 | CCDB-23848_G12 | MW186962 | Tajikistan | Peter I Mts, Mingbulak | This study |
| P. iphigenides zarmitanus | BPAL1390-12 | CCDB-03030_E12 | MW186963 | Uzbekistan | Nuratau Mts, Zarmitan | This study |
| P. iphigenides zarmitanus | BPAL1391-12 | CCDB-03030_F01 | MW186964 | Uzbekistan | Nuratau Mts, Zarmitan | This study |
| P. iphigenides zarmitanus | BPAL1392-12 | CCDB-03030_F02 | MW186965 | Uzbekistan | Nuratau Mts, Zarmitan | This study |
| P. iphigenides zarmitanus | BPAL1394-12 | CCDB-03030_F04 | MW186967 | Uzbekistan | Nuratau Mts, Zarmitan | This study |
| P. iphigenides zarmitanus | BPAL1514-12 | CCDB-03031_H05 | MW186968 | Uzbekistan | Hissar Range, Tamshush | This study |
| P. iphigenides zarmitanus | BPAL1515-12 | CCDB-03031_H06 | MW186969 | Uzbekistan | Hissar Range, Tamshush | This study |
| P. iphigenides zarmitanus | BPAL1533-12 | CCDB-03032_B01 | MW186970 | Uzbekistan | Hissar Range, Sangardak | This study |
| P. iphigenides zarmitanus | BPAL1534-12 | CCDB-03032_B02 | MW186971 | Uzbekistan | Hissar Range, Sangardak | This study |
| P. iphigenides zarmitanus | BPAL1535-12 | CCDB-03032_B03 | MW186972 | Uzbekistan | Hissar Range, Sangardak | This study |
| P. iphigenides zarmitanus | BPAL1536-12 | CCDB-03032_B04 | MW186973 | Uzbekistan | Hissar Range, Sangardak | This study |

Table 1. Specimens of the *Polyommatus* (Agrodiaetus) damone complex used in the DNA-barcode analysis.

| Species and subspecies | Sequence code | Field code | GenBank number | Country | Locality | Reference |
|---|---|----------------|-----------------------|--------------------------|--------------------------------------|----------------------------|
| P. iphigenides zarmitanus | BPAL1544-12 | CCDB-03032_B12 | MW186974 | Uzbekistan | Hissar Range, Tamshush | This study |
| P. iphigenides zarmitanus | AY556853 | DS01001 | AY556853 | Uzbekistan | Kitabsky reserve | Wiemers 2003 |
| P. iphigenides zarmitanus (Holotype) | BPAL1393-12 | CCDB-03030_F03 | MW186966 | Uzbekistan | Nuratau Mts, Zarmitan | This study |
| P. juldusus | BPAL852-11 | CCDB-05724_H08 | MW186985 | Kazakhstan | Almaty Region, Kegen Pass | This study |
| P. juldusus | BPAL870-11 | CCDB-05725_B03 | MW186986 | Kyrgyzstan | Issykkyl, Kadzhisai | This study |
| P. juldusus kasachstanus | AY496759 | n/a | AY496759 | Kazakhstan | Dzhungarian Alatau | Kandul et al. 2004 |
| P. juldusus kirgisorum | BPAL1381-12 | CCDB-03030_E03 | MW186987 | Kyrgyzstan | Shamsi | This study |
| P. karatavicus | BPAL040-10 | RPVL-00040 | MW186975 | Kazakhstan | Karatau Mts, Minzhilgi | This study |
| P. karatavicus | BPAL041-10 | RPVL-00041 | MW186976 | Kazakhstan | Karatau Mts, Minzhilgi | This study |
| P. karatavicus | BPAL042-10 | RPVL-00042 | MW186977 | Kazakhstan | Karatau Mts, Minzhilgi | This study |
| P. karatavicus | BPAL1388-12 | CCDB-03030_E10 | MW186978 | Kazakhstan | Karatau Mts | This study |
| P. karatavicus | AY496760 | n/a | AY496760 | Kazakhstan | Karatau Mts | Kandul et al. 2004 |
| P. mediator habievi | JF343830 | ILL087 | JF343830 | Mongolia | Arshantyn-Nuruu Mts | Vodolazhsky et al. 2011 |
| P. mediator habievi | JF343829 | ILL086 | JF343829 | Mongolia | Bayan Ulegei aimak, Elt Gol river | Vodolazhsky et al. 2011 |
| P. mediator mediator | EF104602 | n/a | EF104602 | Mongolia | Altai Mts, Biger | Kandul et al. 2004 |
| P. phyllides askhabadicus | BPAL864-11 | CCDB-05725_A09 | MW186983 | Iran | Kuh e Sorkh Mts, Fariman | This study |
| P. phyllides askhabadicus | BPAL865-11 | CCDB-05725_A10 | MW186984 | Iran | Kuh e Sorkh Mts, Fariman | This study |
| P. phyllides askhabadicus | AY954011 | n/a | AY954011 | Iran | Khorasan, Chakane | Lukhtanov et al. 2005 |
| P. phyllides kentauensis | BPAL1382-12 | CCDB-03030_E04 | MW186980 | Kazakhstan | Karatau Mts | This study |
| P. phyllides kentauensis | AY496769 | n/a | AY496769 | Kazakhstan | Karatau Mts | Kandul et al. 2004 |
| P. phyllides phyllides | FJ663239 | LOWA633-06 | FJ663239 | Tajikistan | Iskanderkul | Lukhtanov et al. 2009 |
| P. phyllides phyllides | BPAL1328-12 | CCDB-03029_H09 | MW186979 | Uzbekistan | Sairob | This study |
| P. phyllides phyllides | BPAL1578-12 | CCDB-03032_E10 | MW186981 | Tajikistan | Iskanderkul | This study |
| P. phyllides phyllides | BPAL2660-14 | CCDB-17967_H11 | MW186982 | Tajikistan | Sarsaryak | This study |
| P. phyllides phyllides | yllides FJ663240 LOWA571-06 FJ663240 Uzbekistan Nuratau Mts, Zarmitan | | Nuratau Mts, Zarmitan | Lukhtanov et al. 2009 | | |
| P. phyllides phyllides | AY496771 | n/a | AY496771 | Kazakhstan | Karzhantau Mts | Kandul et al. 2004 |
| P. phyllides phyllides | AY496770 | n/a | AY496770 | Kazakhstan | Kirgizski Range | Kandul et al. 2004 |

(MGCL), Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA. Photographs of these specimens, as well as collecting data are available in the Barcode of Life Data System (BOLD), projects Butterflies of Palearctic (BPAL) and Butterflies of Palearctic Part B (BPALB) at http://www.boldsystems.org/.

We also used 31 published *COI* sequences (Wiemers 2003; Kandul et al. 2004, 2007; Lukhtanov et al. 2005, 2009; Vodolazhsky et al. 2011; Vodolazhsky and Stradomsky 2012) which were downloaded from GenBank (Table 1).

Sequences were aligned using the BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference as described previously (Vershinina and Lukhtanov 2010; Przybyłowicz et al. 2014; Lukhtanov et al. 2016). Briefly, the Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burn-in = 0.25, nst = 6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. We checked runs for convergence and proper sampling of parameters [effective sample size (ESS) > 200] using the program tracer v1.7.1 (Rambaut et al. 2018). The first 25% of each run was discarded as burn-in. The consensus of the obtained trees was visualized using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Chromosomal analysis

Karyotypes were studied in 16 adult males representing four species (Table 2) and were processed as previously described (Lukhtanov et al. 2014; Vishnevskaya et al. 2016). Briefly, gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C. Different stages of male meiosis, including metaphase I (MI) and metaphase II (MII) were examined using an original two-phase method of chromosome analysis (Lukhtanov et al. 2006, 2008). Abbreviation *ca* (circa) means that the count was made with an approximation due to an insufficient quality of preparation or overlapping of some chromosomes or bivalents.

Leica DM2500 light microscope equipped with HC PL APO 100×/1.44 Oil CORR CS lens and S1/1.4 oil condenser head was used for bright-field microscopy analysis. Leica DM2500 light microscope equipped with HC PL APO 100×/1.40 OIL PH3 lens was used for phase-contrast microscopy analysis.

Results

DNA-barcode analysis

Phylogenetic analysis revealed six clusters of closely related individuals within the P (A.) *damone* species complex (Fig. 1). Of these clusters, four groups were monophyletic and two groups were paraphyletic. The lineages of P. (A.) *damone* (I) and P. (A.) *karatavicus* Lukhtanov, 1990 (V) were highly supported. The lineage of P. (A.) *phyllides* (Staudinger, 1886) (VI) and the lineage [(P. (A.) *mediator* Dantchenko et Churkin, 2003 + P. (A.) *juldusus kasachstanus* Lukhtanov et Dantchenko, 1994)] (II) had medium support. The clusters III [P. (A.) *iphigenides iphigenides* (Staudinger, 1886) + P. (A.) *iphigenides melanius* (Staudinger, 1886)] and VI (P. (A.) *iphigenides zarmitanus* subsp. nov.) appeared on the tree as two distinct, not closely related paraphyletic taxa.

Chromosomal analysis

Chromosomal analysis of three males of *P*. (*A*.) *damone damone*, of a single male of *P*. (*A*.) *damone tanais* Dantchenko et Pljushtch, 1993, of two males of *P*. (*A*.) *iphigenides iphigenides*, of three males of *P*. (*A*.) *iphigenides melanius*, of a single male of *P*. (*A*.) *phyllides phyllides*, of two males of *P*. (*A*.) *phyllides askhabadicus* (Forster, 1960) and four males of *P*. (*A*.) *iphigenides zarmitanus* subsp. nov. revealed the same (or almost the same) haploid chromosome number n = 66 or n = 67 in all studied taxa (Table 2). The karyotype structure was also found to be identical in all studied individuals, with three large bivalents in the center of metaphase plates (Fig. 2). Bivalent 1 was 1.2–1.5 times larger than bivalent 2, and the latter was 1.2–1.5 times larger than bivalent 3.

| Species | ID number | Chromo- some number | Country | Locality | date | Collector | Reference |
|----------------------------|----------------------|------------------------|--------------|--|---------------------|-----------|-----------------------------------|
| P. damone altaicus | 1987-445 | n = ca65 | Russia | Altai Mts, Tshulyshman River, 500 m | 3–10 August 1987 | VL | Lukhtanov 1989 |
| P. damone altaicus | 1997-1 | n = ca65-67 | Kazakhstan | near Zaisan city | 23 June 1997 | VL | Lukhtanov and Dantchenko 2002a |
| P. damone altaicus | 1997-2 | n = 67 | Kazakhstan | Saur Mts, Saikan | 2–3 July 1997 | VL | Lukhtanov and Dantchenko 2002a |
| P. damone damone | 94001 | n = 66-67 | Russia | Saratov Region, near Volsk | July 1994 | AD | Lukhtanov et al. 1997 |
| P. damone damone | 94002 | n = 67 | Russia | Saratov Region, near Volsk | July 1994 | AD | Lukhtanov et al. 1997 |
| P. damone damone | 94003 | n = 66 | Russia | Saratov Region, near Volsk | July 1994 | AD | Lukhtanov et al. 1997 |
| P. damone damone | 94008 | n = 67 | Russia | Saratov Region, near Volsk | July 1994 | AD | Lukhtanov et al. 1997 |
| P. damone damone | 94010 | n = ca66-67 | Russia | Saratov Region, near Radishevo | July 1994 | AD | This study |
| P. damone damone | 95DG5 | n = ca67 | Russia | South Ural, Kuvandyk | 1995 | AD | This study |
| P. damone damone | 95DG6 | n = ca66-67 | Russia | South Ural, Kuvandyk | 1995 | AD | This study |
| P. damone irinae | AD00P077 | n = ca67 | Russia | Volgograd region | July 2000 | AD | Kandul et al. 2007 |
| P. damone pliushtchi | 95051 | n = 65-67 | Russia | Crimea, Ai-Petri, 1200 m | 14 July 1995 | B & K | Kandul 1997 |
| P damone pljushtchi | 95054 | $n = c_{2}66.68$ | Russia | Crimea Ai Petri 1200 m | 14 July 1995 | B & K | Kandul 1997 |
| D. damone pijushtchi | 95055 | n = ca65.67 | Duccio | Crimea, Ai Petri, 1200 m | 14 July 1995 | B & K | Kandul 1997 |
| T. damone pijusnicm | 95055 | n = ca03-0/ | Russia D. | Crimea, Al-Petri, 1200 m | 14 July 1993 | Dak | Kandul 1997 |
| r. aamone pijusnicm | 96009 | $\Pi = Ca03-00$ | Russia | Crimea, Al-Petri, 1200 m | 10 July 1993 | Dak | Kandul 1997 |
| P. damone pijusntchi | 96010 | n = 6/ | Russia | Crimea, Al-Petri, 1200 m | 10 July 1995 | Dak | Kandul 1997 |
| P. damone pijusntchi | 96011 | n = 65 | Russia | Crimea, Al-Petri, 1200 m | 10 July 1995 | Dak | Kandul 1997 |
| P. damone pljushtchi | 96012 | n = 66-6/ | Russia | Crimea, Ai-Petri, 1200 m | 10 July 1995 | B&K | Kandul 1997 |
| P. damone pljushtchi | 96017 | n = ca66-68 | Russia | Crimea, Ai-Petri, 1200 m | 10 July 1995 | B & K | Kandul 1997 |
| P. damone pljushtchi | 95050 | n = 66-67 | Russia | Crimea, Ai-Petri, 1200 m | 14 July 1995 | B & K | Kandul 1997 |
| P. damone tanais | 95005 | n = ca67 | Ukraine | Don River basin, Shirokaya balka | 26 May 1995 | AD | This study |
| P. iphigenides iphigenides | irkeshtam | n = ca66-67 | Kyrgyzstan | Transalai Mts (east), Irkeshtam | 1996 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides iphigenides | 1996-4 | n = ca66-67 | Kyrgyzstan | Naryn Region, Chaek | 4 July 1996 | VL | This study |
| P. iphigenides iphigenides | 1996-3 | n = ca66 | Kyrgyzstan | Moldatoo Mts, Teke- Uyuk | 30 June 1996 | VL | This study |
| P. iphigenides iphigenides | 1995 – Chiitala | n = 67 | Kyrgyzstan | Alai Mts, Chiitala village, 2300 m | 1995 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides iphigenides | 1994-1 | n = ca66-67 | Tajikistan | Iskanderkul | July 1994 | VL | This study |
| P. iphigenides iphigenides | 95205 | n = ca66-67 | Kyrgyzstan | Alai Mts, Chiitala village, 2300 m | 1995 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides iphigenides | NK00P823 AY496758 | n = ca65-67 | Kazakhstan | Ugamski range | June 2000 | VL | Lukhtanov et al. 2005 |
| P inhigenides melanius | 068K18A | n = 66 | Taiikaistan | Alai Mts Jirgatol | July 2018 | AD | This study |
| P iphigenides melanius | 077K18A | n = 67 | Tajikaistan | Alai Mts Jirgatol | July 2018 | AD | This study |
| P iphigenides melanius | T:002 | n = 66 | Tajikaistan | Peter I Mts | July 20218 | VI | This study |
| P. iphigenides zarmitanus | 94L01 | n = ca66-68 | Uzbekistan | Nuratau Mts, Zarmitan, | 11–13 June | VL | This study |
| P. iphigenides zarmitanus | 94L03 | n = ca68 | Uzbekistan | Nuratau Mts, Zarmitan, 1300 m | 11–13 June 1994 | VL | This study |
| P. iphigenides zarmitanus | 94L04 | n = 67 | Uzbekistan | Nuratau Mts, Zarmitan, 1300 m | 11–13 June 1994 | VL | This study |
| P. iphigenides zarmitanus | 94L54 | n = ca66-67 | Uzbekistan | Hissar Range, Sangardak, 1600 n | 2 July 1994 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides zarmitanus | 94L61 | n = 67 | Uzbekistan | Hissar Range, Tamshush, 1800 n | 5–7 July 1994 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides zarmitanus | 94L64 | n = 66 | Uzbekistan | Hissar Range, Tamshush, 1800 n | 5–7 July 1994 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides zarmitanus | 94L74 | n = ca65-67 | Uzbekistan | Samarkand Region, Aman-Kutan | 7 July 1994 | VL | Lukhtanov and Dantchenko 2002a |
| P. iphigenides zarmitanus | 94L75 | n = ca65-67 | Uzbekistan | Samarkand Region, Aman-Kutan | 7 July 1994 | VL | Lukhtanov and Dantchenko 2002a |

Table 2. Chromosome numbers of species of the *Polyommatus (Agrodiaetus) damone* complex collected by A. Dantchenko (AD), V. Lukhtanov (AV), and Yu. Budashkin and N. Kandul (B & K).

| Species | ID number | Chromo- | Country | Locality | date | Collector | Reference |
|---------------------------|-----------|-------------|------------|------------------------|--------------|-----------|------------------|
| | | some number | | | | | |
| P. iphigenides zarmitanus | 1994-2 | n = 67 | Uzbekistan | Nuratau Mts, Zarmitan, | 11–13 June | VL | This study |
| | | | | 1300 m | 1994 | | |
| P. iphigenides zarmitanus | 1994-3 | n = 67 | Uzbekistan | Tamshush | 1994 | VL | Lukhtanov and |
| | | | | | | | Dantchenko 2002a |
| P. iphigenides zarmitanus | 1994-4 | n = 67 | Uzbekistan | Samarkand Region, | 7 July 1994 | VL | Lukhtanov and |
| | | | | Aman-Kutan | | | Dantchenko 2002a |
| P. juldusus kasachstanus | 1997-3 | n = 67 | Kazakhstan | Dzhungarian Alatau, | June 2000 | VL | Lukhtanov and |
| | | | | Kysylagash | | | Dantchenko 2002a |
| P. karatavicus | 2000-K | n = 67 | Kazakhstan | Karatau Mts, Minzhilgi | June 2000 | VL | Lukhtanov et al. |
| | | | | | | | 2005 |
| P. phyllides askhabadicus | F456 | n = ca66-67 | Iran | Kuh-e-Sorkh Mts., | 7 July 2003 | VL&D | This study |
| | | | | Torbat-e-Heydariyeh | | | |
| P. phyllides askhabadicus | VL03F523 | n = 67 | Iran | Khorasan, Chakane | 9 July 2003 | VL&D | This study |
| | AY954011 | | | | | | |
| P. phyllides phyllides | 95204 | n = ca65-67 | Kyrgyzstan | Naryn Region, Moldatoo | 26 July 1995 | VL | This study |
| | | | | Mts, Chon-Konduk | | | |
| P. phyllides phyllides | NK00P672 | n = ca66-67 | Kazakhstan | Kazakhstan, Kirgizski | June 2000 | VL | Lukhtanov and |
| | AY496770 | | | range, | | | Dantchenko 2002a |
| P. phyllides phyllides | NK00P808 | n = ca66-67 | Kazakhstan | Kazakhstan, Karzhantau | June 2000 | VL | Lukhtanov and |
| | AY496771 | | | mts | | | Dantchenko 2002a |

Discussion

Chromosomal stasis

It has been found that all taxa within *P*. (*A*.) *damone* species complex demonstrate chromosomal stasis and share the same or very similar haploid chromosomal number (n = 66 or n = 67). This result is unexpected since the karyotypes are known to be very diverse and species-specific in the subgenus *Agrodiaetus*.

It is believed that an unusual diversity of karyotypes is the most remarkable characteristic of *Agrodiaetus*. Species of this subgenus exhibit one of the highest ranges in chromosome numbers in the animal kingdom (Vershinina and Lukhtanov 2017). In *Agrodiaetus* haploid chromosome numbers (n) range from n = 10 in *P. (A.) caeruleus* (Staudinger, 1871) to n = 134 in *P. (A.) shahrami* (Skala, 2001) (Lukhtanov et al. 2005). The genus *Polyommatus* as a whole shows numbers from n = 10 to n = 226 (Lukhtanov 2015). Additionally, the subgenus *Agrodiaetus* demonstrates a high level of karyotypic differentiation with respect to chromosome size (Lukhtanov and Dantchenko 2002b) and variation in number of chromosomes bearing ribosomal DNA clusters (Vershinina et al. 2015). These differences provide reliable characters for species delimitation, description and identification (de Lesse 1960, 1963; Lukhtanov and Dantchenko 2002a, b).

The $P_{n}(A_{n})$ damone species complex represents an exception. In this group divergence in several phylogenetic lineages was not accompanied by changes in karyotypes, and the chromosome number n = 66-67 is the synapomorphic character for the species of the group.

DNA-barcode clusters

The DNA-barcode clusters revealed in our study correspond well to traditionally recognized species and certain specific geographic areas (Figs 3, 4). Cluster 1 includes



Figure 1. The Bayesian majority rule consensus tree of the analyzed samples of *Polyommatus (Agrodiaetus)* inferred from *COI* sequences. *Polyommatus icarus* is used to root the tree (not shown). Species and subspecies names, GenBank accession numbers, museum ID numbers and localities are shown to the right of the branches. Bayesian posterior probabilities higher than 0.5 are shown next to the recovered branches. 1–6 are clusters (see explanation in the text).



Figure 2. Karyotypes of *Polyommatus (Agrodiaetus) iphigenides melanius* and *P. (A.) phyllides askhabadicus* **a** *P. (A.) iphigenides melanius*, sample 077K18A, MI, n = 67, phase-contrast **b** *P. (A.) iphigenides melanius*, sample 068K18A, MI, n = 66 **c** *P. (A.) phyllides askhabadicus*, sample F523, MI, n = 67. Scale Bar: 10 µm.

specimens from the Crimea in the west to Altai and Saur-Tarbagatai Mts in the east and corresponds to *P.* (*A.*) *damone*. Cluster 2 includes specimens from NE Kyrgyzstan, SE Kazakhstan and SW Mongolia and corresponds to *P.* (*A.*) *juldusus* + *P.* (*A.*) *mediator*. Cluster 3 includes specimens from western and southern Kyrgyzstan, southern



Figure 3. Distribution areas of the *COI* clusters revealed in this study. Cluster 1 corresponds to *P. damone*. Cluster 2 corresponds to *P. juldusus* + *P. mediator*. Cluster 3 corresponds to *P. iphigenides* (including *P. iphigenides melanius*). Cluster 4 corresponds to *P. zarmitanus*. Cluster 5 corresponds to *P. karatavicus*.



Figure 4. Distribution area of *P. phyllides* (cluster 6).

Kazakhstan and Tajikistan and corresponds to *P.* (*A.*) *iphigenides iphigenides* + *P.* (*A.*) *iphigenides melanius*. Cluster 4 includes specimens from West Hissar in Uzbekistan and western Tajikistan and corresponds to *P.* (*A.*) *iphigenides zarmitanus* subsp. nov. which will be described below. Cluster 5 includes specimens from Karatau Mts in Kazakhstan and corresponds to *P.* (*A.*) *karatavicus*. Cluster 6 (Fig. 4) includes specimens from northeastern Iran to southeastern Kazakhstan and corresponds to *P.* (*A.*) *phyllides*.

Cluster 6 (=*P. phyllides*) is sympatric with cluster 2 (=*P. juldusus*) in northern Kyrgyzstan and southeastern Kazakstan, with cluster 3 (=*P. iphigenides iphigenides+P. iphigenides melanius*) in Kyrgyzstan and Tajikistan, with cluster 4 (=*P. iphigenides zarmitanus*) in Uzbekistan and western Tajikistan, with cluster 5 (=*P. karatavicus*) in Karatau Mts in Kazakhstan (Eckweiler and Bozano 2016; our personal observations).

Taxonomic interpretations

Clusters 1 (P. damone), 2 (P. juldusus + P. mediator) and 5 (P. karatavicus)

We follow previous research (Dantchenko 2000; Dantchenko and Churkin 2003, Lukhtanov et al. 2005) in interpreting clusters 1 (*P. damone*), 2 (*P. juldusus* and *P. mediator*) and 5 (*P. karatavicus*) (see Taxonomic conclusions below). *P.* (*A.*) *mediator* was described as a species which is intermediate between *P.* (*A.*) *damone* and *P.* (*A.*) *juldusus*, but more similar to *P.* (*A.*) *juldusus* due to specific white pubescence of the costal area of the forewings (Dantchenko and Churkin 2003). This conclusion is now supported by molecular data: on the phylogenetic tree it appears as a clade, which also includes *P.* (*A.*) *juldusus kasachstanus*, and as a sister clade to *P.* (*A.*) *juldusus juldusus* + *P.* (*A.*) *juldusus kirgisorum*.

Up to our knowledge there are no data on sympatry of *P. (A.) mediator* and *P. (A.) damone* in Mongolia as it was reported or supposed earlier (Bálint and Johnson 1987; Bálint 1989).

Cluster 3 (P. iphigenides iphigenides + P. iphigenides melanius)

Polyommatus (Agrodiaetus) iphigenides is highly polymorphic with regard to the black suffusion on the wing upperside and the marginal and submarginal part of the wing underside in males as well as the white streak on hindwings in both sexes. In extreme cases, the suffusion can be practically absent resembling the upperside in *P. damone* or may extend almost to the discal spot which is observed as a fixed feature in two other taxa, *P. iphigenides melanius* and *P. juldusus kirgisorum*. The white streak is also very variable from clear visibility to complete absence. The taxa *P. (A.) samusi* Korb, 2017 (syn. nov.) and *P. (A.) melanius komarovi* Korb, 2017 (syn. nov.) are mainly described on the base of such extreme forms of the same population. Therefore, we consider these taxa as junior subjective synonyms of *P. (A.) iphigenides iphigenides*.

Cluster 3 also includes the taxon described as Lycaena kindermanni var. Melania Staudinger, 1886. For a long time, due to lack of material it had been considered to be a melanized form of P. (A.) iphigenides iphigenides (e.g. Forster 1960). But in recent years it has been treated as a separate species P. (A.) melanius with a local, nearly dot-like distribution in the border area between southwestern Kyrgyzstan and eastern Tajikistan in the Kyzylsu/Surkhob River basin (Dantchenko 2000; Eckweiler and Bozano 2016). We found that DNA barcodes of P. (A.) iphigenides and P. (A.) melanius are identical or differ by non-fixed 1–2 nucleotide substitutions. The main feature of P. (A.) melanius, a wide dark marginal border on the fore- and hindwings, is quite stable for the diagnosis of the taxon; however, the tendency towards such a wide border is expressed in different populations of P. (A.) iphigenides, too. Therefore, this trait can be hardly considered a species-specific character. Here we argue that P(A) melanius is rather a subspecies P. (A.) *iphigenides* than a species. However, this is not a final conclusion. There is indirect evidence in favour of a possible species status of P. (A.) *melanius*, e.g. the distribution areas of P. (A.) *iphigenides iphigenides* and P. (A.) *iphigenides melanius* almost touch each other, and an intergradation zone would be expected between them. However, such a zone is still unknown, and specimens of P. (A.) *iphigenides iphigenides* and P. (A.) *iphigenides melanius* and P. (A.) *iphigenides melanius* from very close localities are clearly differentiated. We suppose that genome-wide analysis may be useful to verify the taxonomic status of P. (A.) *iphigenides melanius*.

Cluster 4 (P. iphigenides zarmitanus)

Morphologically this group is close to *P. ipigenides iphigenides*, whereas with regard to mitochondrial DNA it is close to sympatric species *P. phyllides* which is morphologically very different. In our opinion, two alternative evolutionary scenarios can explain this pattern.

Scenario 1

The cluster 4 (*P. iphigenides zarmitanus*) and the lineage 6 (*P. phyllides*) are sister species which recently evolved from a common ancestor by means of sympatric speciation.

Scenario 2

Cluster 3 (*P. iphigenides*) and cluster 4 (*P. iphigenides zarmitanus*) are sister taxa evolved in allopatry; therefore, they share an ancestral type of the wing pattern and coloration, although differentiated with respect to DNA barcodes. The similarity between completely sympatric cluster 4 (*P. iphigenides zarmitanus*) and lineage 6 (*P. phyllides*) is a result of ancient mitochondrial introgression.

Analysis of multiple nuclear markers is required in order to distinguish between these two scenarios. Scenario 2 seems to be more probable since mitochondrial introgression is not a rare phenomenon in butterflies (e.g. Gompert 2008; Cong et al. 2017) and is also documented in the subgenus *Polyommatus (Agrodiaetus)* (Lukhtanov et al. 2015). Therefore, below we describe the new lineage discovered in West Hissar region as a subspecies of *P. iphigenides*.

Cluster 6 (P. phyllides)

There is no doubt that the cluster 6 (*P. phyllides*) is a distinct species, since it is a monophyletic lineage (Fig. 1), which is morphologically and ecologically differentiated (Dantchenko 2000, Eckweiler and Bozano 2016) and sympatric with *P. (A.) iphigenides iphigenides*, *P. (A.) iphigenides melanius*, *P. (A.) iphigenides zarmitanus*, *P. (A.) karatavicus* and *P. (A.) juldusus*.

New subspecies description

Polyommatus (Agrodiaetus) iphigenides zarmitanus subsp. nov. http://zoobank.org/092F10F6-B5E7-46C3-AA26-3A4D82F1F6D7

Holotype. (Fig. 5a, b), male, BOLD process ID BPAL1393-12, field # CCDB-03030_F03, GenBank accession number MW186966; Uzbekistan, Samarqand Region, Nuratau Mts, near Zarmitan village, 40.40°N, 66.69°E, 1300 m, 11–13 June 1994, V. Lukhtanov leg., deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

Paratypes. 39 males, 14 females: Uzbekistan, Samarqand Region, Nuratau Mts, near Zarmitan village, 40.40°N, 66.69°E, 1300 m, 11–13 June 1994, V. Lukhtanov leg. 2 males: Uzbekistan, Qashqadaryo Region (old spelling: Kashkadarya Region), Hissar Range (west), near Tamshush village, 38.98°N, 67.35°E, 1800 m, 18–20 June 1994, V. Lukhtanov leg. 20 males: Uzbekistan, Qashqadaryo Region (old spelling: Kashkadarya Region), Hissar Range (west), near Tamshush village, 38.98°N, 67.35°E, 1800 m, 5-7 July 1994, V. Lukhtanov leg. 11 males, 2 females: Uzbekistan, Surxondaryo Region (old spelling: Surkhandarya Region), Hissar Range (west), Sangardak, 38.55°N, 67.50°E, 1600 m, 2 July 1994, V. Lukhtanov leg. 60 males, 21 females: Uzbekistan, Samarqand Region, Zeravshansky Range, Aman-Kutan, 1800 m, 39.27°N, 66.90°E, 7 July 1994, V. Lukhtanov leg. 13 males: Tajikistan, Sughd Region, Zeravshansky Range, Padzhrud village, 39.37°N, 68.03°E, 1300 m, 17 males, 13 males, 10 June 1994. All above paratypes are deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg). 5 males: Uzbekistan, [Jizzakh region], Usmat vic., 1700 m, 30.06.1988, V. Tshikolovets leg., in State Darwin Museum, Moscow. 15 males: [Uzbekistan], Aman-Kutan near Samarqand, 20 June 1938, A. Tsvetaev leg., in State Darwin Museum, Moscow. 26 males, 1 female: [Tajikistan], Hisar-Alai, Zeravshansky Range, Farob, 2000 m, 4 July 1998, G.D. Samodurov leg., in State Darwin Museum, Moscow. 1 female: Ta-



Figure 5. Males of *Polyommatus (Agrodiaetus) iphigenides* **a**, **b** upperside (**a**) and underside (**b**) of the holotype of *P*. (*A*.) *iphigenides zarmitanus* subsp. nov. **c**, **d** upperside (**c**) and underside (**d**) of *P*. (*A*.) *iphigenides iphigenides*, Tajikistan, Transalai Mts, Shibe village **e**, **f** upperside (**e**) and underside (**f**) of the Lectotype of *P*. (*A*.) *iphigenides iphigenides*, "Namangan", in Museum für Naturkunde, Humboldt-Universität zu Berlin **g**, **h** upperside (**g**) and underside (**h**) of the Lectotype of *P*. (*A*.) *iphigenides melanius*, in Museum für Naturkunde, Humboldt-Universität zu Berlin. Scale Bars: 10 mm.

jikistan, West Hissar, Nofin lake, 2400, 17 July 1993, S. Churkin leg., in State Darwin Museum, Moscow. 32 males: [Uzbekistan], Aman-Kutan near Samarqand, 15–25 June 1938, A. Tsvetaev leg., in Zoological Museum Moscow University, Moscow (ZMMU). 7 males: [Uzbekistan], Aman-Kutan near Samarqand, 20–23 June 1938, G.Pashin leg., in ZMMU. 2 males: [Uzbekistan], Aman-Kutan near Samarqand, 27 July and 5 August 1937, A. G. Pashin leg., in ZMMU. 3 females: [Uzbekistan], Aman-Kutan near Samarqand, 15–26 June 1938, A. Tsvetaev leg., in ZMMU. 8 males: Tajikistan, West Hissar, Khazorchashma lake, 2800, 26 July 1993, S. Churkin leg.; 1 female: Tajikistan, West Hissar, Nofin lake, 2400, 17 July 1993, S. Churkin leg., in coll. Churkin (Reutov, Russia). 2 males, 1 female: Uzbekistan, West Hissar, Boysun Mts, Mochay, 1500 m, 26 June 1980, V. Tuzov leg., in coll. Tuzov (Moscow). 10 males, 1 female: [Uzbekistan], Aman-Kutan near Samarqand, 19–23 June 1938, A. Tsvetaev leg., in coll. Sochivko A. (Moscow). 10 males, 1 female: [Tajikistan], Hissar-Alai, Zeravshansky Range, Farob, 2000 m, 4 July 1998, L. Nikolaevsky leg., in coll. V. Kalinin, Moscow.

Description. Males. Forewing length 15-17 mm.

Upperside: Ground color bright glossy milky blue with narrow black marginal line, marginal part of forewings and hindwings dusted with black scales, discal strokes may be present or absent, veins darkened, costal area of the forewings white, hindwings with antemarginal spots, fringe white.

Underside: Forewing ground color light grey, submarginal row blurred, but clear visible; discoidal strokes black, bordered with white; postdiscal rows of black spots bordered with white, basal black spots absent; hindwing ground color light grey, basal area with strong greenish blue suffusion between wing root and basal spots; basal spots small, bordered with white, discal stroke less prominent than on forewings; postdiscal row of black spots bordered with white, submarginal and antemarginal marking strong and clear visible; submarginal row bordered distally with reddish lunules, more pronounced to anal end of row; white streak not contrasting, often hardly noticeable or absent at all, fringes pale grayish.

Genitalia. The male genitalia have a structure typical for other species of the subgenus *Agrodiaetus* (Coutsis 1986, Eckweiler and Bozano 2016).

Females. (Fig. 6a, b) *Forewing* length 15–17 mm.

Upperside: Ground color brown with slightly darker veins, discal strokes present, submarginal and antemarginal marking almost absent on fore wings and strong and clear visible on hindwings, antemarginal black spots on hindwings bordered with orange lunules, fringe whitish.

Underside: ground color and general design as in males but darker, brownish grey, greenish blue basal suffusion near invisible, white streak on hindwings clear visible, enlarged distally, fringe light greyish.

Diagnosis. The new subspecies is distinguished phenotypically from the most similar *P. iphigenides iphigenides* (Figs 5c–f, 6c, d) by the underside of the hind wing, which has a paler and less contrasting coloration. The white streak is also dim and weakly stands out against the background of the wing, is often reduced or absent. The same can be said about the basal greenish-blue suffusion: it is dim and weakly stands out



Figure 6. Females of *Polyommatus (Agrodiaetus) iphigenides* **a**, **b** upperside (**a**) and underside (**b**) of the paratype of *P*. (*A*.) *iphigenides zarmitanus* subsp. nov. **c**, **d** upperside (**c**) and underside (**d**) of *P*. (*A*.) *iphigenides iphigenides*, Tajikistan, Transalai Mts, Shibe village. Scale Bars: 10 mm.

against the background of the wing; its size, on average, is much smaller than that in *P. iphigenides iphigenides*. As a rule, it is limited by black dots of the basal row, while in *P. iphigenides iphigenides* it usually extends further in the distal direction, sometimes to spots of the discal row. This suffusion itself has a more greenish tint than that in *P. iphigenides iphigenides* (in the latter, it is more blue). The new species always has black dots of the basal row (although they are small), while in another species they are reduced.

The main differences between the species are still in the molecular characters. *Polyommatus iphigenides zarmitanus* can be distinguished from *P. iphigenides iphigenides* by using molecular markers from the *COI* gene. These mitochondrial diagnostic characters are in the following positions in the *COI* barcode region: adenine (A) in position 22, cytosine (C) in position 132, guanine (G) in position 180, cytosine (C) in position 286, guanine (G) in position 468, guanine (G) in position 468, and guanine (G) in position 627.

The new subspecies differs from sympatric (syntopic and synchronous) *P. phyllides* by milky blue (not greenish blue) wing upperside and white pubescence of the costal area of the forewings in males and by light grey color of the wing underside (*P. phyllides* has specific warm pinkish grey color of the wing underside). It also differs from *P. phyllides* by diagnostic nucleotide guanine (G) in position 627 of the *COI* barcode region.

Distribution area (Fig. 7). Uzbekistan: West part of the Hissar Range, Zeravshan Mts, Nuratau Mts, Boysun (= Baisuntau) Mts. Tajikistan: west part of the Zeravshan valley and Zeravshansky Range, West Hisar Range.



Figure 7. Distribution area of *P. (A.) iphigenides zarmitanus.* 1 is the type-locality, Zarmitan in Nuratau Mts. 2 is Padzhrud village in Zeravshansky Range. 3 is Aman-Kutan near Samarqand. 4 is Kitabsky Reserve in Hissar Range. 5 is Tamshush in Hissar Range. 6 is Sangardak in Hissar Range. 7 are Khazorchashma and Nofin lakes in Hissar Range. 8 is Mochay in Boysun Mts.

Habitat and biology. Stony steppe and dry meadows from 1200 up to 2800 m alt. Flight period from late May to first decade of August in a single generation. The new subspecies flies syntopically and synchronously with the second generation of *P*. (*A*.) *phyllides*, but on average about one decade earlier. Host plant is preliminary determined as *Hedysarum* sp. (Fabaceae).

Etymology. The name *zarmitanus* is an adjective of the masculine gender. This name originates from Zarmitan, the village in Uzbekistan.

Taxonomic conclusion

The discovered topology (Fig. 1) can be considered as a signal to taxonomic rearrangement within the group. However, since the volume of the studied material of these taxa is small, we prefer to leave the existing taxonomic hypotheses. Additionally, we assume that the hypothesis of the existence of a species called *P*. (*A*.). *altaiensis* with subspecies *P.* (*A.*) *altaiensis altaiensis*, *P.* (*A.*) *altaiensis bogdoolensis* Dantchenko et Lukhtanov, 1997, *P.* (*A.*). *altaiensis mediator* and *P.* (*A.*) *altaiensis habievi* Yakovlev, 2004 (Eckweiler and Bozano 2016) is speculative and not supported by significant morphological characters.

Based on the stated above, we propose the following taxonomic arrangement of the *P. damone* species complex:

P. (A.) damone (Eversmann, 1841)

- P. (A.) damone pljushtchi (Lukhtanov et Budashkin, 1993)
- P. (A.) damone tanais Dantchenko et Pljushtch, 1993
- P. (A.) damone irinae Dantchenko, 1997
- P. (A.) damone damone (Eversmann, 1841)
- P. (A.) damone altaicus (Elwes, 1899) (= Lycaena damone var. sibirica Staudinger, 1899; = Agrodiaetus carmon altaiensis Forster, 1956)
- P. (A.) damone walteri Dantchenko et Lukhtanov, 1993
- P. (A.) damone bogdoolensis Dantchenko et Lukhtanov, 1997
- P. (A.) damone fabiani Bálint, 1997
- *P.* (*A.*) *mediator* Dantchenko et Churkin, 2003 (= *Agrodiaetus mediator habievi* Yakovlev, 2004)
- P. (A.) juldusus (Staudinger, 1886) (= Lycaena damone var. duplicata A. Bang-Haas, 1910)
- P. (A.) juldusus juldusus (Staudinger, 1886)
- P. (A.) juldusus kirgisorum Lukhtanov et Dantchenko, 1994 (=P. hyrsyz Koçak et Kemal, 2001; = P. kirgisorum khamul Korb, 2009; = P. kirgisorum gorthaur Korb, 2009)
- P. (A.) juldusus kasachstanus Lukhtanov et Dantchenko, 1994
- P. (A.) juldusus rueckbeili Forster, 1960
- P. (A.) juldusus tianchinensis Eckweiler, 2013
- P. (A.) iphigenides (Staudinger, 1886)
- *P.* (*A.*) *iphigenides iphigenides* (Staudinger, 1886) (= *P. ishkashimicus alajanus* Korb, 1997; = *P. samusi* Korb, 2017, syn. nov.; = *P. melanius komarovi* Korb, 2017, syn. nov.)
- P. (A.) iphigenides melanius (Staudinger, 1886)
- P. (A.) iphigenides zarmitanus subsp. nov.
- P. (A.) karatavicus Lukhtanov, 1990

P. (A.) phyllides (Staudinger, 1886)

- P. (A.) phyllides phyllides (Staudinger, 1886)
- P. (A.) phyllides askhabadicus (Forster, 1960)
- P. (A.) phyllides kentauensis Lukhtanov, 1990
- P. (A.) phyllides urumbash Churkin et Zhdanko, 2008

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RESEARCH ARTICLE



A re-examination of the West European species of Boreonectes Angus, 2010, with particular reference to B. multilineatus (Falkenström, 1922) (Coleoptera, Dytiscidae)

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Abstract

The West European species of *Boreonectes* Angus, 2010 are reviewed. *B. multilineatus* (Falkenström, 1922) is shown to be widely distributed in the Pyrenees, where it is the only species known to occur. The chromosomes of all five west European species are found to have, in addition their different numbers of chromosomes, differences in the number and locations of secondary constrictions, and in some cases, the number of chromosomes with clear centromeric C-bands. The level of differences between the chromosomes of the species is in stark contrast with the very slight genetic (DNA) differences between them and this suggests that chromosome differentiation may have been a driver of speciation. Two of the species, *B. griseostriatus* (De Geer, 1774) and *B. multilineatus*, have distributions extending northwards as far as Arctic Scandinavia. It is pointed out that, while these northern areas now constitute the major portions of their ranges, they must be of fairly recent origins as most of the area would have been covered by ice sheets and therefore not habitable during the glacial maximum of the Last Glaciation. This contrasts with the situation in the area of the Central European mountains where fossil faunas, including *Boreonectes*, are known. *B. griseostriatus*, identifiable to species by its parameres, was present in the Woolly Rhinoceros site at Starunia in the Western Ukraine, and this fauna is discussed as well as an English fauna of similar age.

Keywords

Boreonectes, B. multilineatus, chromosomes, Coleoptera, Distributions, Dytiscidae, Pyrenees, Pleistocene fossils

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For Ignacio Ribera, 1963–2020. Taken from us by the Corona virus when he still had so much research to enjoy and to share with the rest of us.

Introduction

Boreonectes Angus, 2010 is a genus of small diving beetles (Dytiscidae) typically found in mountain lakes and in barren pools at lower altitudes in the north. The Palaearctic species were for many years regarded as all belonging to a single species, *B. griseostriatus* (De Geer, 1774) (see Zaitzev 1953 for discussion), though their generic placement was not stable until Angus (2010b) erected the genus *Boreonectes*. Fery and Ribera (2018), in their analysis of the genera of subtribe Deronectina, further stabilised *Boreonectes* by restricting it to the *B. griseostriatus* group plus one other species. However, chromosomal studies started by R. B. Angus in the 1980s began to show a number of different karyotypes. The results of these studies were published by L. A. Dutton and R. B. Angus (2007) and revealed the existence of seven distinct species, five of them new, in Europe. Details of the Palaearctic species are given in Table 1.

B. multilineatus (Falkenström, 1922) is among these chromosomally validated species, and was taken to refer to paler, more distinctly striped material from inland montane areas in Fennoscandia, with *B. griseostriatus* occurring in coastal rocky localities (Nilsson and Holmen 1995). Nilsson and Holmen added that while *B. griseostriatus* was not known outside coastal Fennoscandia, *B. multilineatus* was in all probability the species recorded across northern Eurasia as far east as Kamchatka. However, serious doubt was cast on this view by the discovery by Angus (2008) of pale, strongly lined *B. griseostriatus*, closely resembling *B. multilineatus*, near Sevettijärvi in Finnish Lapland. Not only that, but *B. griseostriatus* is now known to be widely distributed in the northern part of the Alps, from the Col du Petit St Bernard in the west to Bavaria in the east (Angus 2010a, b) and Franck Bameul's discovery of *B. multilineatus* in the Pyrenees (Angus 2010a) suggested that this species might have a more western distribution, a view strengthened by his subsequent discovery of this species widely distributed in the Pyrenees, in the apparent absence there of any other *Boreonectes* species.

In view of these discoveries the known distribution of *B. multilineatus* is reviewed and a detailed chromosomal comparison of all five West European *Boreonectes* is undertaken.

Material and methods

The *B. multilineatus* used in this study is listed in Table 2. The sources of material of the other species are given in the primary references indicated in the captions to the illustrations of their karyotypes.

The methods used for preparing chromosomes and the handling of the data are those used by Dutton and Angus (2007) and subsequent papers on *Boreonectes*. The methods were described in detail by Angus et al. (2020).

| Nº | Species | Distribution |
|-----|--|---|
| 1 | Boreonectes griseostriatus (De Geer, 1774) | Fennoscandia, Alps (northern) |
| | = B. maritimus (Helliesen, 1890) | |
| | = B. g. var. nigrescens Favre, 1890 | |
| 1a | B. griseostriatus griseostriatus (De Geer, 1774) | |
| 1b | B. griseostriatus strandi (Brink, 1943) | Arctic coast of Fennoscandia and Kola |
| 2 | Boreonectes multilineatus (Falkenström, 1922) | Pyrenees, Inland Scandinavia, British Isles, Faroes |
| 3 | Boreonectes emmerichi (Falkenström, 1936) | Tibetan Plateau |
| 4 | Boreonectes macedonicus (Guéorguiev, 1959) | North Macedonia, Crete |
| | = B. creticus (Dutton et Angus, 2007) | |
| 5 | Boreonectes alpestris (Dutton et Angus, 2007) | Alps (southern) |
| 6 | Boreonectes ibericus (Dutton et Angus, 2007) | Mountains of Iberia, Maritime Alps, Corsica, Middle Atlas Mts |
| 7 | Boreonectes inexpectatus (Dutton et Angus, 2007) | France, Hautes Alpes, Lac du Lauzet inférieur |
| 8 | Boreonectes riberae (Dutton et Angus, 2007) | Bulgaria, Turkey (Anatolia) |
| 9* | Boreonectes piochardi (Régimbart, 1878) | Israel/Lebanon, Mt Hermon |
| 10* | Boreonectes palaestinus (Baudi di Selve, 1894) | Palestine, Svria |

Table 1. The Palaearctic species of Boreonectes Angus, 2010.

*Footnote. These two names almost certainly refer to the same species and the type of *B. piochardi* is a *Boreonectes*. From their distributions it seems possible that they are the same species as *B. riberae*, over which they have priority. Chromosome preparations from living material would be needed to resolve this.

| Country | Locality, date & collector | Coordinates | Number examined + reference |
|---------------------|---|--------------------|---|
| SWEDEN | Västerbotten, Åmsele, viii.1990, A.N. Nilsson | 64.528°N, 19.350°E | 3 ♂♂. Dutton and Angus 2007 |
| SCOTLAND | Kirkcudbright, Clatteringshaws Loch, viii.1990, G. N. Foster | 55.067°N, 4.282°W | 4♂♂, 1♀. Dutton and Angus 2007 |
| FRANCE | Lac d'Oncet, 11.ix.2010, F. Bameul. | 42.927°N, 0.1335°E | 2♂♂, 3♀♀. Angus 2010b |
| | Lac d'Anapéou, 30.vii.2011, F. Bameul. | 42.926°N, 0.1284°W | 5♂♂. Angus et al. 2015 |
| | Etangs de Fontargente, 5.ix.2015, F. Bameul. | 42.630°N, 1.710°E | 1♂, 1♀. This paper |
| SPAIN | Ibón de las Isérias, 18.vii.2015, F. Bameul. | 42.745°N, 0.479°W | 3 \bigcirc \bigcirc , 1 \bigcirc . This paper |
| | Ibón de Anayet Este, 12.viii.2017, F. Bameul. | 42.780°N, 0.4405°W | 2♂♂. This paper |
| | Bielsa, "Ibón de Urdicito 1", 29.viii.2015, F. Bameul. | 42.669°N, 0.278°E | 1 \Diamond , 2 \bigcirc \bigcirc . This paper |
| | Chistén, "Ibón de Urdicito 2" Ibones de la Solana de Urdicito, 29.viii.2015, F. Bameul. | 42.666°N, 0.286°E | |
| Northern IRELAND | Antrim, Garron Plateau above Glen Arrif, R. Anderson, 3.vi.2008 | 55.003°N, 6.062°W | 4♀♀. Angus, 2008 |
| IRELAND | Cork, NW Bonane, small lake 451 m a.s.l. on Glenlough Mountain K. Scheers, J. Bergsten & A. N. Nilsson, 10.vi.2018 | 51.746°N, 9.644°W | 1 Å, 299. This paper. |

Table 2. Localities of the *B. multilineatus* material used for chromosome analysis.

Results

B. multilineatus

The Pyrenean distribution of *B. multilineatus* is shown in Fig. 1. The species is widely distributed along the length of the Pyrenees, with localities in both France and Spain (see Table 2). All the specimens were collected by Franck Bameul. Fig. 2 shows the two Irish localities from which material yielding karyotypes has been obtained. Swedish and Scottish localities are listed in Table 2. British and Irish localities for *B. multilineatus* are given by Foster et al. (2016). The only other records for this species are from the Faroe Islands, the material in this case being identified by DNA analysis (Angus et al. 2017). Irish *B. multilineatus* are shown in Fig. 3a, b



Figure 1. Map showing the Pyrenean localities for *B. multilineatus*. For details of the localities see Table 1. All specimens collected by Franck Bameul.



Figure 2. Map showing the Irish localities from which *B. multilineatus* chromosomes have been obtained.



Figure 3. *B. multilineatus* and *griseostriatus*, habitus **a, b** *multilineatus*, Ireland, county Cork **a** \bigcirc **b** \bigcirc **c** *B. griseostriatus*, Switzerland, Le Louché **d** *B. griseostriatus* var *nigrescens* Favre, Switzerland, Le Louché. Scale bar: 1 mm for (**a, b**); **c, d** are field photographs by Dr E. M. Angus.

along with Swiss *B. griseostriatus* and *griseostriatus* var. *nigrescens* Favre. The Irish (Cork) *multilineatus* are much darker than the illustration given by Nilsson and Holmen (1995) as fig. 347, and are even darker than the *griseostriatus* shown as fig. 346. On the other hand, the Swiss *griseostriatus* shown in Fig. 3c is clearly paler, with well-separated dark lines. This specimen was taken along with the *griseostriatus* var. *nigrescens* shown in Fig. 3d.

Mitotic chromosomes, arranged as karvotypes, are shown in Fig. 4a-n, and first metaphase of meiosis is shown in Fig. 4p-r. These cover all the localities from which B. multilineatus chromosomes have been obtained. The karyotype is made up of 28 pairs of autosomes and sex chromosomes which are X0 ($\stackrel{\frown}{\bigcirc}$) and XX ($\stackrel{\bigcirc}{\bigcirc}$). The metric data are as follows: The longest autosomes measured 4.63 µ in the least condensed nucleus measured and 3.3μ in the most condensed. The corresponding values for autosome 27 are 2.13 μ and 1.3 μ , and for autosome 28 (often dot-like) are 1.3 μ and 0.53μ . The X chromosome, the longest in the nucleus, gave values of 7.2 and 5.5 μ . The Relative Chromosome Lengths (RCL, the length of each chromosome expressed as a percentage of the total haploid autosome length in the nucleus) range from 5.5 to about 2.5 in autosome 27 and 1.5 in autosome 28. The RCL of the X chromosome is about 6. Many of the autosomes have very similar RCLs (range about 3-3.8) so that adjacent pairs are often inseparable unless the centromere positions are clearly different (see Table 2). Note that measurement should be taken from simply Giemsa-stained chromosomes as C-banding often alters their apparent length. Five pairs of autosomes appear to have secondary constrictions. Detailed chromosomal comparisons are given in the next section.





Figure 4. *B. multilineatus* – mitotic chromosomes from midgut and testis (**a–o**), metaphase I of meiosis, from testis (**p–r**) **a** \mathcal{J} , Sweden, Åmsele, plain (Giemsa stained), shown as fig. 20 by Dutton and Angus (2007) **b, c** \mathcal{J} , Scotland, Clatteringshaws Loch **b** plain **c** C-banded **d–g** France, Hautes-Pyrénées, Lac d'Oncet **d, e** \mathcal{J} **f, g** \mathcal{Q} **d, f** plain **e, g** C-banded, shown as fig. 3g–j by Angus (2010b) **h, i** \mathcal{J} , France, Hautes-Pyrénées, Lac d'Anapéou **h** plain **i** C-banded, shown as fig. 5k, l by Angus et al. (2015) **j** \mathcal{J} , France, Ariège, Etangs de Fontargente, plain **k** \mathcal{J} , Spain, Ibon de Anayet Este **I, m** \mathcal{J} , Spain, Ibon de Urdecito **I** plain **m** C-banded; One replicate of C-banded autosome 15 has been lost from **m**; **n** \mathcal{Q} , Ireland, Antrim, Garron Plateau above Glen Arrif **o** \mathcal{Q} , Ireland, Cork, NW Bonane **p, q** metaphase I, Spain, Provincia de Huesca, Ibones de Urdicito **o** plain **p** C-banded **r** Ireland, Cork, NW Bonane, Metaphase I, plain. Scale bar: 5 µm.

| Chromosome | B. griseostriatus | B. multilineatus | B. alpestris | B. inexpectatus | B. ibericus |
|------------|--------------------------|------------------|--------------|-----------------|--------------------------|
| 1 | sm | m | m - sm | -C sm | - C m (+ 24), sm (alone) |
| 2 | 2c | 2c | 2c | m | m |
| 3 | sm - m | m - sm | m | sa | sm |
| 4 | m | m | m | sa | -C 2c |
| 5 | 2c | 2c | 2c | m | -C sm |
| 6 | sm | 2c | sm | sm | m |
| 7 | sm - m | m | m | m | 2c |
| 8 | 2c | 2c | sm | 2c | -C m |
| 9 | m | m | m - sm | Sa | m (C very weak) |
| 10 | m | m | m | 2c | -C m |
| 11 | m | 2c | sm - sa | m | -C m |
| 12 | 2c | sm - sa | sm - sa | sa | Sm |
| 13 | sa | m | m | sm | 2c |
| 14 | m | m | m | sm | -C m |
| 15 | sm - sa | m | m | m | 2c (C very weak) |
| 16 | m | 2c | m | m | -C m |
| 17 | m | sa | sm | sm | -C m |
| 18 | m | sa | sm | m | -C sm |
| 19 | m | m - sm | m - sm | m | -C sm/sa |
| 20 | m - sm | m | m | m | -C sm |
| 21 | m - sm | m | m | sa | m (C sometimes weak) |
| 22 | m - sm | m | m | -C m | -C m |
| 23 | sm - sa | m | m | m | -C sm |
| 24 | sa | m | m - sm | sm | -C sa/sm |
| 25 | m | m | m | m | Sm |
| 26 | m | m | m - sm | m | -C sm |
| 27 | m | m | m | sm | - |
| 28 | sm (northern) m (Alpine) | m - sm (dot) | _ | 2c | - |
| 29 | sm (northern) m (Alpine) | _ | - | m | - |
| 30 | sm (dot) | _ | - | - | - |
| Х | m | m | m | m | sm |

Table 3. Chromosome data.

Chromosome comparisons

Karyotypes for comparison of the West European species are shown in Figs 5–7. These are at a higher magnification than Fig. 4, so that it should not be necessary to zoom in on these if viewed as pdfs. Chromosome data are given in Table 3.

Centromere positions from Centromere Indices (CI), the length of the shorter chromosome as a percentage of the total length of the chromosome, based on Sumner (2003) and secondary constrictions (**2c**) of the *Boreonectes* species: m, metacentric, CI 46–50; sm, submetacentric, CI 26–45; sa, subacrocentric, CI 16–25. -C, no centromeric C-band. Note that secondary constrictions are identified by their appearing open in some preparations. No attempt has been made to stain for nucleolus organisers (NORs).

B. griseostriatus and B. multilineatus (Fig. 5).

B. griseostriatus has the highest number of chromosomes of any of the species considered here, with 30 pairs of autosomes and X0/XX sex chromosomes. The metric data are: Measured lengths of autosome 1 2.53–1.99 μ , of autosome 29 1.73–0.87 μ ,

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Figure 5. *Boreonectes griseostriatus* (**a–m**) and *B. multilineatus* (**n–r**) at higher magnification, for detailed comparison. **a–e** northern localities **a** \Diamond , Sweden, Öregrund (shown as fig. 2a by Dutton and Angus 2007, and as fig. 2a by Angus 2008) **b, c** Finland, Sevettijärvi, \heartsuit **b** plain (Giemsa stained) **c** the same nucleus C-banded (shown as fig. 2b, c by Angus 2008) **d, e** *B. g. strandi*, \Diamond , Norway, Bugøynes, **d** plain **e** the same nucleus C-banded (shown as fig. 2d, e by Angus 2008) **f–m** Alpine localities **f, g** \Diamond , Switzerland, Valais, pool by Le Louché **f** plain **g** the same nucleus, C-banded (shown as fig. 3d, e by Angus 2010) **h, i** \Diamond , Germany, Bavaria, Seeonalm **h** plain **i** the same nucleus C-banded (shown as fig. 3f, g by Angus 2010a) **j, k** \Diamond , France, Savoie, pool south of the Lac de Mont Cenis **j** plain **k** the same nucleus C-banded (shown as fig. 3c, d by Angus 2010a) **n–r** *B. multilineatus*, details of the material given in Table 1 **n** \Diamond , Scotland, C-banded **o, p** \Diamond , France, Lac d'Oncet **o** plain **p** C-banded **q, r** \heartsuit , France, Lac d'Oncet **q** plain **r** the same nucleus, C-banded. Missing chromosomes indicated by solid circles. Scale bar: 5 µm.

of autosome 30 (often dot-like) $1.28-0.71 \mu$. The values for the X chromosome are $4.42-2.3 \mu$. The RCLs of the autosomes range from about 4 (autosome 1) to about 2 (autosome 29) and about 1.6 (autosome 30). The RCL of the X chromosome is about 6.5. Other features of the chromosomes are given in Table 2. Four pairs of autosomes (2, 5, 8 and 12) have secondary constrictions (**2c**), but recognition of these requires preparations in which they are open, often more apparent on C-banded preparations as on autosome 2 in Fig. 5k, autosomes 5 and 8 in Fig. 5g. A secondary constriction on autosome 12 is suggested by Fig. 5b, c, d, e, these being Giemsa-stained and C-banded preparations from two specimens. There is a minor difference between northern (Fig. 5a-e) and Alpine populations (Fig. 5f-m) populations in that autosomes 28 and 29 are submetacentric in northern populations but metacentric in southern ones. All the chromosomes have distinct centromeric C-bands.

B. multilineatus has six clear secondary constrictions on autosomes 2, 5, 6, 8, 11 and 16, shown on the C-banded preparation in Fig. 5n and supported by the Giemsa-stained preparations shown in Fig. 4a, b. This is the highest number in this group. The constrictions on autosomes 2, 5, and 8 appear to match those of *B. griseostriatus*, but the one on autosome 11 is unmatched in the *B. griseostriatus* karyotype, though one is present on pair 12. The secondary constriction on autosome 16 of *B. multilineatus* appears completely unmatched in *B. griseostriatus*. The metric data are given in the preceding section. All the chromosomes have distinct centromeric C-bands.

B. alpestris and B. multilineatus (Fig. 6)

B. alpestris has a karyotype at first sight closely resembling that of *B. multilineatus* but has only 27 pairs of autosomes plus the usual X0 or XX sex chromosomes. The metric data are: measured lengths of autosome 1 $3.75-2.68 \mu$, of autosome 26 $2.25-2.02 \mu$, of autosome 27 $1.97-1.66 \mu$ and of the X chromosome $6.25-3.3 \mu$. The RCLs range from about 4 (autosome 1) to about 2.15 (autosome 26) and about 1.8 (autosome 27), while that of the X chromosome ranges from about 8.35-5.79. Other features are given in Table 2. All

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Figure 6. *B. griseostriatus* (**a**), *B. inexpectatus* (**b**, **c**), *B. multilineatus* (**d**) and *B. alpestris* (**e**–**j**) at higher magnification, for detailed comparison **a** *B. griseostriatus*, \Diamond , C-banded, Petit St Bernard (as fig. 2m) **c**, **d** *B. inexpectatus*, paratypes **b** plain (Giemsa stained) **c** C-banded **c** is the nucleus shown as fig. 2i by Dutton and Angus (2007), here somewhat rearranged **d** *B. multilineatus*, \Diamond , Scotland **e–j** *B. alpestris* **e** paratype \Diamond , Italy, Dolomites, Falcade, shown as fig. 2f by Dutton and Angus (2007) **f**, **g** \Diamond , Italy, Gran Paradiso, Colle del Nivolet **e** plain **f** C-banded, shown as fig. 1b, c by Angus et al. (2017) **h**, **i** \heartsuit , Switzerland, Ticino, San Bernardino pass **h** plain **i** C-banded, shown as fig. 1d, e by Angus et al. (2017) **j** paratype \heartsuit , Switzerland, Ticino, Medeglia, plain, shown as fig. 2g by Dutton and Angus (2007). Scale bar: 5 µm.

the chromosomes have clear centromeric C-bands, and only 2 secondary constrictions, on autosomes 2 and 5, are apparent. The decrease in RCL between the 2 smallest autosomes (pairs 26 and 27) is clearly less than between those of *B. multilineatus* (pairs 27 and 28).

B. inexpectatus and B. griseostriatus (Fig. 6)

B. inexpectatus has a karyotype comprising 29 pairs of autosomes and X0, XX sex chromosomes, and thus, in terms of numbers of chromosomes, is the species coming closest to *B. griseostriatus*. The metric data are: measured lengths of autosome 1 2.8-2.4 μ, of autosome 28 1.16-1.06 μ, of autosome 29 0.75-0.63 μ and of the X chromosome 4.16-3.12 µ. The RCLs range from about 5.9 (autosome 1) to about 2.25 (autosome 28) and about 1.4 (autosome 29), while that of the X chromosome is about 7. Other features are given in Table 2. Autosomes 1 and 22 lack centromeric C-bands and autosomes 8, 10 and 28 have secondary constrictions. Autosome pairs 3, 4, 9, 12, 21 and 24 have particularly heavy subacrocentric C-bands, and autosome pair 7 has equally heavy submetacentric ones. The *B. inexpectatus* karyotype appears unlike that of any other species. To date this species is known from only one locality, the Lac de Lauzet inférieur, the smaller and higher of the two Lauzet lakes. Dutton and Angus (2007) mention chromosome preparations from three males, done on November 13th 1998, and a further male and two females done on November 30th that year. The 13th November preparations gave karyotypes agreeing with each other, but the preparations done on the 30th failed – nothing was photographed. No DNA data are available.

B. ibericus (Fig. 7)

B. ibericus has the most distinctive karyotype of any of the West European species. It has 24–26 pairs of autosomes, with a fusion-fission polymorphism involving pairs 1 and 24. The sex chromosomes are X0 (\mathcal{O}), XX (\mathcal{Q}), with the X chromosome clearly submetacentric, as against metacentric in all the other species. 15 pairs of autosomes lack centromeric C-bands, 1 pair (No. 8) may be with or without C-bands, and 3 pairs have them very weak. The metric data are: measured lengths of autosome 1 5.62–2.8 μ (fused with autosome 24), 3.93–2.5 μ (unfused), of autosome 24 1.25–1 μ , of autosome 26 1.45–0.62 μ and of the X chromosome 6.66–3.12 μ . Secondary constrictions are present on autosome pairs 4, 7, 13 and 15.

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Figure 7. *B. ibericus* at higher magnification, for detailed comparison (**a–k**) **a–c** paratypes, Spain, Peña Lara, plain (Giemsa stained) **a** $\overset{\circ}{\supset}$, autosomes 1 and 24 homozygous fused **b** $\overset{\circ}{\supset}$, autosomes 1 and 24 heterozygous for fusion **c** $\overset{\circ}{\ominus}$, autosomes 1 and 24 homozygous unfused; shown as fig. 2j–l by Dutton and Angus (2007) **d, e** paratypes, Italy, Alpi Maritimi: plain **d** $\overset{\circ}{\supset}$, autosomes 1 and 24 homozygous unfused; shown as fig. 2j–l by Dutton and Angus (2007) **d, e** paratypes, Italy, Alpi Maritimi: plain **d** $\overset{\circ}{\supset}$, autosomes 1 and 24 heterozygous for fusion, 1 replicate of autosome 26 lost (from Dutton's unpublished MSc thesis) **f, g** $\overset{\circ}{\supset}$, Corsica, autosomes 1 and 24 heterozygous for fusion **f** plain **g** C-banded. Shown as fig. 3j, k by Angus (2010 a) **h–j** Morocco, Moyen Atlas **h** $\overset{\circ}{\ominus}$, plain, complete, autosomes 1 and 24 heterozygous for fusion **i, j** $\overset{\circ}{\supset}$, lacking 9 autosomes (positions indicated by solid circles), autosomes 1 and 24 heterozygous for fusion (unfused autosome 1 missing) **i** plain **j** C-banded. Shown as fig. 30–q by Angus (2010b) **k** $\overset{\circ}{\ominus}$ paratype, France, upper Maritime Alps (Hautes Alpes), Lac du Lauzet Superieur, C-banded, autosomes 1 and 24 heterozygous for fusion. Shown as fig. 2m by Dutton and Angus (2007). Scale bar: 5 µm.

Discussion

The West European species of *Boreonectes* show a striking diversity of karvotypes, in sharp contrast to their genetic (DNA) differences which are very slight (Angus et al. 2017). The two studied southeast European species (B. macedonicus (Georgiev, 1959) and B. riberae (Dutton et Angus, 2007)) are slightly more distant genetically and the Tibetan B. emmerichi (Falkenström, 1936) much more so, in fact closer to American "griseostriatus", although its karyotype is very similar to that of B. macedonicus (Angus et al. 2015). This suggests that in western Europe chromosome diversification has been a driver of speciation. Five species are involved, of which two, B. griseostriatus and B. multilineatus, have distributions extending into northern Europe. The extent of the northern distribution of *B. griseostriatus* requires clarification, as does the possibility of its existence in the Nearctic. It is very easy to regard *B. griseostriatus* as a primarily northern species with "glacial relict" populations in the Alps. However, as Ignacio Ribera has stressed in conversations, much if not all of these northern parts of its range would have been covered by ice sheets over much of the Last Glaciation, and hence uninhabitable, so the southern populations are likely to be the older ones. In the case of *B. multilineatus*, the eastern extent of its northern range requires clarification and it seems possible that Pyrenees may be the oldest part of its range. Nilsson and Holmen (1995) record B. griseostriatus only from coastal parts of Finnmark (Norway), with the inland parts occupied by B. multilineatus. This inland Finnmark material should be checked in view of the stripy B. griseostriatus taken at Sevettijärvi near Inari in Finnish Lapland. Fortunately, the two species are separable by details of the male genitalia aedeagus and parameres.

There is some fossil evidence for these beetles in central Europe during the Last Glaciation. *Boreonectes* is among the beetles at the famous Woolly Rhinoceros site at Starunia near Lvov in the western Ukraine (Angus 2010a). The material includes a male from which the aedeagus has been dissected. The median lobe and one paramere are present and the paramere is shown, along with those of modern *B. griseostriatus* and *B. multilineatus*, in Fig. 8. The apical parts of these parameres are very prone to shrivelling when dried, and the Irish *B. multilineatus* (Fig. 8a) was transferred to DMHF immediately after dissection and hence shows the true shape of the apex. The



Figure 8. *Boreonectes* parameres. **a** *B. multilineatus*, Ireland, county Cork **b–d** *B. griseostriatus* **b** Starunia fossil **c** Norway, Tjøme **d** Sweden, Öregrund. The apodeme on the inner face is present in **b** and **c** but lost from **a** and **d**; **d** also lacks the basal lobe. Scale bar: 0.5 mm.

two *B. griseostriatus* (Fig. 8c, d) were also treated this way and so show their true shape. The fossil (Fig. 8b) was laterally compressed but has preserved its shape (as often happens with Pleistocene fossils), and very clearly matches *B. griseostriatus* rather than *B. multilineatus*. The elytra are pale with well-separated dark stripes. The bleaching of the darker more sclerotised main part of the parameres is an artefact of its having been preserved in oil (and salt) and is typical of many of the Starunia fossils. The Helophorus Fabricius, 1775 of the Starunia site were investigated by Angus (1973), who listed 9 species, with a mixture of East Siberian and European species. Angus (1982), on the basis of their karyotypes, separated H. aquaticus (Linnaeus, 1758) and H. aequalis Thomson, 1868, both European species, and showed that both were present among the Starunia fossils. Angus (1998) showed that the two Starunia fossils regarded in the 1973 account as being H. glacialis (Villa et Villa, 1833), were in fact H. griseus (Herbst, 1793) and showed how the aedeagi of that European species could be distinguished from Tibetan H. montanus d'Orchymont, 1926. This brings the total number of species to 10. Angus gave the age of the Starunia fauna as about 23,000 years, based on radiocarbon dating of collagen from the Woolly Rhinoceros done in the Smithsonian Institution in Washington DC (SI-642), which would date it is shortly before the maximum extension of the ice sheets of the Last Glaciation. However, subsequent investigations have resulted in a somewhat older date of 33,000-40,000 years (Kuc et al. 2012), which puts it squarely in the Cold-Continental phase which followed on from the somewhat warmer Upton Warren Interstadial. Faunas of this period, in England, can be quite rich and include a mixture of European and East Palaearctic species. Coope (1977) gives an account of temperature fluctuations during the Last (Deven-
sian) Glaciation. The Last (Eemian or Ipswichian) Interglacial dates to about 120,000 years ago and was succeeded by a cold treeless episode. Then, at about 60,000 years ago there was an episode, the Chelford Interstadial, in which Northern Coniferous forest developed in England. This was followed by a return to tundra conditions, which lasted till about 43,000 years ago and was followed by the Upton Warren Interstadial, an episode with warm but treeless conditions and a predominantly European fauna (Girling 1974; Coope and Angus 1975). After the thermal maximum of the Upton Warren Interstadial there was a gradual change to a colder more continental climate with faunas including often abundant Siberian and Tibetan species. This colder phase lasted from about 40,000 to about 25,000 years ago when temperatures lowered still further as the maximum extent of the ice sheets approached. The oldest known of these post thermal maximum faunas was at Queensford gravel pit near Dorchester on Thames and was dated to 39,300 +/- 1350 years ago (Coope 1985). This was a rich fauna apparently dating from the oldest age given for the Starunia site. *Boreonectes* sp. was among the included species. These faunas give an indication of the full-glacial environments of Central Europe in which Boreonectes griseostriatus lived. Unfortunately, there are no identifiable fossils of *B. multilineatus*. There are *Boreonectes* fossils in England, but these are isolated elytra which, although clearly marked with dark stripes on a pale background, could belong to either B. griseostriatus or multilineatus.

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RESEARCH ARTICLE



Cytogenetic characterisation and chromosomal mapping of microsatellite and telomeric repeats in two gecko species (Reptilia, Gekkonidae) from Thailand

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Abstract

Studies of chromosomes of *Cyrtodactylus jarujini* Ulber, 1993 and *C. doisuthep* Kunya et al., 2014 to compare microsatellite and TTAGGG sequences by classical and molecular techniques were conducted in Thailand. Karyological typing from a conventional staining technique of *C. jarujini* and *C. doisuthep* showed diploid chromosome numbers of 40 and 34 while the Fundamental Numbers (NF) were 56 in both species. In addition, we created the chromosome formula of the chromosomes of *C. jarujini* showing that 2n (40) = $L_{1}^{sn} + L_{2}^{sn} + L_{3}^{sn} + M_{1}^{n} + M_{4}^{t} + S_{2}^{n} + S_{5}^{s}$ while that of *C. doisuthep* was 2n (34) = $L_{1}^{sn} + L_{2}^{m} + L_{3}^{n} + M_{1}^{n} + M_{4}^{t} + S_{2}^{n} + S_{5}^{t}$ while that of *C. doisuthep* was 2n (34) = $L_{1}^{sn} + L_{2}^{sn} + L_{3}^{sn} + L_{2}^{n} + L_{3}^{sn} + M_{1}^{n}$ + $M_{2}^{t} + S_{1}^{sn} + S_{1}^{s}$. Age-NOR staining revealed NOR-bearing chromosomes in chromosome pairs 13 and 14 in *C. jarujini*, and in chromosome pairs 9 and 13 in *C. doisuthep*. This molecular study used the FISH technique, as well as microsatellite probes including (A)₂₀, (TA)₁₅, (CGG)₁₀, (GAA)₁₀, (TA)₁₅ and TTAGGG repeats. The signals showed that the different patterns in each chromosome of the Gekkonids depended on probe types. TTAGGG repeats showed high distribution on centromere and telomere regions, while (A)₂₀, (TA)₁₅, (CGG)₁₀, (CGG)₁₀, (CGG)₁₀, (CGG)₁₀, (CGG)₁₀, (CGG)₁₀, (CGG)₁₀, the sequences including chromosomes and some had strong signals on only a pair of homologous chromosomes. These results suggest that the genetic linkages have been highly differentiated between the two species.

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Keywords

Ag-NOR, Cyrtodactylus doisuthep, Cyrtodactylus jarujini, FISH microsatellite, karyotype

Introduction

Bent-toed geckos (genus *Cyrtodactylus* Gray, 1827) in Thailand have been classified into approximately 24 species (Chuaynkern and Chuaynkern 2012). *Cyrtodactylus jarujini* ranges from Nong kai, Bueng Kan and Nakhon Phanom Provinces, Thailand. More recently, Sumontha et al. (2008), found it in two caves on two sandstone hills, Phu Sing and Phu Thok, where it remained by day on the walls and crevices and emerged from the caves at night. Both in Phu Sing and Phu Thok, syntropy was found with the cave-dwelling agamid *Mantheyus phuwuanensis* (Manthey and Nabhitabhata 1991). It has also been recorded from central and northern Laos (Stuart 1999), but the exact identity of the Lao populations has to be re-evaluated (Fig. 1A). In contrast *C. doisuthep* is known only from Doisuthep in the Doi Suthep-Pui Range, Mueang District, Chiang Mai Province, northern Thailand (Fig. 1B).

Only 13% of gekkonid species have been karyotyped (Olmo and Signorino 2005) and were studied with conventional cytogenetic methods, including routine staining, as well as R-, NOR- and C-banding (Moritz 1983; Olmo and Signorino 2005; Shibaike et al. 2009). However, a small number of species were studied by molecular cytogenetic techniques (Kawai et al. 2009). The diploid number amongst gekkonid lizards ranges from 2n = 28 to 46 with most of the karyotypes composed of 28–46 chromosomes (Gorman 1973; Olmo 1986; Schmid et al. 1994). There are five karyotyped Cyrtodactylus species: C. consobrinus 2n = 48, NF = 50, C. pubisulcus 2n = 42, NF = 44 (Ota et al. 1992); C. interdigitalis 2n = 42, NF = 52 and C. kunyai 2n = 40, NF = 52 (Thongnetr et al. 2019a); C. saiyok 2n = 42, NF = 42 (Thongnetr et al. 2019b). The typical karyotype consists of a gradual series of telocentric chromosomes (sometimes with a few metacentric) and there is no distinction between macro- and microchromosomes, the centromere often being subterminal (Gorman 1973). Karyotype evolution within the group is accompanied by fissions and fusions and pericentric inversions (Gorman 1973; Olmo and Signorino 2005). This information on chromosomes is considered important along with other information for identification of the species (Campiranont 2003), especially the identification of related species, because of similarity of shape, appearance and other phenotypic expressions that are presumed to be associated with the genotype. Information from sequences of DNA allows us to understand the creation of a phylogenetic tree (dendrogram), because these characteristics often have a particular pattern. Information on chromosomes can be used to identify the phylogenetic relationship between species and population of animals (Lauhajinda and Taksintum 2006). Therefore, it is necessary to study the karyology of this group. In addition, geckos could be affected by the actions of humans in their use of household objects and agricultural chemicals. Thus, the gecko is one of the important groups of animals that can serve as a model for studying the environmental impact from human actions in the future.

Material and methods

The samples of *C. jarujini* and *C. doisuthep* were collected from the Phu Wua, Ban Phaeng District, Nakhonphanom Province and Doi Suthep-Pui Range, Mueang District, Chiang Mai Province, Thailand, (permission from an ethical committee ID U1-04498-2559). Chromosomes were directly prepared in vivo (Ota et al. 1990) by 0.1% colchicine were injected into the animals' intramuscular and abdominal cavity and left for 8-10 hours. Bone marrow, liver and testis (male) were cut into small pieces and then mixed with 0.075 M potassium chloride (KCl). After discarding all large cell pieces, 15 ml of cell suspension was transferred to a centrifuge tube and incubated 30-40 minutes, then centrifuged at 3,000 rpm for 8 minutes. Cells were fixed in fresh cool fixative of methanol:glacial acetic acid (3:1) and gradually made up to 8 ml before centrifuging again at 3,000 rpm for 8 minutes, whereupon the supernatant was discarded. Fixation was repeated until the supernatant was clear and the pellet was mixed with 1 ml fixative. Using conventional Giemsa staining, a drop of the mixture was added to a clean and cold slide by micropipette followed by the air-dry technique. The slide was conventionally stained with 20% Giemsa solution for 30 minutes (Patawang et al. 2014). Then, the slides were rinsed thoroughly with running tap water to remove excess stain. Afterwards, the slides were allowed to airdry at room temperature. Ag-NOR banding was analysed following the method of Howell and Black (1980). Two drops each of 50% silver nitrate and 2% gelatine solutions were added to slides, respectively. Then, they were sealed with cover glasses and incubated at 60 °C for 5-10 minutes. Afterwards, they were then soaked in distilled water until the cover glasses were separated. Finally, the slides were allowed to air-dry at room temperature and observed under microscope. Metaphase figures were analysed according to the chromosome classification of Chaiyasut (1989) and Turpin and Lejeune (1965). Chromosomes were classified as metacentric (m), submetacentric (sm), acrocentric (a) and telocentric (t). The Fundamental Number (NF: number of chromosome arms) is obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to acrocentric chromosomes. The use of microsatellite probes described by Kubat et al. (2008) was followed here with slight modifications. These sequences were directly labelled with Cy3 at the 5'-terminal during synthesis by Sigma (St. Louis, MO, USA). Fluorescence In Situ Hybridization (FISH) was performed under highly stringent conditions on mitotic chromosome spreads (Pinkel et al. 1986). After denaturation of chromosomal DNA in 70% formamide/ 2×SSC at 70 °C, spreads were incubated in 2×SSC for 4 min at 70 °C. The hybridization mixture (2.5 ng/µL probes, 2 µg/µL salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37 °C in a moist chamber containing 2×SSC. The post hybridization wash was carried out with 1×SSC for 5 min at 65 °C. A final wash was performed at room temperature in 4×SSCT for 5 min. Finally, the chromosomes were counterstained with DAPI (1.2 µg/mL), mounted in antifading solution (Vector, Burlingame, CA, USA), and analyzed in fluorescence microscope Nikon ECLIPSE.

Results

The diploid chromosome number and fundamental number

The diploid numbers in *C. jarujini* and *C. doisuthep*, were 40 and 34, respectively (Fig. 1C, E), whereas NF was 56 in both species (Fig. 1G, I). The type chromosomes of metacentric, submetacentric, acrocentric and telocentric were 8-4-4-24 and 14-6-2-12. There are no sex-related chromosomal heteromorphisms in the two species here studied.

The karyological characteristics

The karyotype of *C. jarujini* consists of two large metacentric, four large submetacentric, six large telocentric, two medium metacentric, eight medium telocentric, four small metacentric, four small acrocentric and ten small telocentric chromosomes. The karyotype formula for *C. jarujini* is as follows: $2n (40) = L_2^m + L_6^m + M_2^m + M_8^m$

| | C | | 2 | ö ð 3 | 6 4 | 8 9 5 | 6 | G A 7 |
|--------------------------------------|--------|------------------------------------|-------------------------------------|---|-----------------------------------|--------------------------|------------------------------|-----------------------------------|
| A | V 46 × | 8 8 8 | 8 8 9 | 10 | 6 6 11 | 8 8 12 | 1 3 | 1 4 |
| Charles and | 10 0,0 | 15 | 16 | 17 | * * 18 | 19 | 20 | |
| 3 cm | D | | 5 | 3 | 88 | 80 5 | 6 | H 7 |
| Cyrtodactylus jarujini | | 8 | 9 | 10 | 88 11 | 12 | 13 | 8 1 14 |
| | | 1 5 | • • 16 | 17 | 18 | 19 | 20 | |
| | | | | | | | | |
| | E | 88 | 2 | 1 3 | 88 | 5 | 88 6 | 00 7 |
| B | E | 3 1 9 8 | 2 2 8 # 9 | 1 3 0 10 | 88 4 80 11 | 5 12 | 6 13 | 7 7 14 |
| B | E | 8 1 8 8 15 | 2 9 16 | 1 3 10 17 | 88 4 80 11 | 5 12 | 6 13 | 7 7 14 |
| B 3 cm | F | 8 1 8 15 15 | 88 2 88 9 16 88 2 | 3 10 17 18 3 | 88 4 00 11 | 88 5 12 81 5 | 6 13 6 | 7 14 I4 J |
| B 3 cm Cyrtodactylus doisuthep | F | 88 1 15 13 1 8 8 | 2 9 16 16 16 | 3 10 17 17 3 A 10 10 | 38 4 40 11 | 5 22 12 12 | 6 13 6 4 6 13 | I 7 14 J 7 7 14 |

Figure 1. The *C. jarujini* specimen (**A**), metaphase chromosome plate and karyotypes (**A–G**) by conventional technique, (**D–H**) by Ag-NOR banding technique. The *C. doisuthep* specimen (**B**), metaphase chromosome plate and karyotypes (**E–I**) by conventional technique, (**F–J**) by Ag-NOR banding technique. Arrows indicated Ag-NORs regions. Scale Bar: 5 μm.

+ S_{4}^{n} + S_{4}^{i} + S_{10}^{t} or 2n (40) = 8m + 4sm + 4a + 24t. The karyotype of *C. doisuthep* comprises four large metacentric, six large submetacentric, six large telocentric, two medium metacentric, four medium telocentric, eight small metacentric, two small acrocentric and two small telocentric chromosomes. The karyotype formula for *C. doisuthep* is as follows: 2n (34) = L_{4}^{m} + L_{6}^{m} + L_{6}^{t} + M_{2}^{m} + M_{4}^{t} + S_{8}^{m} + S_{2}^{a} + S_{2}^{t} or 2n (34) = 14m + 6sm + 2a + 12t.

Ag-NOR banding

This technique highlighted active NORs on pairs 13 and 14 of *C. jarujini* (Fig. 1D, H) and pairs 9 and 13 of *C. doisuthep* (Fig. 1F, J).

Microsatellite pattern

Microsatellites $(A)_{20}$, $(TA)_{15}$, $(CAG)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$ and $(TA)_{15}$ abundantly distributed in some chromosomes, usually in telomeric regions of both species studied. FISH with the telomeric probe TTAGGG revealed hybridization signals on each telomere of all chromosomes (Fig. 2).

Discussion

Karyological data of the genus Cyrtodactylus

The species in the *Cyrtodactylus* exhibited a variable chromosome number, ranging from 34 to 42, however, the most frequent numbers were 40 and 42. The present study showed that the chromosome numbers of C. jarujini and C. doisuthep were 40 and 34, respectively. The fundamental number was 56 in both species. These results showed difference and accordance with others *Cyrtodactylus* that have been reported (Table 1). The karyological characteristics of C. jarujini and C. doisuthep obtained in the present study are the first report of chromosome sizes and the chromosome types in these species. In different species of Cyrtodactylus, different karyological characteristics can be found. However, overall, of these karyotypes of C. jarujini and C. doisuthep resemble those of other Cyrtodactylus species and other gekkonids, which comprised many mono-armed (telocentric) and few bi-armed chromosomes (meta- or submetacentric). For those gekkonid chromosomes which have been reported previously, most species showed that the karyotype comprises of many mono-armed chromosomes and few bi-armed chromosomes. The present results of C. jarujini and C. doisuthep agreed with the chromosomal evolution line hypothesis within the gekkonid group (Trifonov et al. 2011). The karyotype of C. jarujini and C. doisuthep showed the gradient of most telocentrics, while comprising of a few bi-armed chromosomes. These features conform to the hypothesis of re-arrangement from ancestral karyotype by Robertsonian fissions, fusions or pericentric inversions (Gorman 1973; King 1987).



Figure 2. Karyotypes of two geckos presenting the patterns of microsatellite $(A)_{20}$, $(CAG)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$, $(TA)_{15}$ and TTAGGG; *C. jarujini* (**A–F**), *C. doisuthep* (**G–L**). Scale Bars: 10 µm.

Active NOR sites

Nucleolus organiser regions (NORs) are chromosome sites which contain the 18S and 28S ribosomal RNA genes. If these regions were active during the interphase prior to mitosis, they can be detected by silver nitrate staining (Howell and Black 1980). In the present study, the chromosome markers of both *Cyrtodactylus* are determined by using

| Species | 2n | NF | Karyotype formula | NORs | Location | Reference |
|----------------------------------|----|----|---|---------|----------|---------------------------|
| Cyrtodactylus consobrinus | 48 | 50 | 2bi-arm+46t | - | Malaysia | Ota et al. (1992) |
| (Peters, 1871) | | | | | | |
| C. doisuthep Kunya et al., 2014 | 34 | 56 | 14m+6sm+2a+12t | P9, 13 | Thailand | Present study |
| C. interdigitalis Ulber, 1993 | 42 | 52 | 4m+2sm+4a+32t | P12 | Thailand | Thongnetr et al. (2019a) |
| C. jarujini Ulber, 1993 | 40 | 56 | 8m+4sm+4a+24t | P13, 14 | Thailand | Present study |
| C. kunyai Pauwels et al., 2014 | 40 | 52 | 8m+4sm+6a+22t | P12 | Thailand | Thongnetr et al. (2019a) |
| C. pubisulcus Inger, 1958 | 42 | 44 | 2bi-arm+40t | - | Malaysia | Ota et al. (1992) |
| C. saiyok Panitvong, 2014 | 42 | 42 | 42t | P15 | Thailand | Thongnetr et al. (2019b) |
| Gekko chinensis Gray 1842 | 40 | 46 | 6bi-armed+34uni-armed | - | China | Lau et al. (1997) |
| G. gecko (Linnaeus, 1758) | 38 | 50 | 12bi-armed+26uni-armed | - | - | Cohen et al. (1967) |
| | 38 | 50 | Lm2 + Lsm4 + Lt4 + Mt6 + Sm4 + Sa2 + St16 | P4 | Thailand | Patawang et al. (2014) |
| G. hokouensis Pope, 1928 | 38 | 56 | 4m+6sm+20t+8bi-armed | P(L)19 | China | Chen et al. (1986) |
| G. monarchus (Schlegel, 1836) | 38 | 46 | _ | - | Malaysia | Ota et al. (1990) |
| G. petricolus Taylor, 1962 | 38 | 54 | _ | - | _ | Ota (1989) |
| G. shibatai Toda et al., 2008 | 38 | 58 | 4m+8sm+18t+8bi-armed | P(L)19 | Japan | Shibaike et al. (2009) |
| G. tawaensis Okada, 1956 | 38 | 58 | 4m+8sm+18t+8bi-armed | P(L)19 | Japan | Shibaike et al. (2009) |
| G. taylori | 42 | _ | _ | - | Thailand | Ota and Nabhitabhata |
| Grossmann et Ulber, 1990 | | | | | | (1991) |
| G. vertebralis Toda et al., 2008 | 38 | 62 | 4m+14sm+14t+6bi-armed | P(L)19 | Japan | Shibaike et al. (2009) |
| Hemidactylus brookii | 40 | 44 | 4bi-armed+36t | - | - | Bhatnagar (1962) |
| Gray, 1854 | | | | | | |
| H. flaviviridis Rüppell, 1835 | 40 | 60 | 20bi-armed+20t | - | - | Asana and Mahabale (1941) |
| | 46 | 46 | 46t | - | - | Makino and Momma (1949) |
| | 40 | 52 | 12bi-armed+28t | - | - | Branch (1980) |
| H. frenatus Schlegel, 1836 | 46 | 46 | 46t | - | - | Makino and Momma (1949) |
| | 40 | 54 | 14bi-armed+26t | P3 | - | King (1978) |
| | 40 | 46 | 6bi-armed+34t | - | _ | Darevsky et al. (1984) |
| H. mabouia | 42 | 56 | 14bi-armed+28t | - | - | Becak et al. (1972) |
| (Moreau de Jonnès, 1818) | 42 | 54 | 12bi-armed+30t | _ | _ | McBee et al. (1987) |

Table 1. Karyotype reviews in the genera Cyrtodactylus, Gekko Laurenti, 1768 and Hemidactylus Gold-fuss, 1820 (Gekkonidae, Squamata).

Remarks: 2n = diploid chromosome number, NORs = nucleolus organiser regions, SCR = subcentromeric regions, NF = fundamental number (number of chromosome arms), bi-arm = bi-armed chromosome, m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, L = large, S = small, P = chromosome pair and – = not available.

the Ag-NOR banding technique as shown in Table 1. *C. jarujini* had the acrocentric chromosome pair 13 and metacentric chromosome pair 14, which were the NOR-bearing chromosome. Pair 13 NORs were located on the short arm near the telomere (telomeric NOR) and the pair 14 NORs were located on the short arm near the centromere (centromeric NOR). *C. doisuthep* had the metacentric, two chromosome pair 9 and pair 13 which were the NOR-bearing chromosomes. Pair 9 NORs were located on the arm near the telomere (telomeric NOR) on both sides and the pair 13 NORs were located on the arm near the telomere (telomeric NOR) on both sides and the pair 13 NORs were located on the arm near the telomere (telomeric NOR).

The NORs in both species of genus *Cyrtodactylus* exhibited at the telomeric region on the long arm and short arm and are similar to the previous reports of the gekkonids for the Gekkonidae family by King (1978) and Moritz and King (1985). The NORs of *Dixonius siamensis* (Boulenger, 1898), *G. gecko*, *G. hokouensis*, *G. shibatai*, *G. tawaensis*, *G. vertebralis*, *H. frenatus* and *H. platyurus* were found at all regions on the short arm



Figure 3. Idiograms represent the $(A)_{20}$, $(CAG)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$, $(TA)_{15}$ and TTAGGG mapping on the chromosomes of *C. jarujini* (**A**) and *C. doisuthep* (**B**).

and that agrees with those previous reported (Asana and Mahabale 1941; Makino and Momma 1949; Bhatnagar 1962; Cohen et al. 1967; Becak et al. 1972; King 1978; Branch 1980; Darevsky et al. 1984; Chen et al. 1986; McBee et al. 1987; Ota 1989; Ota et al. 1990; Ota and Nabhitabhata 1991; Lau et al. 1997; Ota et al. 2001; Shibaike et al. 2009; Patawang et al. 2014; Trifonov et al. 2011; Trifonov et al. 2015).

Microsatellite pattern

Microsatellites or simple sequence repeats (SSRs) are oligonucleotides of 1–6 base pairs in length, forming excessive tandem repeats of usually 4 to 40 units (Tautz and Renz 1984; Ellegren 2004; Chistiakov et al. 2006). They show abundant distribution throughout eukaryotic genomes, being dispersed or clustered both in euchromatin or heterochromatin. They are highly polymorphic regarding copy number variations (Ellegren 2004). In our present study both species exhibited the same general hybridisation pattern for some applied probes with the motif TAAGGG repeat showing abundance at the telomeric ends of all chromosomes (Fig. 3), corroborating findings from other gekko groups studied to date (Srikulnath 2015). Otherwise, the dinucleotides (A)₂₀, (CAG)₁₀, (CGG)₁₀, (GAA)₁₀ and (TA)₁₅ accumulated exclusively in telomeric and subtelomeric chromosomal regions. However, the results clearly indicate that the microsatellite repeats are in high copy number on some chromosome pairs, according to previous reports on reptile groups (Pokorná et al. 2011; Matsubara et al. 2013).

Conclusions

In this study, the comparison of the cytogenetic maps of two *Cyrtodactylus* species (*C. jarujini* and *C. doisuthep*) enabled us to delineate the process of chromosomal reorganisation in this group. This is the first report in Thailand for the study of cytogenetics of both species. Therefore, the cytogenetic data obtained can be used to benefit cytotaxonomy and the study of evolution of geckos, as well as being an essential prerequisite for future genome projects of gecko groups.

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RESEARCH ARTICLE



A comparative analysis of the karyotypes of three dolphins – Tursiops truncatus Montagu, 1821, Tursiops australis Charlton-Robb et al., 2011, and Grampus griseus Cuvier, 1812

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Abstract

The aim of this study is to produce G-banded karyotypes of three dolphin species, *Tursiops truncatus* Montagu, 1821, *Tursiops australis* Charlton-Robb et al., 2011, and *Grampus griseus* Cuvier, 1812, and to determine if any differences between the species can be observed. Monolayer skin cultures were established and processed for chromosome study by trypsin banding. The results indicate that the three species here investigated have the same diploid number (2n = 44) and very similar gross chromosome morphology, however G-banding allows distinction between each species. Chromosome 1 in *G. griseus* is significantly different from the other 2 species, and chromosome 2 in *T. australis* is subtly different from the other 2 species. This result is of potential significance in taxonomic studies, and can provide an unequivocal answer in the assessment of suspected hybrids between these species.

Keywords

Burrunan, chromosome, Common Bottlenose, G-band, Risso's dolphin

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Introduction

The family Delphinidae contains 37 recognized species, excluding *Tursiops australis* Charlton-Robb et al., 2011, whose status has not been conclusively resolved (Committee on Taxonomy 2020). The first karyotype of a dolphin, *Tursiops truncatus* Montagu,1821, was published by Walen and Madin (1965), and since then a total of at least seven species have been studied cytogenetically (Atlas of Mammalian chromosomes 2020). It has been concluded that the studied species have similar karyotypes, the majority of apparent variation being associated with differing accumulation of heterochromatic regions, as demonstrated in a study by G- and C-banding of the karyotypes of *Stenella clymene* Gray, 1850, *Lagenorhynchus albirostris* Gray, 1846, and *Phocoena phocoena* Linnaeus, 1758 (Arnason et al. 1980). There has, however, been no detailed comparative G-banding analysis of karyotypes within this family. This may in part be due to the use of differing banding techniques, the varying banding resolutions achieved, and use of differing karyogram templates, in addition to a general lack of availability of appropriate study material.

The three species of dolphin investigated here belong to the subfamily Delphininae, but it has been proposed that Grampus griseus Cuvier, 1812, should be attributed to the subfamily Globicephalinae, based on cytochrome b sequencing studies (LeDuc et al. 1999). The karyotype of T. truncatus has been published on several occasions, initially by Arnason (1974), and more recently with an ideogram by Bielec et al. (1997). The aim of this study is to describe the karyotype of T. australis and G. griseus, not yet described in the scientific literature, to enable use of the karyological characteristics of these species to identify putative hybrids between these species, and to help clarify the specific/sub-specific status of T. australis. The identification of hybrids is of interest because T. truncatus and G. griseus are the species most frequently noted as the origin of hybrids in captivity (Espada et al. 2019). There are conflicting views as to whether the recently described species T. australis (Charlton-Robb et al. 2011) should be categorized as such, or as a subspecies. It was considered on morphological grounds by Jedensjö et al. (2020) that T. australis falls within the species T. truncatus. A molecular study by Moura et al. (2020) provides evidence of a monophyletic origin of the genus Tursiops Gervais, 1855, but they conclude that their data indicate that T. australis is best considered as a subspecies within T. aduncus Ehrenberg, 1833 (refer to the phylogenetic network presented as Figure 2 in that paper, which clearly positions T. australis as a clade within T. aduncus). The molecular evidence for determining that T. australis is a separate species has been described as weak, and to include inappropriate analysis (WoRMS editorial board. 2020), and the morphological evidence has been criticized on the grounds that the sample size was small, interspecies comparison was limited and there was overlap occurring in all metric characters (Atlas of Mammalian chromosomes 2020).

Material and methods

Tissue source and cell establishment

The tissue samples available for this study were from a male and female common bottlenose dolphin (*T. truncatus*), a male and a female Burrunan dolphin (*T. australis*), and a female Risso's dolphin (*G. griseus*). Skin samples from *T. truncatus* and *T. australis* were obtained from captive individuals at SeaWorld, Queensland, Australia during routine vaccinations. The tissue was taken from the tail using a biopsy punch. One female *T. truncatus* (CB01) was wild caught in 1994 and is approximately 33 years old. The other (CB02) was a male wild caught in 1985 and is approximately 43 years old. Both individuals of *T. australis* were born in captivity; one male aged 40 was transferred to Sea World in 1990 from Marineland, South Australia (BD01), and the other was a female aged 10 born at Sea World (BD04). A lung sample from a stranded *G. griseus* was provided by Dolphin Marine Conservation Park, Coffs Harbour, New South Wales, Australia (RD01). All tissue samples were immediately placed in DMEM media with 10% fetal bovine serum, 1% penicillin/streptomycin (10,000 U/mL stock) and 1% amphotericin B (250 µg/mL stock) and kept at 4 °C until processing.

Samples were washed several times with DMEM media (as described above) and cut into 1–3 mm pieces in fresh media. Tissue pieces were transferred to 25 cm² flasks, arranged evenly on the lower surface of the flask. The flasks were incubated in an inverted position at 37 °C, 5% CO₂ for 24 hours. Five mL of media was introduced, and then the flasks were returned to the incubator in an upright orientation. When cells reached ~70% confluence, tissue pieces were detached and removed. Cells were cryopreserved in liquid nitrogen at a concentration of 1 × 10⁶ cells/mL in DMEM media supplemented with 10% dimethyl sulfoxide, until ready to be used (Arsham et al. 2016).

Species identification

The Qiagen DNeasy Blood and Tissue kit was used to isolate DNA from -2×10^6 cells, according to the manufacturer's protocol for cultured cells. The resulting DNA was forwarded to the DNA Sequencing Facility at Griffith University, for confirmation of species. Around 660 bp of the mitochondrial COI gene was used for amplification by Platinum taq DNA polymerase (Invitrogen). The following primers were used – forward 5–3' ATTCAACCAATCATAAAGATATTGG, reverse 5–3' TAAACTTCTG-GATGTCCAAAAAATCA (Hebert et al. 2004). ExoSap-IT (Applied Biosystems) was then used to clean the PCR amplicons, which were then bi-directionally sequenced. The Barcode of Life Database (v4, BOLD http://www.boldsystems.org/) was then used as a reference to classify the resulting sequences.

Karyotyping

A flask of cells for each dolphin at various passages (CB01: P7; CB02: P6; BD01: P6; BD04: P6; RD01: P6) was forwarded to the cytogenetics laboratory at Sullivan Nicolaides Pathology. Here, the cells were either incubated overnight at 37 °C prior to initiation of harvest, or sub-cultured into 25 cm² flasks in Amniomax II medium (Gibco), then incubated at 37 °C until ready for harvest. The cells were harvested when approximately 80% confluent. This was initiated by adding colchicine (100 µg/mL,Sigma) for 2 hours, suspending the cells in the medium with trypsin (Trypsin/EDTA 1×, Sigma), and swelling the cells by treatment with hypotonic solution (0.075 M potassium chloride) at 37 °C for 10 minutes. A 10% prefix solution of 3% acetic acid was then added before methanol/glacial acetic acid (3:1) fixation. The resulting cell suspension was used to prepare slides by dropping via a glass pipette onto clean dry slides (Arsham et al. 2016). After overnight incubation at 60 °C, the slides were G-banded using a modification of the method of Seabright (1971). Wright's/Giemsa stain (Kinetik) was used to stain the slides.

A Metafer slide scanner (Metasystems) was used to select cells for processing, and the Ikaros karyotyping system (Metasystems) was used to produce karyograms.

The template employed for chromosome grouping is consistent with that used by Bielec et al. (1997), and their chromosome assignments have been followed as far as possible, given the difficulty sometimes caused by differences in appearance between replication banded and trypsin banded chromosomes. The chromosomes are arranged according to position of the centromere, pairs 1–2 are subtelocentric, pairs 3–11 submetacentric, 12–17 metacentric and 18–21 acrocentric, noting that some chromosomes could be categorized within different groups, but the template has been followed.

Results

Species identification

Species identification confirmed both CB01 and CB02 to be *T. truncatus* with a 99.27% and 99.85% match of COI gene sequence, respectively. BD01 and BD04 were confirmed to be *T. australis* with a 99.71% COI gene sequence match for both individuals. RD01 was confirmed to be *G. griseus* with a 99.85% match of COI gene sequence.

Karyotype

The diploid number of all 3 species is 44. In all individuals studied, the karyotype consists of 2 large subtelocentric pairs (1–2), 9 submetacentric pairs (3–11), 6 smaller metacentric/submetacentric pairs (12–17), 4 acrocentric pairs (18–21), an X chromosome which closely resembles that observed in many mammalian species, and in the

2 males studied, a small Y chromosome. Five to 22 karyotypes per individual were prepared, depending on the availability of suitable metaphases, and these showed a consistent karyotype in each case. A representative karyogram from each of the five individuals studied is presented in Figs 1–5.

There are a number of heterochromatic variants visible in these individuals. In the male *T. truncatus* there is a size polymorphism in the distal short arm of chromosome 6, the chromosome on the right has a larger G-negative band, and in the female, the short arm of chromosome 3 of the chromosome on the right has a larger pale band between the two dark bands, and the proximal long arm of chromosome 4 has a larger G-negative band just below the centromere. In the female *T. australis*, there are variant heterochromatic regions in the distal short arm of chromosome 2, where the chromosome on the right has a larger grey band distally, and the short and long arms of chromosome 4, where the chromosome on the right has a smaller pale band at the end of the short arm, and a smaller pale region just below the centromere. *G. griseus* has a variant on the proximal long arm of chromosome 18, the G-band negative region being larger in the chromosome on the right.

Apart from the size polymorphisms attributable to heterochromatin variants, the results show that chromosome 1 in *G. griseus* has a significantly different morphology



Figure 1. G-banded karyotype of male *T. truncatus* (CB02). Note the size polymorphism in the distal short arm of chromosome 6.



Figure 2. G-banded karyotype of female *T. truncatus* (CB01). Note the size polymorphism in the short arm of chromosome 3 and the proximal long arm of chromosome 4.



Figure 3. G-banded karyotype of male *T. australis* (BD01).



Figure 4. G-banded karyotype of female *T. australis* (BD04). Note the size polymorphism in the distal short arm of chromosome 2, and the short and long arms of chromosome 4.



Figure 5. G-banded karyotype of female *G. griseus* (RD01). Note the size polymorphism in the proximal long arm of chromosome 18.



Figure 6. A chromosome 1 from **A** *T. truncatus* **B** *G. griseus* **C** idiogram of chromosome 1 from *T. truncatus* to the left, *G. griseus* to the right **D** chromosome 2 from *T. truncatus* **E** *T. australis*, with arrows indicating the position of the centromere **F** idiogram of chromosome 2 from *T. truncatus* to the left, *T. australis* to the right, with arrows indicating possible breakage points of a pericentric inversion.



Figure 7. Composite karyogram of the 3 studied species, with 1 homologue of each chromosome presented. Chromosomes from male *T. truncatus* are to the left, female *T. australis* in the middle, and female *G. griseus* to the right.

from the two *Tursiops* species. In the *Tursiops* karyograms, the short arm consists essentially of a proximal dark and distal light band, with a pale centromeric region, and a prominent dark band on the proximal long arm. In the *G. griseus* karyogram, the short arm has a darker distal region and a thin dark band in the proximal region, and it is also slightly longer. The centromeric region of *G. griseus* is not as distinctly pale, and there

is no proximal dark band on the long arm. The remainder of the long arm is similar, but not completely identical. Overall, the chromosome is slightly shorter in *G. griseus*. Figure 6A–C compares an example of chromosome 1 from *T. truncatus* and *G. griseus*, together with an ideogram showing the differences in banding pattern.

In both male and female karyograms of *T. australis*, the dark band on the proximal long arm of chromosome 2 in *T. truncatus* and *G. griseus* is present on the proximal short arm. Figure 6D–F compares an example of chromosome 2 from *T. truncatus* and *T. australis*, and an ideogram indicating the banding pattern of the chromosomes. Figure 7 shows a composite karyogram with one homologue from each of the 3 species.

Discussion

The karyotypes of the three species of dolphin studied here are very similar, all having the same chromosome number (2n = 44) and gross morphology. It is only when studying the detail of the G-banding pattern that differences become apparent. This can be readily visualized by referring to Figure 7, in which the banding pattern of the combined karyograms is apparently identical, with the exception of the chromosomes 1 and 2. The level of banding achieved is, compared to human karyotyping, standard resolution, so greater resolution would allow more precise identification of potential areas of difference. To achieve G-bands, we have used a modification of the trypsin method (Seabright 1971), which produces a banding pattern where GC rich DNA stains pale, and AT rich DNA is dark. The replication method used by Bielec et al. (1997), stains early replicating DNA pale, and late replicating DNA dark, so while the results are broadly consistent, there are differences, for example, heterochromatin can be pale by G-bands, but is dark using replication banding, so this has to be taken into account when comparing karyograms prepared by the two methods.

As the number of individuals available is limited, reasons other than interspecific differences for the observed variation need to be considered. The presence of isolated populations can be a source of intraspecific variation, however in the karyotypes of the individual pairs studied, there was no heteromorphism that could not be assigned to heterochromatic size, relating the variant regions to the C-banded karyogram of *Tursiops gilli* Dall, 1873, now reclassified as *T. truncatus*, depicted in Arnason (1974).

Chromosome 1 appears very similar in *T. truncatus* and *T. australis*, and also, from the literature, in the delphinids *S. clymene*, and *L. albirostris*, and in the harbor porpoise *P. phocoena* (in the latter karyogram the short arm is smaller, lacking the prominent dark band, and the distal C-band positive region is lacking) (Arnason 1980), but is significantly different in the individual of *G. griseus* here analysed. Examination of the karyotypes of apparently related species may assist in determining whether the banding pattern of this chromosome is unique to *G. griseus*, or present in other species, which would indicate an evolutionary relationship.

The proximal dark band on chromosome 2 is on the long arm in *S. clymene, L. al*birostris, *P. phocoena* (Arnason 1980) and *T. truncatus* (Bielec et al. 1997), and in *G. griseus* in this study. The pericentric region of this chromosome does not appear to contain a significant C-band positive block, although it has heterochromatic regions in proximity on either side (Arnason 1980), so pericentric inversion of heterochromatin would not explain the different morphology. The simplest explanation is a small pericentric inversion in *T. australis*, however a more complex rearrangement cannot be excluded. Pericentric inversions can occur and be inherited within a species, but are very rarely homozygous in one individual. In this instance, the two animals, although both captive, originated from different locations, and both were homologous for the rearrangement, so a population variant appears unlikely. This finding thus may provide a marker which differentiates *T. australis* and *T. truncatus*. It may also confirm a relationship between *T. australis* and *T. aduncus*, if *T. aduncus* is shown to have the same banding pattern of chromosome 2 as that of *T. australis*, could therefore clarify the taxonomic position of *T. australis*.

Hybrids between dolphin species occur rarely in the wild, more frequently in captive animals. In captivity, the most frequently observed hybrids result from crosses between *T. truncatus* and *G. griseus* (Espada et al. 2019). Our preliminary observations of banding pattern in these species indicate that hybrids would be recognizable cytogenetically, and the degree of difference in chromosome 1 structure in the two species suggests that meiotic pairing, and thus fertility of a hybrid, would be unlikely.

Conclusion

The three species of dolphin species described here can be distinguished by their banding pattern, these differences being consistent in all cells within the individuals studied. The small number of individuals analysed makes it premature to draw firm conclusions, but it appears that these differences may potentially have use as an additional tool in determining the species of a particular animal where this is unclear, and in assessment of hybrids. Study of further individuals of these species, and of other dolphins, would enable karyotypic variation to be added to molecular and morphological differences in establishing the evolutionary relationships within this group. In the light of the study by Moura et al. (2020), the morphology of chromosome 2 of *T. aduncus* would be of particular interest in establishing the lineage of *T. australis*.

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RESEARCH ARTICLE



Cytogenetic analysis of Hypomasticus copelandii and H. steindachneri: relevance of cytotaxonomic markers in the Anostomidae family (Characiformes)

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Abstract

Recent phylogenetic hypotheses within Anostomidae, based on morphological and molecular data, resulted in the description of new genera (*Megaleporinus* Ramirez, Birindelli et Galetti, 2017) and the synonymization of others, such as the reallocation of *Leporinus copelandii* Steindachner, 1875 and *Leporinus steindachneri* Eigenmann, 1907 to *Hypomasticus* Borodin, 1929. Despite high levels of conservatism of the chromosomal macrostructure in this family, species groups have been corroborated using banding patterns and the presence of different sex chromosome systems. Due to the absence of cytogenetic studies in *H. copelandii* (Steindachner, 1875) and *H. steindachneri* (Eigenmann, 1907), the goal of this study was to characterize their karyotypes and investigate the presence/absence of sex chromosome systems using different repetitive DNA probes. Cytogenetic techniques included: Giemsa staining, Ag-NOR banding and FISH using 18S and 5S rDNA probes, as well as microsatellite probes (CA)₁₅ and (GA)₁₅. Both species had

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2n = 54, absence of heteromorphic sex chromosomes, one chromosome pair bearing Ag-NOR, 18S and 5S rDNA regions. The (CA)₁₅ and (GA)₁₅ probes marked mainly the subtelomeric regions of all chromosomes and were useful as species-specific chromosomal markers. Our results underline that chromosomal macrostructure is congruent with higher systematic arrangements in Anostomidae, while microsatellite probes are informative about autapomorphic differences between species.

Keywords

Anastomid, coastal basins, cytogenetics, endemic species, fluorescence *in situ* hybridization, freshwater fishes, repetitive sequences

Introduction

Within the order Characiformes, the family Anostomidae encompasses around 150 valid species distributed throughout South America (Froese and Pauly 2019; Fricke et al. 2020). Fish of this family carry out annual reproductive migrations and constitute a large part of the fish biomass in several aquatic habitats, representing an important resource for human activities (Garavello and Britski 2003). Up to now, seven anostomid species are considered endangered and many others need urgent assessment of their conservational status (reviewed in Birindelli et al. 2020). In many cases, original type series are composed of more than one species, such as the case of *Leporinus copelandii* Steindachner, 1875 (Birindelli et al. 2020).

Recently, phylogenetic hypotheses based on morphological and molecular data have suggested the creation of the new genus *Megaleporinus* Ramirez, Birindelli et Galetti, 2017 (Ramirez et al. 2016, 2017), and the synonymization of others, such as the reallocation of *L. copelandii* and *Leporinus steindachneri* Eigenmann, 1907 to *Hypomasticus* Borodin, 1929 (Birindelli et al. 2020). Even with these proposed changes, both *Leporinus* Agassiz, 1829 and *Hypomasticus* are still not monophyletic, requiring further taxonomic investigations.

Cytogenetic studies in this group have revealed a conserved karyotype macrostructure of 2n = 54 and fundamental number (NF) = 108 (Table 1). Regardless of this conservatism, the cytogenetic banding patterns, the differential accumulation of repetitive DNA, and the presence/absence of sex chromosome systems have been useful to help species identification in this family (reviewed in Barros et al. 2017). Both *Hypomasticus copelandii* (Steindachner, 1875) and *Hypomasticus steindachneri* (Eigenmann, 1907) had an early divergence in the phylogeny of the family (Ramirez et al. 2016, 2017; Birindelli et al. 2020), and were never analyzed cytogenetically. Therefore, the goal of this paper was to characterize their karyotypes and to investigate the presence/absence of sex chromosome systems using different repetitive DNA probes in these two species from Brazilian southeastern coastal basins in order to identify potential cytotaxonomic markers. We also provided a review of the cytogenetic data available for the family Anostomidae.

| Species | 2 <i>n</i> | Karyotype | Sex-System | Ag-NOR | 18S | 55 | References |
|-----------------------------|------------|--------------------|---|--------|-----|----------|--|
| Abramites hypselonotus | 54 | - | no | - | 2 | - | Silva et al. 2013 |
| A. solaria | 54 | - | no | 2 | - | - | Martins et al. 2000 |
| Anostomus ternetzi | 54 | - | no | 2 | - | - | Martins et al. 2000 |
| Hypomasticus copelandii | 54 | 28m+26sm | no | 2 | 2 | 2 | Present Study |
| H. steindachneri | 54 | 30m+24sm | no | 2 | 2 | 2 | Present Study |
| Laemolyta taeniata | 54 | 28m+26sm | no | 2 | 2 | 2† | Barros et al. 2017 |
| Leporellus vittatus | 54 | 28m+26sm | no | 2 | 2 | 2–4 † | Galetti Jr et al. 1984; Dulz et al. 2019 |
| Leporinus agassizi | 54 | 28m+26sm | no | 2 | 2 | 2 | Barros et al. 2017 |
| L. amblyrhyncus | 54 | - | no | 2 | - | - | Galetti Jr et al. 1991 |
| L. fasciatus | 54 | 28m+26sm | no | 2 | 2 | 2 | Barros et al. 2017 |
| L. friderici | 54 | 28m+26sm/32m+22sm | no | 2 | 2 | 2-4 | Martins and Galetti Jr., 1999; Silva et al. 2012; Borba et al. 2013; Barros et al. 2017; Ponzio et al. 2018; Dulz et al. 2019; Crepaldi and Parise- Maltempi 2020 |
| L. lacustris | 54 | 30m+24sm | no | 2 | 2 | - | Galetti Jr et al. 1981; Galetti Jr et al. 1984; Mestriner et al. 1995; Silva et al. 2012, 2013; Borba et al. 2013 |
| L. multimaculatus | 54 | 26m+28sm | ZZ/ZW | 2 | - | - | Barros et al. 2018; Venere et al. 2004 |
| L. octofasciatus | 54 | - | no | 2 | - | - | Galetti Jr et al. 1984 |
| L. piau | 54 | - | no | 2 | - | - | Galetti Jr et al. 1991 |
| L. striatus | 54 | - | no | 2 | 2 | - | Galetti Jr et al. 1991; Silva et al. 2012, 2013; Borba et al. 2013; Ponzio et al. 2018 |
| L. taeniatus | 54 | - | no | 2 | - | - | Galetti Jr et al. 1991 |
| Megaleporinus conirostris ‡ | 54 | - | ZZ/ZW | 2 | - | - | Galetti Jr et al. 1995 |
| M. elongatus ‡ | 54 | | $\begin{array}{c} Z_{1}Z_{1}Z_{2}Z_{2}/\\ Z_{1}W_{1}Z_{2}W_{2} \end{array}$ | 2 | 2 | 4 | Martins and Galetti Jr. 2000; Parise-Maltempi et al. 2007, 2013; Marreta et al. 2012; Silva et al. 2012, 2013; Borba et al. 2013; Ponzio et al. 2018; Crepaldi and Parise-Maltempi 2020 |
| M. macrocephalus ‡ | 54 | _ | ZZ/ZW | - | 2 | - | Galetti Jr and Foresti 1986; Galetti Jr et al. 1995; Silva et al. 2012, 2013; Borba et al. 2013; Ponzio et al. 2018; Utsunomia et al. 2019; Crepaldi and Parise-Maltempi 2020 |
| M. obtusidens ‡ | 54 | 26m+28sm/ 28m+26sm | ZZ/ZW | 2 | 2 | 2-4 | Galetti Jr et al. 1981; Galetti Jr et al. 1995; Martins and Galetti Jr. 2000; Silva et al. 2012, 2013; Borba et al. 2013; Utsunomia et al. 2019; Dulz et al. 2020 |
| M. reinhardti ‡ | 54 | 28m+26sm | ZZ/ZW | - | 2 | 2 | Galetti Jr and Foresti 1986; Galetti Jr et al. 1995; Dulz et al. 2020 |
| M. trifasciatus ‡ | 54 | 26m+28sm | ZZ/ZW | 2-3 | 6§ | 2† | Galetti Jr et al. 1995; Barros et al. 2017 |
| Pseudanos trimaculatus | 54 | - | no | 2 | - | - | Martins et al. 2000 |
| Rhytiodus microlepis | 54 | 28m+26sm | no | 2 | 4§ | 2 | Barros et al. 2017 |
| Schizodon altoparanae | 54 | - | no | 2 | - | 4 | Martins and Galetti Jr. 2000 |
| S. borellii | 54 | - | no | 2 | 2 | 4 | Martins and Galetti Jr. 2000; Silva et al. 2012, 2013; Ponzio et al. 2018 |
| S. fasciatus | 54 | 28m+26sm | no | 2 | 22§ | 2 † | Barros et al. 2017 |
| S. intermedius | 54 | - | no | 2 | - | - | Martins and Galetti Jr. 1997 |
| S. isognathus | 54 | - | no | 2 | 2 | 4 | Martins and Galetti Jr. 2000; Silva et al. 2012, 2013; Ponzio et al. 2018 |

Table 1. Cytogenetic data available on the Anostomidae species regarding their chromosome number (2n), karyotype description, presence or absence of sex-chromosome systems, number of chromosomes marked by the Ag-NOR banding technique, and also 18S and 5S rDNA probes.

† indicates synteny between 18S and 5S rDNA clusters. ‡ Species were assigned to the new genus *Megaleporinus* according to Ramirez et al. (2017). § Barros et al. (2017) did not exclude the possibility of technical artifacts and suggested that the expansion of the rDNA sites should be confirmed with supplementary analysis.

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Martins and Galetti Jr. 2000

Martins and Galetti Jr. 2000

Martins and Galetti Jr. 2000

no

no

no

S. knerii

S. nasutus

S. vittatus

54

54

54

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Material and methods

Sample collection

Hypomasticus copelandii was collected from Glória (Paraíba do Sul River Basin), Itabapoana (Itabapoana River Basin), Matipó (Doce River Basin) and Mucuri (Mucuri River Basin) rivers, covering its full range of distribution in southeastern Brazil. *Hypomasticus steindachneri* was collected from Tiririca Lake (Doce River Basin) (Table 2). Collection permit of the Instituto Chico Mendes de Biodiversidade (ICMBio) (SISBIO14975-1) was issued to Jorge Abdala Dergam. Species identification followed Garavello (1979) and the sex identification was made through histological analysis. Voucher specimens were deposited in the scientific collection of the Museu de Zoologia João Moojen in Viçosa, Minas Gerais, Brazil (Table 2).

Cytogenetic analyses

The specimens were anesthetized with clove oil 300 mg. L^{-1} (Lucena et al. 2013) as approved by the Universidade Federal de Vicosa Animal Welfare Committee (CEUA authorization 08/2016). Mitotic chromosomes were obtained from a direct method using kidney (Bertollo et al. 1978) and the following cytogenetic techniques were used: conventional staining with Giemsa 5% diluted in Sorensen buffer (0.06M, pH 6.8) for basic karyotypic analysis, identification of the argyrophilic nucleolar organizer regions through Ag-NOR banding technique (Howell and Black 1980), and fluorescence in situ hybridization (FISH) following the protocol outlined in Pinkel et al. (1986) using 18S and 5S rDNA probes, as well as (CA)₁₅ and (GA)₁₅ microsatellite probes. The ribosomal probes were obtained through polymerase chain reaction (PCR) using the following primers: 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010); 5Sa (5'-TAC GCC CGA TCT CGT CCG ATC-3') and 5Sb (5'-CAG GCT GGT ATG GCC GTA AGC-3') (Martins et al. 2006). The ribosomal genes were labeled with digoxigenin-11-dUTP (Roche Applied Science) and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science), whereas the microsatellite probes were synthesized and labeled with Cy3 fluorochrome at the 5' end (Sigma).

Table 2. Locales and sample size of *Hypomasticus copelandii* and *Hypomasticus steindachneri* from southeastern Brazil.

| Species | Voucher | Locality | GPS coordinates | Sample size |
|----------------------------|----------------------|--|------------------------|-------------|
| | | | | Male/Female |
| Hypomasticus copelandii | MZUFV4500 MZUFV 4504 | Glória River, Paraíba do Sul River Basin | 21°05'21"S, 42°20'30"W | 01/02 |
| | MZUFV4503 MZUFV 4504 | Itabapoana River, Itabapoana River | 20°59'26"S, 41°42'56"W | 02/02 |
| | | Basin | | |
| | MZUFV4502 | Matipó River, Doce River Basin | 20°06'59"S, 42°24'14"W | 04/04 |
| | MZUFV4354 | Mucuri River, Mucuri River Basin | 17°42'21"S, 40°45'42"W | 0/1 |
| Hypomasticus steindachneri | MZUFV3596 MZUFV3607 | Tiririca Lake, Doce River Basin | 19°18'51"S, 42°24'13"W | 4/4 |
| | MZUFV3635 MZUFV4658 | | | |

Digital images were captured in a BX53F Olympus microscope equipped with DP73 and MX10 Olympus camera for classical and molecular techniques respectively, both using the CellSens imaging software. Chromosomes were measured with the Image-Pro Plus software and classified according to their size and arm ratios as metacentric (m) or submetacentric (sm) (Levan et al. 1964). At least five metaphases from each individual were analyzed in order to determine the chromosomal patterns.

Results

Our results showed 2n = 54 in all *H. copelandii* populations, karyotype of 28m + 26sm and NF = 108, no heteromorphic sex chromosomes were detected, and Ag-NOR was located at the terminal region of chromosome pair 4 (Fig. 1).



Figure 1. Giemsa-stained karyotypes of *Hypomasticus copelandii* and *Hypomasticus steindachneri*. Ag-NORs are shown in the boxes. Scale bar: 10 µm.



Figure 2. Cytogenetic FISH patterns on *Hypomasticus copelandii* (**A**, **B**) and *Hypomasticus steindachneri* (**C**, **D**). Left column (CA)₁₅ probe (**A–C**). Right column (GA)₁₅ probe (**B–D**). 18S and 5S rDNA probes are shown in the boxes. Scale bar: 5 μ m.

H. steindachneri showed 2n = 54, karyotype of 30m + 24sm and NF = 108, also without heteromorphic sex chromosomes, and Ag-NOR was located at the terminal region of chromosome pair 8 (boxes in Fig. 1). The 18S rDNA signals were detected at the terminal region of chromosome pair 4 in *H. copelandii* and pair 8 in *H. steindachneri*, whereas the 5S rDNA signals were detected at the interstitial region of chromosome pair 8 in *H. copelandii* and pair 7 in *H. steindachneri* (boxes in Fig. 2).

The microsatellite $(CA)_{15}$ was detected in both arms of all chromosomes in *H. copelandii*, whereas microsatellite $(GA)_{15}$ showed the same pattern with the exception of submetacentric pair 18 that showed signals in the interstitial region of the short arm (Fig. 2). Probes $(CA)_{15}$ and $(GA)_{15}$ exhibited the same general pattern in *H. steindachneri*, terminal markings in both arms of all chromosomes, except for metacentric pair 11, which showed interstitial signals in the short arm with both probes (Fig. 2). These distinctive markings obtained with the microsatellites were consistently observed in both sexes.



Figure 3. Phylogenetic tree of the Anostomidae family adapted from Ramirez et al. (2017) and Birindelli et al. (2020) including all cytogenetic information available regarding presence or absence of sex chromosome systems. AB: Absent; UN: Unknown.

Discussion

The conserved Anostomidae karyotype macrostructure is observed in both *H. copelandii* and *H. steindachneri*, i.e. 2n = 54 and NF = 108, with some differences in the karyotypic formula regarding the number of metacentric and submetacentric chromosomes (Table 1). The absence of heteromorphic sex chromosomes reflects their early divergence in the phylogeny of the family (Ramirez et al. 2016, 2017; Birindelli et al. 2020). This is the first cytogenetic report for the genus *Hypomasticus* indicating that the absence of a sex chromosome system constitutes a plesiomorphic trait within Anostomidae (Fig. 3).

Ramirez et al. (2017) proposed the creation of *Megaleporinus* based on morphological, molecular and cytogenetic data, synonymizing some *Leporinus* and *Hypomasticus* species, and considering the ZZ/ZW sex system as a synapomorphic trait of this new genus. This hypothesis has been corroborated by other studies, which also included *Megaleporinus elongatus* (Valenciennes, 1850) with a Z_1Z_2/W_1W_2 multiple sex chromosome system (Parise-Maltempi et al. 2007, 2013; Marreta et al. 2012; Barros et al. 2018; Crepaldi and Parise-Maltempi 2020). However, not all current *Megaleporinus* species have been karyotyped (Fig. 3), and a ZZ/ZW system has also been observed in *Leporinus multimaculatus* Birindelli, Teixeira et Britski, 2016, which may have arisen independently (Venere et al. 2004; Barros et al. 2018). The inclusion of this species in the phylogenetic analyzes will help to elucidate this question, as well as the cytogenetic characterization of the remaining *Megaleporinus* spp.

Although Ag-NOR number is conserved for most anastomid species with only two markings (Table 1), the chromosome locus characterizes each species, comprising a species-specific character useful as an efficient cytotaxonomic marker (Galetti Jr et al. 1984, 1991; Barros et al. 2017). High correlation between Ag-NOR banding and 18S rDNA FISH technique is also a conserved pattern in the family, with only three exceptions (Table 1). Barros et al. (2017) acknowledged that this discrepancy observed on these three species could be due to technical artifacts and suggested that the expansion of the 18S rDNA sites in Anostomidae should be verified with supplementary analysis. The 18S and 5S rDNA probes were not co-located in neither *H. copelandii* nor *H. steindachneri*, as observed in most species of the family (Table 1), although it remains to be confirmed with double-FISH analysis, as syntenic sites have been observed in other species of the family, such as in *Megaleporinus trifasciatus* (Steindachner, 1876), *Laemolyta taeniata* (Kner, 1858), *Schizodon fasciatus* Spix et Agassiz, 1829 (Barros et al. 2017), and *Leporellus vittatus* (Valenciennes, 1850) (Dulz et al. 2019).

In Anostomidae, 5S rDNA variation is restricted to two or four markings and, interestingly, with intraspecific variation among populations in a few species (Table 1). These intraspecific variations call attention to the importance of populational studies to highlight species genetic diversity, important to delineate conservational strategies (Paiva et al. 2006; Abdul-Muneer 2014). Specially in the cases of migratory species, where the highly fragmented habitats could cause isolation of gene flow (Santos et al. 2013). The identical cytogenetic patterns observed in all *H. copelandii* populations, covering its full distribution range, indicate absence of genetic structure.
Microsatellite $(CA)_{15}$ and $(GA)_{15}$ probes marked the terminal region of both arms in most of the chromosomes in both species, a pattern that is observed in the autosomes of species with sex chromosome systems, whereas the heteromorphic sex chromosomes have specific accumulation patterns of distinct repetitive DNA classes (Parise-Maltempi et al. 2007; Cioffi et al. 2012; Marreta et al. 2012; Poltronieri et al. 2014; Utsunomia et al. 2019; Dulz et al. 2020). The differential interstitial markings, observed in both male and female chromosome complements, can be used as an additional cytotaxonomic marker to distinguish *H. copelandii* from *H. steindachneri* (Fig. 2), and also from species with heteromorphic sex chromosomes (Cioffi et al. 2012; Poltronieri et al. 2012; Poltronieri et al. 2014).

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RESEARCH ARTICLE



Chromosomal mapping of repetitive DNA in Melipona seminigra merrillae Cockerell, 1919 (Hymenoptera, Apidae, Meliponini)

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Abstract

Melipona Illiger, 1806 is represented by 74 known species of stingless bees, distributed throughout the Neotropical region. Cytogenetically it is the most studied stingless bee genus of the tribe Meliponini. Member species are divided in two groups based on the volume of heterochromatin. This study aim was to analyze the composition and organization of chromatin of the stingless bee subspecies *Melipona seminigra merrillae* Cockerell, 1919 using classical and molecular cytogenetic techniques, so contributing to a better understanding of the processes of chromosomal changes within the genus. We confirm that *M. seminigra merrillae* has a chromosome number of 2n = 22 and n = 11, results that differ from those reported for the genus in the absence of B chromosomes. The heterochromatic pattern revealed a karyotype composed of chromosomes with a high heterochromatin content, which makes it difficult to visualize the centromere. Silver nitrate impregnation (Ag-NOR) showed transcriptionally active sites on the second chromosomal pair. Staining of base-specific fluorophores DAPI-CMA₃ indicated a homogeneous

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distribution of intensely DAPI-stained heterochromatin, while CMA₃ markings appeared on those terminal portions of the chromosomes corresponding to euchromatin. Similar to Ag-NOR, fluorescence in situ hybridization (FISH) with 18S ribosomal DNA probe revealed distinct signals on the second pair of chromosomes. Microsatellite mapping (GA)₁₅ showed markings distributed in euchromatic regions, while mapping with (CA)₁₅ showed marking patterns in heterochromatic regions, together with a fully marked chromosome pair. Microsatellite hybridization, both in heterochromatic and euchromatic regions, may be related to the activity of transposable elements. These are capable of forming new microsatellites that can be dispersed and amplified in different regions of the genome, demonstrating that repetitive sequences can evolve rapidly, thus resulting in within-genus diversification.

Keywords

Cytogenetics, fluorescence in situ hybridization (FISH), heterochromatin, stingless bee

Introduction

Bees of the genus *Melipona* Illiger, 1806 are highly social insects, with collective offspring care, division into castes and one or more overlapping generations between adult colony members also within castes there are fully reproductive, poorly reproductive and sterile individuals (Wilson and Hölldobler 2005; Michener 2007). Members of the tribe Meliponini are commonly called stingless bees, and their species are distributed throughout the Neotropical region. The Meliponini tribe comprise 33 genera with approximately 417 known species (Camargo and Pedro 2013). Of these genera, *Melipona* is the most species-rich represented by 76 valid species, of which 43 occur in Brazil (Camargo and Pedro 2013; Pedro 2014).

With 23 species with described karyotypes, Melipona has the largest number of cytogenetically studied members (Tavares et al. 2017). According to karyotypic analyses, most species of the genus have a chromosome number of 2n = 18 (queens/workers) and n = 9 (drones). However, there are some exceptions. The species *Melipona quinquefas*ciata Lepeletier, 1836 and M. rufiventris Lepeletier, 1836 which have B chromosomes (Rocha et al. 2007; Lopes et al. 2008), while M. seminigra merrillae Cockerell, 1919, M. seminigra pernigra Moure & Kerr, 1950 and M. seminigra abunensis Cockerell, 1912 have 2n = 22 and n = 11 chromosomes (Francini et al. 2011; Andrade-Souza et al. 2018; Cunha et al. 2018). Melipona has a unique distribution pattern of constitutive heterochromatin (CH), which differentiates it from other Meliponini (Hoshiba and Imai 1993; Rocha et al. 2003; Cunha et al. 2018). Based on the distribution pattern/quantity of CH, species in the genus can be divided into two groups: Group I - composed of species with a low amount of CH, present only in pericentromeric regions, and Group II - composed of species with a high amount of CH, present along almost the entire length of each chromosome (Rocha and Pompolo 1998; Rocha et al. 2002; Lopes et al. 2011).

The objective of this study was to use a combination of classical cytogenetics and molecular tools to obtain information on the composition and organization of the chromatin of *Melipona seminigra merrillae*, an Amazonian stingless bee.

Material and methods

Larvae of *M. seminigra merrillae* were collected in colonies maintained in the Instituto Nacional de Pesquisas da Amazônia (INPA) Meliponary, Manaus, Amazonas, Brazil. Mitotic chromosomes were obtained using the protocol given by Imai et al. (1988) with modifications: cerebral ganglia were removed from larvae in the post-defecation stage and dissected in 1% sodium citrate solution containing 0.005% colchicine. The cerebral ganglia were then dissected using entomological pins to expose cellular contents. The material containing metaphasic chromosomes were mounted on air-dried slides, which had been previously treated with three sequential fixatives: first (water: ethanol: acetic acid, 4:3:3), second (ethanol: acetic acid, 1:1), third (100% acetic acid). Slides were then stained with 5% Giemsa solution in Sörensen buffer (0.06 M, pH 6.8) for 20 minutes.

To analyze constitutive heterochromatin, slides with chromosome-bearing material were subjected to the C-banding technique, using Sumner's (1972) protocol with increased treatment time. Slides were treated in 0.2 M hydrochloric acid (HCl) solution for 6 minutes, washed in distilled water and incubated for 9 minutes in 5% barium hydroxide solution freshly prepared, filtered and maintained at 60 °C. Barium hydroxide action was halted by immersing slides for 1 minute in 0.2 M HCl solution at room temperature. Slides were then incubated in 2xSSC solution (sodium chloride 0.3M and 0.03M trisodium citrate, pH 7.0) in a water bath at 60 °C for 12 minutes, washed in running water and then stained with 5% Giemsa solution in Sörensen buffer (0.06 M, pH 6.8).

The active Nucleolus Organizer Regions (NORs) were detected with silver nitrate impregnation (Ag-NOR), following the protocol proposed by Howell and Black (1980). Sequential staining with fluorochromes chromomycin A_3 (CMA₃) and 4',6-diamidino-2-phenylindole (DAPI) was carried out following the methodology of Schweizer (1980).

Fluorescence *in situ* hybridization (FISH) was performed following Pinkel's et al. (1986) protocol. Products obtained via PCR (18S ribosomal DNA probe) were labeled by biotin-14-dATP nick translation (Biotin Nick Translation mix; Invitrogen) and digoxigenin11-dUTP nick (Dig-Nick Translation mix; Roche Applied Science) following the manufacturer's instructions. This probe was obtained by PCR amplification using the primers 18SF1 (5'-GTCATATGTTGTCTCAAAGA-3') and 18SF2 (5' – TCT AAT TTT TTC AAA GAT AAC GC – 3') designed for *Melipona quinquefasciata* (Pereira 2006). The PCR reaction was performed in a thermocycler with a final volume of 20 μ L (2 μ L of dye 10X + 1.2 μ L of MgCl₂ 25 mM + 0.2 μ L of dNTPs + 1 μ L of primer 18SF1 + 1 μ L of primer 18SF2 + 1 μ L of DNA template + 13.4 μ L of milli-Q water). The amplification cycle had the following steps: 3 minutes at 94 °C (initial denaturation), 1 minute at 95 °C (denaturation), 1 minute at 55 °C (priming cycle), 2 minutes at 72 °C (extension), 5 minutes at 72 °C (final extension). The microsatellites (GA)₁₅, (CA)₁₅ were labelled directly with Cy3 in the 5' regions (Sigma, St. Louis, MO, USA).

Images of metaphase chromosomes were captured with a Leica DM 2000 epifluorescence photomicroscope, using a 100× immersion objective. Slides stained with fluorochromes (CMA₃ and DAPI) were analyzed using 450–480 nm (CMA₃) and 330–385 nm (DAPI) excitation filters. Adobe Photoshop 7.0 CS4 software was used to assemble karyotype images of mitotic metaphase chromosomes. Each chromosome was virtually cut and paired according to its size, following a decreasing order of size. In this study, 240 larvae were analyzed using 10 metaphases for each individual, and about 40 individuals produced satisfactory results.

Results

After analysis, we found that *M. seminigra merrillae* presented chromosomal numbers 2n = 22 and n = 11 (Fig. 1a, b). C-banding technique revealed a karyotype with a high heterochromatic content for all chromosomes, making it difficult to accurately visualize the position of the centromere. Therefore, the morphological identification was less precise or even impossible (Fig. 1c). Silver nitrate impregnation (Ag-NOR) in



Figure 1. Representative karyotype of *Melipona seminigra merrillae* with Giemsa-stained chromosomes **a** female karyotype with 2n = 22 **b** male karyotype with n = 11 **c** C-banding **d** Ag-NOR-banding of the second chromosome pair. Scale bar: 10 µm.

M. seminigra merrillae showed transcriptionally active ribosomal sites on the second pair of chromosomes (Fig. 1d).

Regarding base-specific fluorophores, DAPI stained almost the entire length of all chromosomes evenly, except for the weakly stained terminal regions (Fig. 2a). In contrast, CMA_3 marked terminal regions (Fig. 2b) 18S ribosomal DNA sequence mapping showed two terminal markers on the second chromosomal pair, with a difference in size between homologues (Fig. 3).

The microsatellite probe $(GA)_{15}$ labeled only euchromatic regions (Fig. 4a), while $(CA)_{15}$ revealed signals spread almost along the entire length of all chromosomes except for terminal regions; however, a particular chromosome pair was fully labeled (Fig. 4b).



Figure 2. Fluorochrome staining of *Melipona seminigra merrillae* chromosomes **a** DAPI, with uniform staining on almost every chromosome **b** CMA_3 , showing euchromatin in terminal regions of all chromosomes. Scale bars: 10 µm.



Figure 3. Distribution pattern of 18S rDNA sites on *Melipona seminigra merrillae* chromosomes. Additionally, size heteromorphism between homologues is also evident in the second pair **a** metaphase plate **b** karyotype with paired chromosomes. Scale bar: $10 \mu m$.



Figure 4. Repetitive DNA mapping on *Melipona seminigra merrillae* chromosomes **a** probe $(GA)_{15}$ hybridized with euchromatic regions and **b** $(CA)_{15}$ hybridized with heterochromatic regions. Arrows indicate fully marked chromosomes. Scale bar: 10 µm.

Discussion

Our findings confirm the observations of Francini et al. (2011) on the chromosome number of *M. seminigra merrillae*. Similar karyotype numbers have been reported for *M. seminigra abunensis* and *M. seminigra pernigra* (Andrade-Souza et al. 2018; Cunha et al. 2018). Such results differ from those already described for the genus, which generally has 2n = 18 and n = 9. Chromosome number variations have been also recorded for *M. rufiventris* and *M. quinquefasciata*; however, changes observed in these species were due to the presence of B chromosomes (Rocha et al. 2002; Capoco 2016; Tavares et al. 2017), which were not recorded in *M. seminigra merrillae*. Chromosome number increase in the studied species is due to centric fission with a subsequent addition of heterochromatin (Cunha et al. 2020).

Francini et al. (2011) classified *M. seminigra merrillae* as belonging to the low heterochromatic content group (group I) during previous cytogenetic studies. However, our analyses showed that this species has heterochromatin distributed almost throughout the entire length of chromosomes, with euchromatic regions restricted to the terminal regions. Consequently, the positions of the centromeres are difficult to determine, which supports the categorization of this species in the high heterochromatic content group (group II) (Rocha and Pompolo 1998; Rocha et al. 2002, 2003; Lopes et al. 2006, 2011; Cunha et al. 2018).

According to Tavares et al. (2010), the amount of heterochromatin in the different groups of *Melipona* is directly related to the size of the genome. Thus, group II species tend to have more genomic DNA than species in group I. The chromosome number of *M. seminigra merrillae* is higher (2n = 22). At present, we do not know whether this species has more DNA compared to those with 2n = 18 and the same heterochromatin distribution pattern. These aspects should be a subject of future investigations.

The use of the Ag-NOR staining method to detect active NORs in *Melipona* normally does not show active sites, however, in *M. seminigra merrillae* it was possible to observe these regions which were clearly seen every time in the second chromosome pair. Similar results were also obtained for two other species, *M. asilvai* Moure, 1971 and *M. marginata* Lepeletier, 1836 (Maffei et al. 2001).

Results of chromosome staining by base-specific fluorophores in *M. seminigra merrillae* were similar to that described in a number of other Meliponini species belonging to group II (Rocha et al. 2003; Miranda et al. 2013; Andrade-Souza et al. 2018; Cunha et al. 2018). Several authors have suggested that CMA_3^+ markings in Meliponini have a strong correlation with NORs (Rocha et al. 2007; Lopes et al. 2011; Lopes et al. 2012). Studies of the genus *Melipona* frequently report a chromosome pair strongly marked by CMA_3 . However, this was not the case in *M. seminigra merrillae*. Although the nucleolus organizing regions are characterized by rich concentration of CG bases, use of CMA_3 demonstrates that, in this species, such regions do not always coincide, a fact also reported for other Meliponini (Duarte et al. 2009; Godoy et al. 2013).

Heteromorphism in size of a particular chromosome pair was found among all metaphases analyzed for homologous chromosomes by mapping 18S rDNA sites. Apparently this is a recurrent characteristic in Meliponini (Rocha et al. 2007; Menezes et al. 2014; Andrade-Souza et al. 2018), and it is due to the repetitive nature of ribosomal DNAs, that results in errors during duplication of the genetic material or uneven crossing-over followed by deletion of a small part of the chromosome (Araújo et al. 2002; Mampumbu 2002).

In general, heterochromatic regions of chromosomes are characterized by large amounts of repetitive DNA (Cioffi et al. 2011). Although *M. seminigra merrillae* chromosomes carry large amounts of heterochromatin, the repetitive DNA probe $(GA)_{15}$ hybridized with euchromatic regions, a pattern also observed in chromosome mapping of other Meliponini: in *M. scutellaris* Latreille, 1811 by Piccoli et al. (2018), and in *M. interrupta* Latreille, 1811 by Travenzoli et al. (2019a). This result may be associated with the presence of a family of satellite DNA or even with transposable elements (TEs) that could be linked to gene regulation. Among the bees, TEs have been reported for the genus *Apis* Linnaeus, 1758 (Lampe et al. 2003) and for some *Melipona* species (Cunha et al. 2020).

The presence of positive microsatellite $(CA)_{15}$ signals scattered along the chromosomes of *M. seminigra merrillae* is similar to the pattern revealed by C-banding. Considering that microsatellites, or simple sequence repeats (SSRs), are notable components of constitutive heterochromatin, such repeats probably play an important role in chromosomal organization, regulation of gene expression, dissemination of heterochromatin and, in some cases, in increasing the size of the genome. These functions of SSRs have been demonstrated both for bees and other organisms (Cioffi et al. 2011; Milani and Cabral-de-Melo 2014; Biscotti et al. 2018; Cunha et al. 2020).

Our results indicate that the chromatin of *M. seminigra merrillae* has specific distribution patterns for each type of repeat, a characteristic that may be associated with the occurrence of chromosomal rearrangements in this species. Distribution of SSRs in heterochromatic and euchromatic regions in *Melipona* can also be explained by their relationship with transposable elements, which may have certain sites predisposed to the formation of new microsatellites. This would, in turn, favor dispersion and amplification of these microsatellites between different genomic regions (Milani and Cabralde-Mello 2014; Travenzoli et al. 2019b).

Conclusions

Considering the chromosome number and heterochromatic content, our results are similar to those already revealed for other subspecies of *Melipona seminigra*. As a result, the nature of the euchromatin, together with distribution of NOR sites and the 18S rDNA, is similar to that in other species of *Melipona* that belong to the group II. This study also highlights the existence of possible chromosomal rearrangements in *M. seminigra merrillae*. *Finally*, use of the above-mentioned microsatellite probes for mapping repetitive DNA can expand our knowledge of this type of SSRs in Amazonian stingless bees in the future.

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