CompCytogen 16(1): 1–17 (2022)
doi: 10.3897/compcytogen.v16.i1.76260
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative cytogenetics on eight Malagasy Mantellinae (Anura, Mantellidae) and a synthesis of the karyological data on the subfamily

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Academic editor: Rafael Noleto | Received 7 October 2021 | Accepted 20 December 2021 | Published 11 February 2022

http://zoobank.org/40F08D44-8889-470C-A4D7-94FF17C3CCEA

Citation: Mezzasalma M, Andreone F, Odierna G, Guarino FM, Crottini A (2022) Comparative cytogenetics on eight Malagasy Mantellinae (Anura, Mantellidae) and a synthesis of the karyological data on the subfamily. Comparative Cytogenetics 16(1): 1–17. https://doi.org/10.3897/compcytogen.v16.i1.76260

Abstract

We performed a molecular and cytogenetic analysis on different Mantellinae species and revised the available chromosomal data on this group to provide an updated assessment of its karyological diversity and evolution. Using a fragment of the mitochondrial 16S rRNA, we performed a molecular taxonomic identification of the samples that were used for cytogenetic analyses. A comparative cytogenetic analysis, with Giemsa's staining, Ag-NOR staining and sequential C-banding + Giemsa + CMA + DAPI was performed on eight species: *Gephyromantis* sp. Ca19, *G. striatus* (Vences, Glaw, Andreone, Jesu et Schimmenti, 2002), *Mantidactylus* (*Chonomantis*) sp. Ca11, *M.* (*Brygoomantis*) *alutus* (Peracca, 1893), *M.* (*Hylobatrachus*) *cowanii* (Boulenger, 1882), *Spinomantis* prope *aglavei* "North" (Methuen et Hewitt, 1913), *S. phantasticus* (Glaw et Vences, 1997) and *S.* sp. Ca3. *Gephyromantis striatus*, *M.* (*Brygoomantis*) *alutus* and *Spinomantis* prope *aglavei* "North" have a karyotype of 2n = 24 chromosomes while the other species show 2n = 26 chromosomes. Among the analysed species we detected differences in the number and position of telocentric elements, location of NOR loci (alternatively on the 6th, 7th or 10th pair) and in the distribution of heterochromatin, which shows species-specific patterns. Merging our data with those previously available, we propose a karyotype of 2n = 26 with all biarmed elements and loci of NORs on the 6th chromosome pair as the ancestral state in the whole family Mantellidae. From this putative ancestral

condition, a reduction of chromosome number through similar tandem fusions (from 2n = 26 to 2n = 24) occurred independently in *Mantidactylus* Boulenger, 1895 (subgenus *Brygoomantis* Dubois, 1992), *Spinomantis* Dubois, 1992 and *Gephyromantis* Methuen, 1920. Similarly, a relocation of NORs, from the putative primitive configuration on the 6th chromosome, occurred independently in *Gephyromantis*, *Blommersia* Dubois, 1992, *Guibemantis* Dubois, 1992, *Mantella* Boulenger, 1882 and *Spinomantis*. Chromosome inversions of primitive biarmed elements likely generated a variable number of telocentric elements in *Mantella nigricans* Guibé, 1978 and a different number of taxa of *Gephyromantis* (subgenera *Duboimantis* Glaw et Vences, 2006 and *Laurentomantis* Dubois, 1980) and *Mantidactylus* (subgenera *Brygoomantis*, *Chonomantis* Glaw et Vences, 1994, *Hylobatrachus* Laurent, 1943 and *Ochthomantis* Glaw et Vences, 1994).

Keywords
Amphibia, chromosome evolution, karyotype, Madagascar, NORs

Introduction

Madagascar is one of the richest biodiversity hotspots and an ideal region to study evolutionary dynamics (Myers et al. 2000; Ganzhorn et al. 2001; Vences et al. 2009). The native amphibians of Madagascar belong to four distinct anuran families: Hyperoliidae, Mantellidae, Microhylidae and Ptychadenidae (Glaw and Vences 2007). Among them, the family Mantellidae includes ca 230 described species (AmphibiaWeb 2021; Frost et al. 2021), representing the most species-rich amphibian group of the island.

Mantellidae are characterized by an extraordinary ecological and morphological diversity (Glaw and Vences 2007; Wollenberg et al. 2011; AmphibiaWeb 2021) and are subdivided into three subfamilies: Laliostominae with an overall low species diversity, including the genera *Laliostoma* Glaw, Vences et Böhme, 1998 (1 species) and *Aglyptodactylus* Boulenger, 1919 (6 species); Boophinae, a species-rich clade of about 80 described species of tree frogs, all belonging to the genus *Boophis* Tschudi, 1838, and Mantellinae, which is by far the most species-rich group including nine genera and more than 140 described species (Glaw and Vences 2007; AmphibiaWeb 2021).

The last three decades have seen the flourishing of the use of molecular techniques, with numerous taxonomic and systematic studies that clarified the relationships among the major groups within this subfamily (Glaw et al. 1998; Vences et al. 1998; Richards et al. 2000; Glaw and Vences 2006; Wollenberg et al. 2011; Kaffenberger et al. 2012). Similarly, these tools have been used in the identification of candidate species (Vieites et al. 2009; Perl et al. 2014) and have later contributed to the formal description of many of them (e.g. Andreone et al. 2003; Crottini et al. 2011a; Cocca et al. 2020; AmphibiaWeb 2021).

However, in contrast to the fast-growing amount of molecular data on Mantellidae, the available chromosomal data remain limited, leaving the karyological diversification of the family mostly unexplored. In particular, available cytogenetic data on the subfamily Mantellinae, obtained using different methods, come from a handful of studies (Morescalchi 1967; Blommers-Schlösser 1978; Pintak et al. 1998; Odierna et

al. 2001; Andreone et al. 2003), together providing the description of the karyotype of ca. 40 species. These studies revealed the occurrence of a conserved chromosome number in most genera (2n = 26), but a marked difference in chromosome morphology, location of NORs and heterochromatin distribution (see Odierna et al. 2001 and Andreone et al. 2003). Differences in chromosome number (2n = 24) were also identified, with five species of the subgenus *Brygoomantis* all sharing this state, thus suggesting that the state of 2n = 24 is a derived feature of the group (Blommers-Schlösser 1978).

Comparative cytogenetics, especially when linked to phylogenetic inference, offers the possibility to identify plesio- and apomorphic states, and recognizes different evolutionary lineages (see e.g. Mezzasalma et al. 2013, 2014, 2017a). However, both the limited taxon sampling and the outdated taxonomy used in most previous works limited the possibility to draw robust comparisons and consistent hypotheses on the evolution of chromosomal diversification in the subfamily.

In this study we performed a comparative cytogenetic analysis on eight mantellid species belonging to the genera *Gephyromantis* Methuen, 1920, *Mantidactylus* Boulenger, 1895 (subgenera *Chonomantis*, *Brygoomantis* and *Hylobatrachus*) and *Spinomantis* Dubois, 1992, using a combination of standard coloration and banding methods. We coupled cytogenetic analyses with the molecular taxonomic identification of the samples and synthesized previously available information on this subfamily to produce an overview of their chromosomal diversity. This, enable us to propose a hypothesis on the chromosome diversification in mantelline frogs.

Material and methods

Sampling

We studied 13 samples of eight mantelline species belonging to the genera *Gephyromantis*, *Mantidactylus* (subgenera *Chonomantis*, *Brygoomantis* and *Hylobatrachus*) and *Spinomantis*. These samples were collected between 1999 and 2004 and conserved as cell suspensions at the University of Naples Federico II.

The list of samples used in this study is provided in Table 1. To provide an overview of the chromosomal data on Malagasy mantelline frogs, we reviewed previously published karyotypes of the subfamily. A complete list of all the considered taxa and karyotypes is provided in Table 2.

Molecular taxonomic identification

DNA was extracted from cell suspensions following Sambrook (1989). A 3' fragment of ca. 550 bp of the mitochondrial 16S rRNA gene was amplified using the primer pair 16Sa (CGCCTGTTTATCAAAAACAT) and 16Sb (CCGGTCTGAAACTCAGATCAGT) (Palumbi et al. 1991). This marker proved to be suitable for amphibian identification (Vences et al. 2005) and has been widely used for Malagasy amphib-

Table 1. Specimens analysed in this study. MRSN = Museo Regionale di Storia Naturale (Turin, Italy); ZMA = Zoological Museum Amsterdam (Amsterdam, Netherlands); FN and FAZC, field numbers of Franco Andreone; GA field numbers of Gennaro Aprea; FG/MV, field numbers of Frank Glaw and Miguel Vences.

Species	Field Number	Sex	Locality
Gephyromantis striatus	MRSN A1988 (FN 7645)	female	Ambatoledama Corridor: Beanjada
Gephyromantis sp. Ca19	MRSN A2109 (FN 7630)	male	Ambatoledama Corridor: Beanjada
Gephyromantis sp. Ca19	MRSN A2075 (FN 7903)	male	Ambatoledama Corridor: Andasin'i Governera
Gephyromantis sp. Ca19	MRSN A2112 (FN 7890)	male	Ambatoledama Corridor: Andasin'i Governera
Gephyromantis sp. Ca19	MRSN A2108 (FN 7566)	female	Ambatoledama Corridor: Beanjada
Mantidactylus (*Brygoomantis*) *alutus* (Peracca, 1893)	MRSN A3639 (FN 7945)	female	Ankaratra: Manjakatompo
Mantidactylus (*Chonomantis*) sp. Ca11	MRSN A3708 (FN 7545)	male	Ambatoledama Corridor: Beanjada
Mantidactylus (*Hylobatrachus*) *cowanii* (Boulenger, 1882)	MRSN A2612 (FAZC 11370)	female	Antoetra: Soamazaka
Mantidactylus (*Hylobatrachus*) *cowanii*	GA 720	male	Mandraka
Spinomantis prope *aglavei* "North" (Methuen et Hewitt, 1913)	MRSN A3563 (FN 7543)	male	Ambatoledama Corridor: Beanjada
Spinomantis phantasticus (Glaw et Vences, 1997)	ZMA 19627 (FG/MV 2002-970)	male	Vohidrazana
Spinomantis sp. Ca3	MRSN A3998 (FN 7567)	male	Ambatoledama Corridor: Beanjada
Spinomantis sp. Ca3	MRSN A3999 (FN 7629)	male	Ambatoledama Corridor: Beanjada

ians (e.g. Vieites et al. 2009; Rosa et al. 2012; Crottini et al. 2011b, 2014; Penny et al. 2017). Amplification conditions were: initial denaturation at 94 °C for 5 min, 36 cycles at 94 °C for 30 s, 50 °C for 45s and 72 °C for 45 s, followed by a final step at 72 °C for 7 min. Amplicons were sequenced on an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator 3.1 (ABI). Chromatograms were checked and edited using Chromas Lite 2.6.6 and BioEdit 7.2.6.1 (Hall 1999). All newly determined sequences were deposited in GenBank (accession numbers: OL830846–OL830858). For taxonomic attribution we compared newly generated sequences with a curated database of reference sequences of the 3' terminus of the 16S gene for all lineages of Malagasy mantellid frogs (Cocca 2020). Taxonomic attribution was performed using a local BLAST analysis against this reference database.

Chromosomal analysis

Cell suspensions were obtained from tissue samples as described in Mezzasalma et al. (2013). In brief, tissues were incubated for 30 min in hypotonic solution (KCl 0.075 M + sodium citrate 0.5%, 1:1) and fixed for 15 min in methanol-acetic acid, 3:1. Fixed tissues were stored at 4 °C and dissociated manually on a steel sieve. Chromosomes were obtained using the air-drying method and stained with conventional col-

Table 2. Available karyological data on mantelline frogs. M = metacentric pairs; sm = submetacentric pairs; st = subtelocentric pairs; t = telocentric pairs; AN = arm number; [#] = NOR bearing chromosome pair; CB = C-banding; F = Fluorochrome; R = references; (1) = Morescalchi (1967); (2) = Blommers-Schlösser (1978); (3) = Pintak et al. (1998); (4) = Odierna et al. (2001); (5) = Andreone et al. (2003); (6) = this study. Nomenclature follows Vieites et al. (2009), updated in Perl et al. (2014).

Genus/subgenus	Species	Karyotype	Banding	R
Mantella Boulenger, 1882	*aurantiaca* Mocquard, 1900	2n = 26 10m 3sm; AN = 52		(1)
	aurantiaca	2n = 26 10m 3sm; AN = 52		(2)
	haraldmeieri Busse, 1981	2n = 26 9m 4sm; AN = 52		(2)
	ebenaui (Boettger, 1880)	2n = 26 11m 2sm; AN = 52		(2)
	aurantiaca	2n = 26 11m 2sm; AN = 52	CB	(3)
	crocea Pintak et Böhme, 1990	2n = 26 11m 2sm; AN = 52	CB	(3)
	baroni Boulenger, 1888	2n = 26 11m 2sm; AN = 52	CB	(3)
	haraldmeieri	2n = 26 11m 2sm; AN = 52	CB	(3)
	ebenaui	2n = 26 11m 2sm; AN = 52	CB	(3)
	viridis Pintak et Böhme, 1988	2n = 26 11m 2sm; AN = 52	CB	(3)
	laevigata Methuen et Hewitt, 1913	2n = 26 11m 2sm; AN = 52	CB	(3)
	baroni	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	ebenaui	2n = 26 11m 1sm; AN = 52	Ag-NOR [2], CB	(4)
	betsileo (Grandidier, 1872)	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	cowanii	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	expectata Busse et Böhme, 1992	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	laevigata	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	madagascariensis (Grandidier, 1872)	2n = 26 11m 1sm; 1st AN = 52	Ag-NOR [2], CB	(4)
	nigricans Guibé, 1978	2n = 26 10m 2sm; 1t AN = 48	Ag-NOR [2], CB	(4)
	pulchra Parker, 1925	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	viridis	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
	aurantiaca	2n = 26 11m 2sm; AN = 52	Ag-NOR [2], CB	(4)
Blommersia Dubois, 1992	*blommersae* (Guibé 1975)	2n = 26 12m 1sm; AN = 52		(2)
	galani Vences, Köhler, Pabijan, et Glaw 2010	2n = 26 12m 1sm; AN = 52		(2)
	grandisonae (Guibé, 1974)	2n = 26 10m 3sm; AN = 52	Ag-NOR [1], CB, F	(5)
Gephyromantis				
Asperomantis	*asper* (Boulenger, 1882)	2n = 26 6m 3sm 4t; AN = 44		(2)
Duboimantis	*granulatus* (Boettger, 1881)	2n = 26 8m 4sm 1t; AN050	Ag-NOR [8], CB, F	(5)
Duboimantis	*leucomaculatus* (Guibé, 1975)	2n = 26 6m 6sm 1t; AN = 50	Ag-NOR [6], CB, F	(5)
Duboimantis	*luteus* (Methuen et Hewitt, 1913)	2n = 26 6m 4sm 1st 2t; AN = 48		(2)
Duboimantis	prope *luteus* Methuen et Hewitt, 1913	2n = 26 6m 2sm 1st 4t; AN = 42	Ag-NOR [11], CB, F	(5)
Duboimantis	prope *moseri* "Masoala" Glaw et Vences, 2002	2n = 26 6m 6sm 1t; AN = 52		(5)
Duboimantis	sp. Ca19	2n = 26 8m 5sm; AN = 52	Ag-NOR [6], CB, F	(6)
Duboimantis	*redimitus* (Boulenger, 1889)	2n = 26 7m 5sm 1t; AN = 50	Ag-NOR [6], CB, F	(5)
Duboimantis	*salegy* (Andreone, Aprea, Vences et Odierna, 2003)	2n = 26 5m 7sm 1st; AN = 52	Ag-NOR [6], CB, F	(5)
Duboimantis	*zavona* (Vences, Andreone, Glaw et Randrianirina, 2003)	2n = 26 9m 4sm; AN = 52	Ag-NOR [6], CB, F	(5)
Laurentomantis	*striatus*	2n = 24 6m 1sm 5t; AN = 38	Ag-NOR [10], CB, F	(6)
Phylacomantis	*pseudoasper* (Guibé, 1974)	2n = 26 7m 7sm; AN = 52	Ag-NOR [9], CB, F	(5)
Guibemantis Dubois, 1992				
Guibemantis	*depressiceps* (Boulenger, 1882)	2n = 26 10m 3sm; AN = 52		(2)
Guibemantis	*timidus* (Vences et Glaw, 2005)	2n = 26 11m 2sm; AN = 52		(2)
Pandanusicola	*methueni* (Angel, 1929)	2n = 26 11m 2sm; AN = 52		(2)
Pandanusicola	*bicalcaratus* (Boettger, 1913)	2n = 26 11m 2sm; AN = 52	Ag-NOR [1], CB, F	(4)
Pandanusicola	prope *bicalcaratus* (Boettger, 1913)	2n = 26 9m 4sm; AN = 52		(2)
Pandanusicola	*liber* (Peracca, 1893)	2n = 26 11m 2sm; AN = 52		(2)

Genus/subgenus	Species	Karyotype	Banding	R
Pandanusicola	*pulcher* (Boulenger, 1882)	2n = 26 9m 4sm; AN = 52		(2)
Pandanusicola	prope *punctatus* (Blommers-Schlösser, 1979)	2n = 26 10m 3sm; AN = 52	Ag-NOR [1], CB, F	(4)
Pandanusicola	*punctatus* (Blommers-Schlösser, 1979)	2n = 26 9m 4sm; AN = 52		(2)
Mantidactylus				
Brygoomantis	*alutus*	2n = 24 12m; AN = 48	Ag-NOR [6], CB, F	(6)
Brygoomantis	*ambohimitombi* Boulenger 1918	2n = 24 9m 3sm; AN = 48		(2)
Brygoomantis	*betsileanus* (Boulenger, 1882)	2n = 24 5m 6sm 1t; AN = 46		(2)
Brygoomantis	prope *biporus* (Boulenger, 1889	2n = 24 8m 4sm; AN = 48		(2)
Brygoomantis	sp. Ca19	2n = 24 7m 5sm; AN = 48		(2)
Brygoomantis	prope *ulcerosus* (Boettger, 1880)	2n = 24 8m 2sm 1st 1t; AN = 46		(2)
Chonomantis	prope *aerumnalis* (Peracca, 1893)	2n = 26 10m 2sm 1t; AN = 50		(2)
Chonomantis	sp. Ca11	2n = 26 10m 2sm 2t; AN = 50		(6)
Chonomantis	*paidroa* Bora, Ramilijaona, Raminosoa et Vences, 2011	2n = 26 6m 7sm; AN = 52		(2)
Hylobatrachus	*cowanii* (Boulenger, 1882)	2n = 26 12m 1t; AN = 50	Ag-NOR [6], CB, F	(6)
Hylobatrachus	*lugubris* (Duméril, 1853)	2n = 26 9m 3sm 1t; AN = 50		(2)
Mantidactylus	*guttulatus* (Boulenger, 1881)	2n = 26 11m 2sm; AN = 52		(2)
Ochthomantis	prope *femoralis* (Boulenger, 1882)	2n = 26 9m 3sm 1t; AN = 50		(2)
Spinomantis				(2)
	aglavei (Methuen et Hewitt, 1913)	2n = 24 9m 3sm; AN = 48		(2)
	prope *aglavei* "North"	2n = 24 10m 2sm; AN = 48	Ag-NOR [7], CB, F	(6)
	peraccae (Boulenger, 1896)	2n = 26 7m 6sm; AN = 48		(2)
	phantasticus	2n = 26 13m; AN = 52		(6)
	sp. Ca3	2n = 26 12m 1sm: AN = 52	Ag-NOR [6], CB, F	(6)

orations (5% Giemsa solution at pH 7), Ag-NOR staining (Howell and Black 1980), C-banding according to Sumner (1972) and sequential C-banding + Fluorochromes (CMA+DAPI) following Mezzasalma et al. (2015). Ag-NOR and C-banding staining were not performed on *M*. sp. Ca11 and *S. phantasticus*, because quantity and quality of metaphase plates were not adequate for additional staining methods. Karyotype reconstruction was performed using at least five plates per sample.

Results

Molecular taxonomic identification

The selected 16S fragment was successfully amplified and sequenced from all analysed samples. All newly generated sequences showed identity scores > 97% with homologous sequences available in the mantellid frogs database generated in Cocca (2020). We followed the nomenclature used in Vieites et al. (2009), updated in Perl et al. (2014) (see Table 1).

Cytogenetic analysis

The studied specimen of *Gephyromantis striatus*, *Mantidactylus* (*Brygoomantis*) *alutus* and *Spinomantis* prope *aglavei* "North" have a karyotype of 2n = 24 chromosomes, with the first six pairs distinctively larger than the other six pairs (Fig. 1; Table 3). In

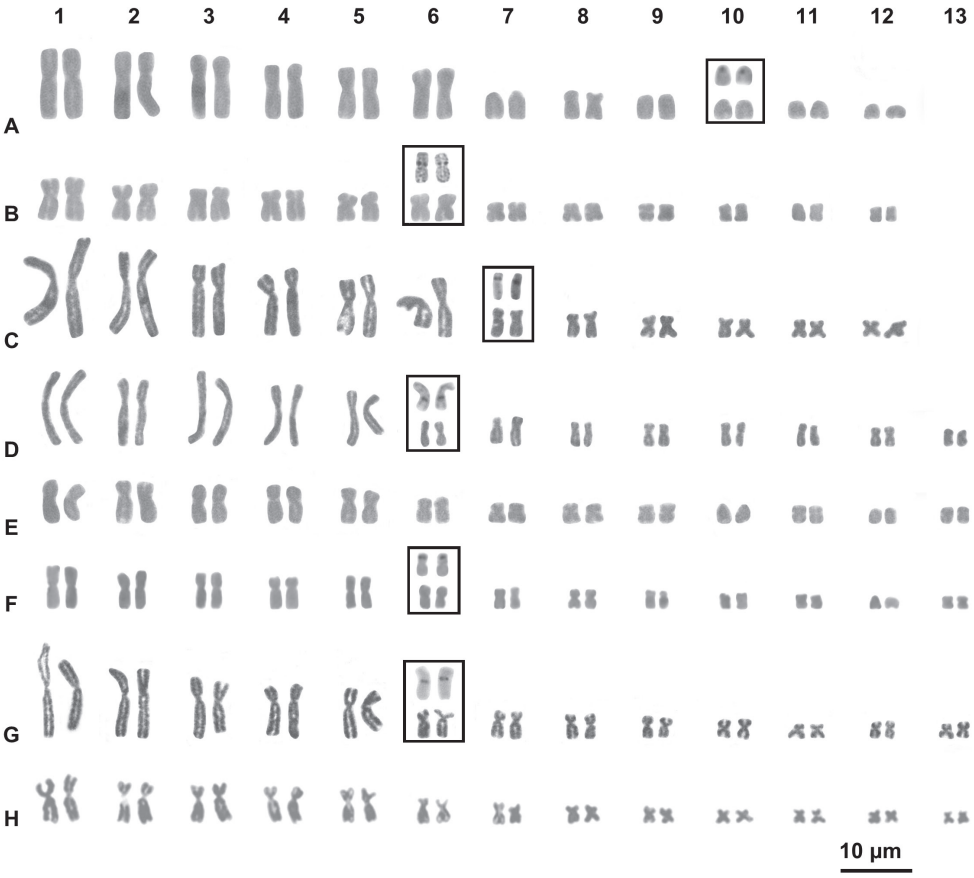

Figure 1. Giemsa stained karyotypes of **A** *Gephyromantis striatus* (FN 7645) **B** *Mantidactylus* (*Brygoomantis*) *alutus* (FN 7945) **C** *Spimomantis* prope *aglavei* "North" (FN 7543) **D** *Gephyromantis* sp. Ca19 (FN 7630) **E** *Mantidactylus* (*Chonomantis*) sp. Ca11 (FN 7545) **F** *Mantidactylus* (*Hylobatrachus*) *cowanii* (FAZC 11370) **G** *Spinomantis* sp. Ca3 (FN 7567) and **H** *Spinomantis phantasticus* (FG/MV 2002-970). Insets represent NOR-bearing pairs stained with Giemsa (down in the insets) and Ag-NOR method (up in the insets).

G. striatus, the pairs 1–6, 8 are biarmed while the other pairs are telocentric, with the pair 10 bearing the NOR loci (Fig. 1A; Table 3). In *M.* (*Brygoomantis*) *alutus* and *S.* prope *aglavei* "North" all pairs are biarmed and NOR loci were detected on the 6th and 7th pair (Fig. 1B, C), respectively.

The samples of the other five species (*G.* sp. Ca19, *M.* (*Chonomantis*) sp. Ca11, *M.* (*Hylobatrachus*) *cowanii*, *S. phantasticus* and *S.* sp. Ca3) presented a karyotype of 2n = 26 chromosomes, with the first five pairs distinctively larger than the remaining eight pairs (Fig. 1D–H). In these species, all chromosome pairs resulted biarmed, with the exception of *M. cowanii* and of *M.* (*Chonomantis*) sp. Ca11, whose karyotype showed one (pair 12) and two pairs (10 and 12) composed of telocentric elements, respectively (Fig. 1E, F). The sixth pair is the NOR bearing one in *G.* sp. Ca19, *M. cowanii* and *S.* sp. Ca3 (Fig. 1D, F, G).

Table 3. Chromosome morphometric parameters of the study species. LR%= % Relative Length (length of a chromosome/total chromosome length*100); CI = centromeric index (ratio between short arm/chromosome length*100). Sh = chromosome shape (m = metacentric; sm = submetacentric; t = telocentric).

Sp.	*G. striatus*	*M. alutus*	*S.* prope *aglavei*	*G.* sp. Ca19	*M.* sp Ca11	*M. cowanii*	*S.* sp. Ca11	*S. phantasticus*
Chr.	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)	LR%-CI (sh)
1	16.8–41.6 (m)	15.1–44.0 (m)	16.9–40.7 (m)	15.0–46.3 (m)	12.3–39.3 (m)	18.6–48.8 (m)	16.1–37.8 (m)	16.2–38.5 (m)
2	12.7–36.9 (m)	11.8–48.5 (m)	14.0–32.0 (sm)	13.7–35.6 (sm)	12.0–34.9 (sm)	12.9–42.3 (m)	14.2–42.8 (m)	13.8–30.9 (sm)
3	11.8–36.7 (sm)	11.6–34.1 (sm)	12.1–26.0 (sm)	12.4–40.8 (m)	11.2–43.9 (m)	12.8–37.2 (sm)	12.4–38.2 (m)	11.5–34.8 (sm)
4	10.9–39.0 (m)	10.6–41.1 (m)	11.9–34.3 (sm)	11.3–42.8 (m)	11.1–38.4 (m)	11.3–40.0 (m)	12.1–30.6 (sm)	11.4–38.5 (m)
5	10.2–45.2 (m)	10.2–44.6 (m)	9.7–44.7 (m)	10.6–36.1 (sm)	10.0–41.7 (m)	19.2–44.8 (m)	9.1–36.0 (sm)	10.4–35.1 (sm)
6	9.7–48.7 (m)	10.1–48.2 (m)	9.7–42.6 (m)	6.4–31.1 (sm)	6.2–44.7 (m)	5.3–47.3 (m)	5.5–38.2 (m)	6.2–33.2 (sm)
7	6.0–0 (t)	5.9–49.0 (m)	4.5–33.0 (m)	5.0–40.1 (m)	6.1–46.2 (m)	5.3–49.3 (m)	5.5–38.7 (m)	6.2–42.9 (m)
8	5.6–39.0 (m)	5.9–41.4 (m)	4.1–47.0 (m)	4.8–29.3 (sm)	6.1–41.0 (m)	4.8–49.6 (m)	5.1–39.8 (m)	5.9–44.5 (m)
9	5.4–0 (t)	5.8–45.8 (m)	3.9–47.0 (m)	4.4–48.8 (m)	5.9–43.8 (m)	4.4–34.4 (sm)	4.9–43.9 (m)	4.4–48.8 (m)
10	4.6–0 (t)	4.9–43.0 (m)	3.5–39.3 (m)	4.3–42.9 (m)	5.5–0 (t)	4.3–41.7 (m)	4.2–44.1 (m)	3.8–48.8 (m)
11	3.4–0 (t)	4.1–45.0 (m)	3.3–49.0 (m)	4.3–37.4 (sm)	5.5–47.5 (m)	4.2–40.8 (m)	3.6–41.7 (m)	3.7–44.1 (m)
12	2.9–0 (t)	4.0–46.3 (m)	3.1–47.4 (m)	4.2–37.4 (sm)	4.2–0 (t)	4.0–0 (t)	3.4–38.0 (m)	3.5–49.6 (m)
13				3.6–43.5 (m)	4.1–42.6 (m)	3.8–38.2 (m)	3.2–46.1 (m)	3.0–43.8 (m)

In *G. striatus*, NOR associated heterochromatin was C-banding positive (CMA + and DAPI -) and tiny centromeric C-bands were present on some chromosome pairs (Fig. 2A, A', A"). *Mantidactylus alutus* and *Spimomantis* prope *aglavei* "North" showed centromeric and telomeric C-bands and NOR associated heterochromatin which were positive to CMA and DAPI negative (Fig. 2B, B', B" and C, C', C"). *Mantidactylus* (*Brygoomantis*) *alutus* also presented an additional bright centromeric band on the chromosomes of pair nine. *Gephyromantis* sp. Ca19 showed centromeric and telomeric C- bands, which were CMA and DAPI positive (Fig. 2D, D', D"). *Spinomantis* sp. Ca3 showed solid telomeric C-bands and NOR associated heterochromatin, which resulted CMA positive and DAPI negative (Fig. 2E, E', E"). *Mantidactylus* (*Hylobatrachus*) *cowanii* had centromeric C-bands on all chromosomes, which were CMA and DAPI negative (Fig. 2F, F', F"). No heteromorphic or completely heterochromatic chromosome were found in any of the studied samples.

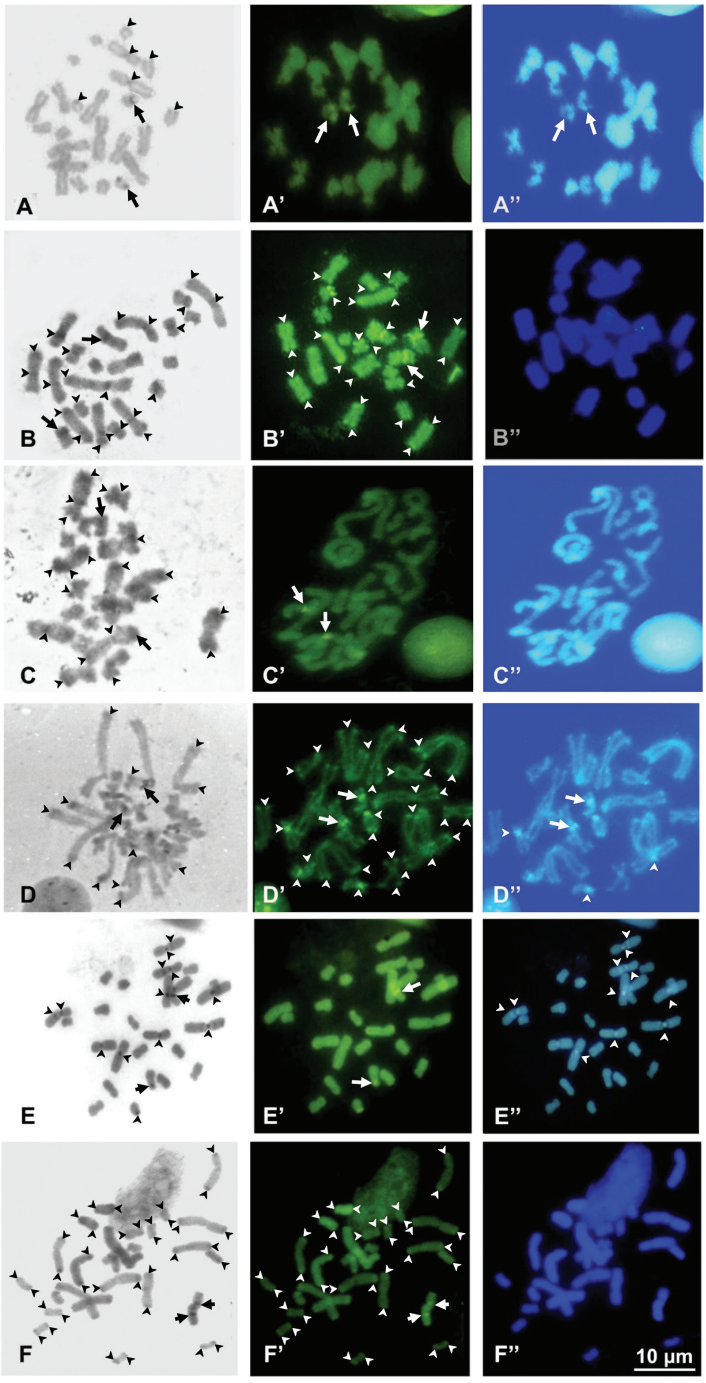

Figure 2. Metaphase plates of *Gephyromantis striatus* (**A, A', A''**), *Mantidactylus* (*Brygoomantis*) *alutus* (**B, B', B''**), *Spinomantis* prope *aglavei* "North" (**C, C', C''**), *Gephyromantis* sp. Ca19 (**D, D', D''**), *Spinomantis* sp. Ca3 (**E, E', E''**) and *Mantidactylus* (*Hylobatrachus*) *cowanii* (**F, F', F''**) stained with C-banding + Giemsa (**A–F**), + CMA (**A'–F'**) + DAPI (**A''–F''**). Arrows point at NORs while arrowheads highlight other heterochromatin blocks.

Discussion

We here provide new karyological data on eight frog species belonging to the subfamily Mantellinae and discuss the available chromosome data on this subfamily to provide a first comprehensive assessment of its karyological diversity.

Available data on representatives of the other two Mantellidae subfamilies (Boophininae and Laliostominae) highlight the occurrence of a conserved karyotype structure in terms of chromosome number and morphology. In particular, the first karyological studies by Blommers-Schlössers (1978) on 12 species of *Boophis* (Boophininae) and on *Aglyptodactylus madagascariensis* (Duméril, 1853) (Laliostominae) revealed a conserved karyotype of 2n = 26 with all biarmed chromosomes.

Following studies by Aprea et al. (1998, 2004) expanded the knowledge on the karyological uniformity to the position of NORs loci, invariably on the sixth chromosome pair both in *Boophis* and *A. madagascariensis*, but evidenced different patterns of heterochromatin composition and distribution. Similar karyological characters were described also in different species of the genus *Mantella* (belonging to the subfamily Mantellinae), all showing a karyotype of 2n = 26 with all biarmed chromosomes (Blommers-Schlössers 1978; Odierna et al. 2001). A karyotype of 2n = 26 with all biarmed elements should thus be considered the primitive condition in the whole family Mantellidae, as it is highly conserved in all subfamilies, genera and most subgenera (see Blommers-Schlösser 1978; Aprea et al. 1998, 2004; Odierna et al. 2001, see Table 2). Nevertheless, species of other genera of the subfamily Mantellinae show a wider karyological variability, both concerning chromosomes number, morphology, localizations of NORs loci and heterochromatin composition and distribution (Blommers-Schlösser 1978; Odierna et al. 2001; present study) (see also Table 2).

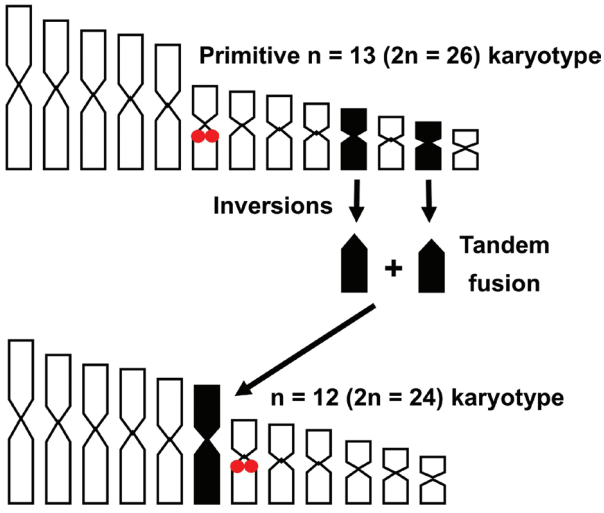

Figure 3. Hypothesized general model of chromosome reduction in Mantellinae from n = 13 (2n = 26) to n = 12 (2n = 24) by means of chromosome fusions. Red dots highlight the NOR bearing chromosome.

Concerning the variability of the chromosome number, a 2n = 26 karyotype is still the most common chromosomal configuration, but karyotypes with a reduced chromosome complement (2n = 24) have been documented in 9 species of three different genera (6 species of *Mantidactylus* (subgenus *Brygoomantis*), 2 *Spinomantis* and *Gephyromantis striatus*) (See Fig. 1 and Table 2). Furthermore, while the 2n = 26 configuration occurs in all three subfamilies of the family Mantellidae (Mantellinae, Boophinae and Laliostominae) (e.g. Aprea et al. 1998, 2004; present study), karyotypes with 2n = 24 seem to occur in just a few phylogenetically lineages (genus *Gephyromantis*, *Mantidactylus* and *Spinomantis*), where the 2n = 26 configuration is also present (Blommers-Schlösser 1978; present study). In turn, the subfamily Boophinae, with all the species showing a 2n = 26 karyotype (Aprea et al. 1998, 2004), has been depicted as a basal group in the Mantellidae radiation (see e.g. Wollenberg et al. 2011). These evidences suggest that a reduction of the chromosome number from 2n = 26 to 2n = 24 occurred repeatedly and independently in different lineages of the subfamily Mantellinae, probably involving chromosome inversions and a fusion (translocation) between two elements of the smallest pairs (6–13), giving rise to an additional large (6th) chromosome pair in several species (e.g. *G. striatus*, *M.* (*Brygoomantis*) *alutus*, and *S.* prope *aglavei* "North") (Fig. 3; Table 3). Interestingly, a similar reduction of the chromosome number driven by tandem fusions (from 2n = 26 to 24) has been documented also in the family Ranidae (Miura et al. 1995).

Other than tandem fusions, chromosome inversions of primitive biarmed elements also had a significant role in the morphological chromosome diversity observed in mantelline frogs. These mechanisms generated a variable number of telocentric elements in different evolutionary lineages (see Figs 1, 3 and Table 2).

Considering the position of the loci of NORs, our results and available literature data (Aprea et al. 1998, 2004; Odierna et al. 2001; Andreone et al. 2003), show that NORs occurrence on the sixth chromosome pair can be considered a primitive state, as it is described for all analysed species belonging to the genus *Boophis*, *A. madascariensis* and most *Gephyromantis*, *Mantidactylus* (subgenus *Brygoomantis*), and *Spinomantis*. On the other hand, a derivate configuration of NOR loci seems to have emerged multiple times in distinct lineages. The different positions of NOR loci in mantelline frogs suggest that these elements were also differently involved in the hypothesized chromosome fusions from 2n = 26 to 2n = 24, providing further support to multiple, independent rearrangements leading to similar karyotype configurations. In fact, while in *M.* (*Brygoomantis*) *alutus* the sixth large chromosome pair likely derived from a fusion involving the primitive NOR bearing pair and another smaller pair, in *G. striatus* and *S.* prope *aglavei* "North" the pair 6 does not include NOR loci, which are found on the 7th and 10th chromosome pair, respectively (see Fig. 1). In other species of *Gephyromantis*, *Blommersia*, *Guibemantis* and *Mantella* the relocation of NORs involved different pairs (1st, 2nd, 8th, 9th, 10th or 11th) (Odierna et al. 2001; Andreone et al. 2003; this study). It should be noted that Ag-NOR staining only evidences active NORs, and the existence of different inactive sites in the karyotypes of the studied species cannot be excluded based only on this analysis. However, we found correspondence in NOR

location using both Ag-NOR and C-banding + CMA (in Figs 1, 2), which also has the power to uncover rDNA clusters (Schmid 1982; Zaleśna et al. 2017).

Various mechanisms may be responsible for NOR relocation, such as cryptic structural rearrangements, minute insertions, reintegration of rDNA genes amplified during ovogonial auxocytosis or the activation of silent sites (Nardi et al. 1977; Schmid 1978; King 1980; Mahony and Robinson 1986; Schmid and Guttenbach 1988; Mezzasalma et al. 2018). These mechanisms may be independent to other rearrangements, despite the resulting change in the configuration of NORs is a significant indicator of lineage divergence at different taxonomic level (e.g. Pardo et al. 2001; Mezzasalma et al. 2015, 2018, 2021).

Sequential C-banding did not evidence the occurrence of any sex-specific, largely heterochromatic chromosomes (generally related to differentiated heterogametic sex chromosomes, a condition not yet documented in the family Mantellidae), B chromosomes, or interchromosomal rearrangements leading to heteromorphic autosome pairs (e.g. Mezzasalma et al. 2014, 2016, 2017b; Sidhom et al. 2020). Nevertheless, C-banding showed a heterogeneous heterochromatin distribution in Mantellidae (see also Aprea et al. 1998, 2004; Odierna et al. 2001; Andreone et al. 2003), highlighting the occurrence of species-specific banding patterns. For example, *G. striatus* and *M.* (*Hylobatrachus*) *cowanii* show different amount and location of C-banding positive heterochromatin in comparison with closely related species with the same chromosome number and similar morphology (e.g. *G.* sp. Ca19 and *M.* (*Brygoomantis*) *alutus*). Interspecific variations in heterochromatin are generally due to different levels of amplification of highly repetitive DNA (Charlesworth et al. 1994). These differences mostly occurred without modifications of the chromosome morphology in Mantellidae (see also Aprea et al. 1998, 2004; Odierna et al. 2001; Andreone et al. 2003), probably by means of symmetrical addition/deletion of heterochromatin. The occurrence of distinctive species-specific banding patterns may be useful in evolutionary cytogenetic and cytotaxonomic studies in the subfamily, but comprehensive comparative analyses would benefit from more banding data on species of different genera and subgenera.

Finally, we also highlight the importance of a preliminary molecular taxonomic identification of mantellid frogs for a consistent karyotype attribution, and that future cytogenetic studies should focus on *Laliostoma* Glaw et al., 1998, *Wakea* Glaw et Vences, 2006, *Boehmantis* Glaw et Vences, 2006 and *Tsingymantis* Glaw et al., 2006, as well as on different undersampled genera and subgenera.

Conclusions

We provide new chromosomal data on eight species belonging to the subfamily Mantellinae, advancing the knowledge on their karyotype diversity, and suggesting that a reduction in the chromosome number and the relocation of NORs loci occurred repeatedly and independently in different genera of this subfamily. We hypothesize a

karyotype of 2n = 26 with all biarmed elements and loci of NORs on the 6th chromosome pair as the ancestral state in the whole family Mantellidae and propose a model for the reduction of the chromosome number from 2n = 26 to 2n = 24 by means of tandem fusions.

Acknowledgements

We are grateful to Malagasy authorities for granting research and export permits. Numerous colleagues helped us in the field: Gennaro Aprea, Frank Glaw, Miguel Vences. Portuguese National Funds through FCT (Foundation for Science and Technology) support the 2020.00823.CEECIND/CP1601/CT0003 contract to AC and the IC-ETA 2020-37 contract to MM. This work was supported by National Funds through FCT under the PTDC/BIA-EVL/31254/2017 project.

References

AmphibiaWeb (2021) University of California, Berkeley, CA, USA. https://amphibiaweb.org [Last Accessed 01 March 2021]

Andreone F, Aprea G, Vences M, Odierna G (2003) A new frog of the genus *Mantidactylus* from the rainforests of north-eastern Madagascar, and its karyological affinities. Amphibia-Reptilia 24(3): 285–303. https://doi.org/10.1163/156853803322440763

Andreone F, Rosa GM, Noël J, Crottini A, Vences M, Raxworthy CJ (2010) Living within dry palm leaves: the discovery of an unknown *Blommersia* (Mantellidae: Anura) reveals a new reproductive strategy in the amphibians of Madagascar. Naturwissenschaften 97(6): 525–543. https://doi.org/10.1007/s00114-010-0667-x

Aprea G, Andreone F, Capriglione T, Odierna G (1998) Chromosome banding in several Malagasy anuran species belonging to the genera *Aglyptodactylus*, *Boophis* and *Mantidactylus*. Abstracts of the 9th Ordinary General Meeting of the Societas Herpetologica Europaea. Chambéry, August 25–29, 1998, Chambéry, 21–21.

Aprea G, Andreone F, Capriglione T, Odierna G, Vences M (2004) Evidence for a remarkable stasis of chromosome evolution in Malagasy treefrogs (*Boophis*, Mantellidae). Italian Journal of Zoology 71(2): 237–244. https://doi.org/10.1080/11250000409356641

Blommers-Schlösser RMA (1978) Cytotaxonomy of the Ranidae, Rhacophoridae, Hyperoliidae (Anura) from Madagascar with a note on the karyotype of two amphibians of the Seychelles. Genetica 48(1): 23–40. https://doi.org/10.1371/journal.pbio.0060118

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371: 215–220. https://doi.org/10.1038/371215a0

Cocca W (2020) Studying the processes of species diversification using the adaptive radiation of the mantellid frogs of Madagascar (Anura: Mantellidae) as a model system. Ph.D. Dissertation, Porto, Portugal, University of Porto. https://repositorio-aberto.up.pt/handle/10216/130002?locale=en

Cocca W, Andreone F, Belluardo F, Rosa GM, Randrianirina JE, Glaw F, Crottini A (2020) Resolving a taxonomic and nomenclatural puzzle in mantellid frogs: synonymization of *Gephyromantis azzurrae* with *G. corvus*, and description of *Gephyromantis kintana* sp. nov. from the Isalo Massif, western Madagascar. ZooKeys 951: 133–157. https://doi.org/10.3897/zookeys.951.51129

Crottini A, Glaw F, Casiraghi M, Jenkins RKB, Mercurio V, Randrianantoandro JC, Randrianirina JE, Andreone F (2011a) A new *Gephyromantis* (*Phylacomantis*) frog species from the pinnacle karst of Bemaraha, western Madagascar. ZooKeys 81: 51–71. https://doi.org/10.3897/zookeys.81.1111

Crottini A, Barbuto M, Casiraghi M, Andreone (2011b) A rapid amphibian survey at Itremo-Ambatofinandrahana, central Madagascar, with confirmed absence of chytrid fungus and recommendations for future monitoring activities. North-Western Journal of Zoology 7(2): 346–351. http://biozoojournals.ro/nwjz/content/v7n2/nwjz.111214.Crottini.pdf

Crottini A, Bollen A, Weldon C, Dalton DL, Kotze A, Noël J, Iambana B, Andreone F (2014) Amphibian survey and current absence of *Batrachochytrium dendrobatidis* (Bd) in Ivoloina Park, Toamasina (eastern Madagascar). African Journal of Herpetology 63(1): 70–78. https://doi.org/10.1080/21564574.2013.833994

Frost DR (2021) Amphibian species of the world: an online reference. Version 6.1. Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. American Museum of Natural History, New York, USA. https://doi.org/10.5531/db.vz.0001

Ganzhorn JU, Lowry II PP, Schatz GE, Somme S (2001) The biodiversity of Madagascar: one of the world's hottest hotspots on its way out. Oryx 35(4): 346–348. https://doi.org/10.1046/j.1365-3008.2001.00201.x

Glaw F, Vences M (2006) Phylogeny and genus-level classification of mantellid frogs (Amphibia, Anura). Organisms, Diversity and Evolution 6(3): 236–253. https://doi.org/10.1016/j.ode.2005.12.001

Glaw F, Vences M (2007) A Field Guide to the Amphibians and Reptiles of Madagascar. 3rd edn. Vences M and Glaw F, Verlags GbR, Köln, 496 pp.

Glaw F, Vences M, Böhme W (1998) Systematic revision of the genus *Aglyptodactylus* Boulenger, 1919 (Amphibia: Ranidae), and analysis of its phylogenetic relationships to other Madagascan ranid genera (*Tomopterna*, *Boophis*, *Mantidactylus*, and *Mantella*). Journal of Zoological Systematics and Evolutionary Research 36(1–2): 17–37. https://doi.org/10.1111/j.1439-0469.1998.tb00775.x

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1 step method. Experientia 36(8): 1014–1015. https://doi.org/10.1007/BF01953855

Kaffenberger N, Wollenberg KC, Köhler J, Glaw F, Vieites DR, Vences M (2012) Molecular phylogeny and biogeography of Malagasy frogs of the genus *Gephyromantis*. Molecular Phylogenetics and Evolution 62(1): 555–560. https://doi.org/10.1016/j.ympev.2011.09.023

King M (1980) C-banding studies on Australian hylid frogs: Secondary constriction structure and the concept of euchromatin transformation. Chromosoma 80(2): 191–217. https://doi.org/10.1007/BF00286300

Mahony MJ, Robinson ES (1986) Nucleolar organiser region (NOR) location in karyotypes of Australian ground frogs (Family Myobatrachidae). Genetica 68(2): 119–127. https://doi.org/10.1007/BF02424409

Mezzasalma M, Andreone F, Aprea G, Glaw F, Odierna G, Guarino FM (2017a) Molecular phylogeny, biogeography and chromosome evolution of Malagasy dwarf geckos of the genus *Lygodactylus* (Squamata, Gekkonidae). Zoologica Scripta 46(1): 42–54. https://doi.org/10.1111/zsc.12188

Mezzasalma M, Andreone F, Aprea G, Glaw F, Odierna G, Guarino FM (2017b) When can chromosomes drive speciation? The peculiar case of the Malagasy tomato frogs (genus *Dyscophus*). Zoologischer Anzeiger 268: 41–46. https://doi.org/10.1016/j.jcz.2017.04.006

Mezzasalma M, Andreone F, Branch WR, Glaw F, Guarino FM, Nagy ZT, Odierna G, Aprea G (2014) Chromosome evolution in pseudoxyrhophiine snakes from Madagascar: a wide range of karyotypic variability. Biological Journal of Linnean Society London 112(3): 450–460. https://doi.org/10.1111/bij.12280

Mezzasalma M, Andreone F, Glaw F, Petraccioli A, Odierna G, Guarino FM (2016) A karyological study of three typhlopid species with some inferences on chromosome evolution in blindsnakes (Scolecophidia). Zoologischer Anzeiger 264: 34–40. https://doi.org/10.1016/j.jcz.2016.07.001

Mezzasalma M, Andreone F, Glaw F, Odierna G, Petraccioli A, Guarino FM (2018) Chromosome aneupolyploidy in an endemic Malagasy gecko (Gekkonidae: *Geckolepis*). Salamandra 54(1): 56–62. http://www.salamandra-journal.com

Mezzasalma M, Andreone F, Glaw F, Guarino FM, Odierna G, Petraccioli A, Picariello O (2019) Changes in heterochromatin content and ancient chromosome fusion in the endemic Malagasy boid snakes *Sanzinia* and *Acrantophis* (Squamata: Serpentes). Salamandra 55(2): 140–144. http://www.salamandra-journal.com

Mezzasalma M, Glaw F, Odierna G, Petraccioli A, Guarino FM (2015) Karyological analyses of *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* provide new insights into the chromosome evolution in the anuran family Pipidae. Zoologischer Anzeiger 258: 47–53. https://doi.org/10.1016/j.jcz.2015.07.001

Mezzasalma M, Guarino FM, Aprea G, Petraccioli A, Crottini A, Odierna G (2013) Karyological evidence for diversification of Italian slow worm populations (Squamata, Anguidae). Comparative Cytogenetics 7(3): 217–227. https://doi.org/10.3897/compcytogen.v7i3.5398

Mezzasalma M, Odierna G, Petraccioli A, Veith M, Guarino FM (2021) Karyological diversification in the genus *Lyciasalamandra* (Urodela: Salamandridae). Animals 11(6): 1709. https://doi.org/10.3390/ani11061709

Miura I, Nishioka M, Borkin LJ, Wu Z (1995) The origin of the brown frogs with 2n = 24 chromosomes. Experientia 51(2): 179–188. https://doi.org/10.1007/BF01929366

Morescalchi A (1967) Le relazioni tra il cariotipo di anuri diplasioceli: 1. I1 corredo cromosomico di alcuni Ranidae. Caryologia 20(1): 65–85. https://doi.org/10.1080/00087114.1967.10796248

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hot-spots for conservation priorities. Nature 403(6772): 853–858. https://doi.org/10.1038/35002501

Nardi I, Barsacchi-Pilone G, Batistoni R, Andronico F (1977) Chromosome location of the ribosomal RNA genes in *Triturus vulgaris meridionalis* (Amphibia, Urodela). II. Intraspecific variability in number and position of the chromosome for 18S and 28S ribosomal RNA. Chromosoma 64(1): 67–84. https://doi.org/10.1007/BF00292889

Odierna G, Vences M, Aprea G, Lötters S, Andreone F (2001) Chromosome data for Malagasy poison frogs (Amphibia: Ranidae: *Mantella*) and their bearing on taxonomy and phylogeny. Zoological Science 18(4): 505–514. https://doi.org/10.2108/zsj.18.505

Palumbi SR, Martin A, Romano S, Mcmillan WO, Stice L, Grabowski G (1991) The Simple Fool's Guide to PCR, Version 2.0. Privately published, University of Hawaii.

Pardo BG, Bouza C, Castro J, Martínez P, Sánchez L (2001) Localization of ribosomal genes in Pleuronectiformes using Ag-, CMA_3-banding and in situ hybridization. Heredity 86(5): 531–536. https://doi.org/10.1046/j.1365-2540.2001.00802.x

Penny SG, Crottini A, Andreone F, Bellati A, Rakotozafy LMS, Holderied MW, Schwitzer C, Rosa GM (2017) Combining old and new evidence to increase the known biodiversity value of the Sahamalaza Peninsula, Northwest Madagascar. Contribution to Zoology 86(4): 273–296. https://doi.org/10.1163/18759866-08604002

Perl RGB, Nagy ZT, Sonet G, Glaw F, Wollenberg KC, Vences M (2014) DNA barcoding Madagascar's amphibian fauna. Amphibia-Reptilia 35(2): 197–206. https://doi.org/10.1163/15685381-00002942

Pintak T, Vences M, Glaw F, Böhme W (1998) Comparative chromosome morphology of Malagasy poison frogs (Amphibia: Ranidae: *Mantella*). Folia Zoologica 47(3): 197–204.

Richards CM, Nussbaum RA, Raxworthy CJ (2000) Phylogenetic relationships within the Madagascan boophids and mantellids as elucidated by mitochondrial ribosomal genes. African Journal of Herpetology 49(1): 23–32. https://doi.org/10.1080/21564574.2000.9650013

Rosa GM, Andreone F, Crottini A, Hauswaldt JS, Noël J, Rabibisoa NH, Randriambahiniarime MO, Rebelo R, Raxworthy CJ (2012) The amphibians of the relict Betampona low-elevation rainforest, eastern Madagascar: an application of the integrative taxonomy approach to biodiversity assessments. Biodiversity and Conservation 21(6): 1531–1559. https://doi.org/10.1007/s10531-012-0262-x

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Lab Press, New York.

Schmid M (1978) Chromosome banding in Amphibia. II. Constitutive heterochromatin and nucleolar organizer regions in Ranidae, Microhylidae and Rhacophoridae. Chromosoma 77: 83–103. https://doi.org/10.1007/BF00287145

Schmid M (1982) Chromosome Banding in Amphibia VII. Analysis of the Structure and Variability of NORs in Anura. Chromosoma 87: 327–344. https://doi.org/10.1007/BF00327634

Schmid M, Guttenbach M (1988) Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97(2): 101–114. https://doi.org/10.1007/BF00327367

Sidhom M, Said K, Chatti N, Guarino FM, Odierna G, Petraccioli A, Picariello O, Mezzasalma M (2020) Karyological characterization of the common chameleon (*Chamaeleo chamaeleon*) provides insights on the evolution and diversification of sex chromosomes in Chamaeleonidae. Zoology 141: 125738. https://doi.org/10.1016/j.zool.2019.125738

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75(1): 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Vences M, Glaw F (2005) A new species of *Mantidactylus* from the East Coast of Madagascar and its molecular phylogenetic relationships within the subgenus *Guibemantis*. Herpetological Journal 15(1): 37–44.

Vences M, Glaw F, Mausfeld P, Böhme W (1998) Comparative osteology of Malagasy poison frogs of the genus *Mantella* (Amphibia: Ranidae: Mantellinae). Bonner zoologische Beiträge 48: 205–215. https://www.biodiversitylibrary.org/page/44945242#page/221/mode/1up

Vences M, Thomas M, van der Meijden A, Chiari Y, Vieites DR (2005) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Frontiers in Zoology 2(1): 5. https://doi.org/10.1186/1742-9994-2-5

Vences M, Wahl-Boos G, Hoegg S, Glaw F, Spinelli Oliveira E, Meyer A, Perry S (2007) Molecular systematics of mantelline frogs from Madagascar and the evolution of their femoral glands. Biological Journal of the Linnean Society 92(3): 529–539. https://doi.org/10.1111/j.1095-8312.2007.00859.x

Vences M, Wollenberg KC, Vieites DR, Lees DC (2009) Madagascar as a model region of species diversification. Trends in Ecology and Evolution 24(8): 456–465. https://doi.org/10.1016/j.tree.2009.03.011

Vieites DR, Wollenberg KC, Andreone F, Kohler J, Glaw F, Vences M (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. Proceedings of the National Academy of Sciences of the United States of America 106(20): 8267–8272. https://doi.org/10.1073/pnas.0810821106

Wollenberg KC, Vieites DR, Glaw F, Vences M (2011) Speciation in little: the role of range and body size in the diversification of Malagasy mantellid frogs. BMC Evolutionary Biology 11(1): 217. https://doi.org/10.1186/1471-2148-11-217

Zaleśna A, Florek M, Rybacki M, Ogielska M (2017) Variability of NOR patterns in European water frogs of different genome composition and ploidy level. Comparative Cytogenetics 11(2): 249–266. https://doi.org/10.3897/CompCytogen.v11i2.10804

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CompCytogen 16(1): 19–38 (2022)
doi: 10.3897/compcytogen.v16.i1.79182
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotype characteristics and gene COI sequences of *Chironomus bonus* Shilova et Dzhvarsheishvili, 1974 (Diptera, Chironomidae) from the South Caucasus (Republic of Georgia, Paravani river)

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Academic editor: Vladimir Gokhman | Received 10 December 2021 | Accepted 2 February 2022 | Published 1 March 2022

http://zoobank.org/981493F9-8804-4910-B040-067A49CBC066

Citation: Karmokov MK (2022) Karyotype characteristics and gene COI sequences of *Chironomus bonus* Shilova et Dzhvarsheishvili, 1974 (Diptera, Chironomidae) from the South Caucasus (Republic of Georgia, Paravani river). Comparative Cytogenetics 16(1): 19–38. https://doi.org/10.3897/compcytogen.v16.i1.79182

Abstract

The study presents data on the karyotype characteristics and the mitochondrial gene COI sequences of the non-biting midge *Chironomus bonus* Shilova et sDzhvarsheishvili, 1974 (Diptera, Chironomidae) from the South Caucasus. The species belongs to the *Ch. plumosus* group of sibling species, one of the most widespread and successful groups in the genus *Chironomus* Meigen, 1803. The karyotype of the studied population is monomorphic. The morphological and chromosomal characteristics of *Ch. bonus* from the Caucasus are similar to those previously described for this species (Kiknadze et al. 1991a). In the phylogenetic tree based on the COI gene sequences, one can observe several clear clusters. We named them Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch.suwai*, and Nearctic *Ch. entis-Ch.plumosus* clusters. The calculated K2P genetic distances within each cluster have not exceeded the 3% threshold for the genus *Chironomus*. Contrary to this, the distances between the clusters exceed this range and correspond to separate species. The *Ch. bonus* sequences belong to the cluster consisting of *Ch. plumosus* (Linnaeus, 1758) sequences from European populations, and do not form a separate clade of the phylogenetic tree. One can suppose that the origin of the *Ch. plumosus* group of sibling species dates back to 5.75–3.43 million years ago (Mya), the epochs of Late Miocene (7,3–5,3 Mya) and early Pliocene (5,3–2,58 Mya). On the other hand, Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch.suwai*, and Nearctic *Ch. entis-Ch. plumosus* clusters appeared relatively recently in the Middle Pleistocene, 1.288–0.307 Mya. The possible relationship between the climate changes in the Pliocene and the origin of the *Ch. plumosus* group are discussed.

Keywords
Chironomidae, *Chironomus bonus*, COI gene, Diptera, mitochondrial DNA, phylogeny, polytene chromosomes, South Caucasus

Introduction

Shilova and Dzhvarsheishvili first described *Chironomus bonus* Shilova et Dzhvarsheishvili, 1974 from Paravani Lake in the Republic of Georgia (Shilova and Dzhvarsheishvili 1974). According to the Fauna Europaea web source (Pape and Beuk 2016), the species is known in Europe from the French mainland, Switzerland, and Bulgaria. The species has also been found in the Republic of Armenia (Sevan Lake) (Kiknadze et al. 2016).

The species *Ch. bonus* belongs to the *Ch. plumosus* group of sibling species, one of the most widespread and successful groups in the genus *Chironomus* Meigen, 1803. According to Shobanov (2000), the group of sibling species is a quasi-taxonomic category that unites species which are similar in morphology and karyotype. Often, there are no clear diagnostic criteria for groups of species, and the association is based on the principle of relative similarity. Shobanov (1989) and Kiknadze et al. (1991) developed the morphological characteristics of the *Ch. plumosus* group. These characteristics include several key features. In general, the larvae are relatively large, ranging from 16 to 30 mm. The larvae of most species belong to the *plumosus*-type, with the so-called sculpturing on the outer (ventral) side of the ventromental plates. Most species in the group prefer lowland rivers with slow current and high sediment silt. In addition, they are widely present in different types of ponds and lakes, of both natural and artificial origin. Several species of the group (at least *Ch. plumosus* (Linnaeus, 1758) and *Ch. borokensis* Kerkis, Filippova, Schobanov, Gunderina et Kiknadze, 1988, see below) can tolerate low oxygen concentrations for an extended period (Shobanov 2001).

According to Kiknadze et al. (2016), the group consists of 14 species: *Ch. agilis* Schobanov et Djomin, 1988; *Chironomus* sp. prope *agilis* (syn. *Ch. agilis* 2) Kiknadze, Siirin et Filippova, 1991; *Ch. balatonicus* Dévai, Wülker et Scholl, 1983; *Ch. bonus* Shilova et Dzhvarsheishvili, 1974; *Ch. borokensis* Kerkis, Filippova, Shobanov, Gunderina et Kiknadze, 1988; *Ch. entis* Shobanov, 1989; *Ch. muratensis* Ryser, Scholl et Wülker, 1983; *Ch. nudiventris* Ryser, Scholl et Wülker, 1983; *Ch. plumosus*; *Ch. sinicus* Kiknadze, Wang, Istomina et Gunderina, 2005; *Chironomus* sp. J Kiknadze, 1991; *Chironomus* sp. K Golygina et Ueno, 2008; *Ch. suwai* Golygina et Martin, 2003; and *Ch. usenicus* Loginova et Belyanina, 1994. The identification of these species can only be done through karyological analysis (reviewed in Kiknadze et al. 1996; Butler et al. 1999). Most of them often occur sympatrically in the same body of water, which can severely complicate the identification process.

The majority of the species in the *Ch. plumosus* group have a Palearctic distribution. Only two of them, *Ch. plumosus* and *Ch. entis*, are also found in the Nearctic, and they can therefore be considered as Holarctic species (Butler et al. 1999; Kiknadze et al. 2000; Golygina and Kiknadze 2001). Adult morphology suggests that Palearctic

Ch. plumosus has a very wide distribution range from Western Europe to the Far East (Linevich and Sokolova 1983). However, karyological analysis has shown that *Ch. borokensis* and *Chironomus* sp. prope *agilis* replace *Ch. plumosus* in Eastern Siberia and the Far East (Kiknadze et al. 1996; Golygina et al. 2003). As indicated before, the karyotype study is the only reliable method for recognizing species in this group.

The karyotype of *Ch. bonus* has been described by Kerkis et al. (1989) and Kiknadze et al. (1991a). A short communication about the *Ch. bonus* karyotype was presented by Belyanina (1983). Some information on the karyotype and external morphology of *Ch. bonus* from Bulgaria was given by Michailova (1994). The biggest DNA databases, GenBank and BOLD, do not contain any DNA data on *Ch. bonus*, including sequences of the COI gene.

The aim of the work is to present the description of the karyotype and gene COI sequences of *Ch. bonus* from the South Caucasus, as well as to compare the karyotype characteristics and DNA data of *Ch. bonus* with the corresponding information available for other species of the *Ch. plumosus* group.

Methods

For both DNA and karyological studies, we used fourth-instar larvae of *Ch. bonus*. We collected larvae from a particular site in the Republic of Georgia (South Caucasus): 18.07.17, 41°19.305'N, 43°45.563'E, Ninotsminda district in the region of Samtskhe-Javakheti, one of the branches of the Paravani river, just 0.6 km north of Saghamo settlement, altitude of ca. 2000 m a.s.l. The maximum depth of the river is about 1 m, and the salinity of the water is about 40 ppm. The collection site is marked on the map with a dark circle (Fig. 1). The geographic division of the Caucasus follows Gvozdetsky (1963).

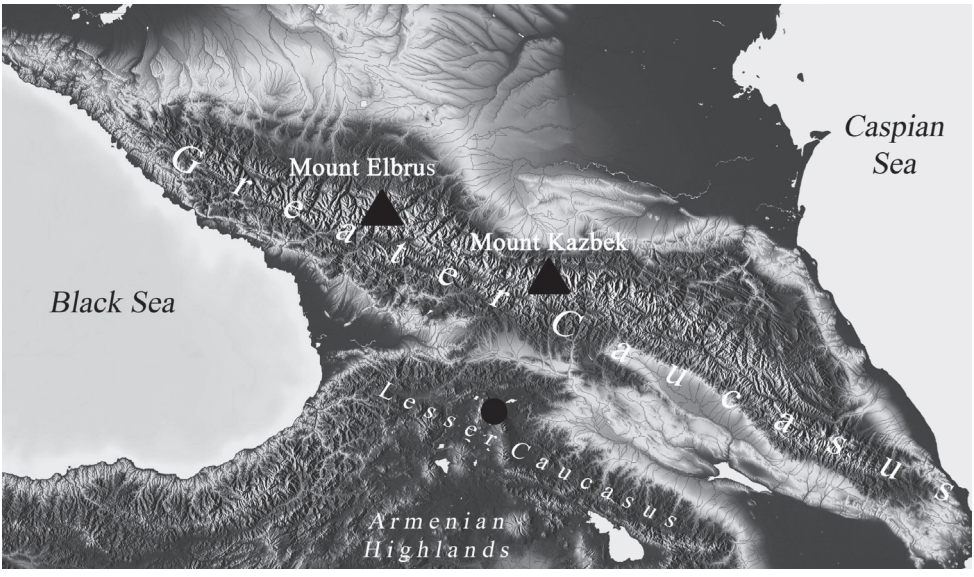

Figure 1. Collection site of *Ch. bonus* in South Caucasus. The collection site is marked with dark circle.

The head capsules and bodies of six larvae were slide-mounted in Faure-Berlese medium. The specimens have been deposited at the Tembotov Institute of Ecology of the Mountain Territories RAS in Nalchik, Russia. We studied the karyotype of all six larvae from the Caucasus region.

For karyological study, we fixed the larvae in an ethanol-glacial acetic acid solution (3:1). The preparations of the chromosomes were made using the ethanol-orcein technique (see Dyomin and Ilyinskaya 1988; Dyomin and Shobanov 1990). The banding sequences were designated as per the accepted convention, specifying the abbreviated name of the species, the symbol of the chromosome arm, and sequence number, as h'bonA1, h'bonB1, etc. (Keyl 1962; Wülker and Klötzli 1973).

We performed the identification of chromosome banding sequences for arms A, E, and F using photomaps by Kiknadze et al. (1991a, 2016) in the system of Keyl (1962) and chromosome mapping for arms C and D as per Kiknadze et al. (1991a, 2016) in the system of Dévai et al. (1989). The chromosome preparations were studied using a Carl Zeiss Axio Imager A2 microscope.

DNA extraction, amplification and sequencing

We used four karyologically studied larvae of *Ch. bonus* for further DNA extraction. DNA was extracted from the larvae and preserved in 96% ethanol using a Diatom DNA Prep 100 kit (Izogen Laboratory Ltd, Moscow, Russia) according to the manufacturer's protocol. DNA extraction was performed on vacuum-dried samples without prior homogenization. Samples were incubated in a lysis buffer at a temperature of 55.5 °C for 16 h. After the extraction, the head capsules were retrieved for dry mounting. The resulting DNA solutions were stored at -18 °C. The amplification of the mitochondrial COI gene was conducted using the MasterMix X5 kit (Dialat Ltd, Moscow).

To amplify the mitochondrial COI gene's barcoding region, primers 911 (5′-TTTCTACAAATCATAAAGATATTGG-3′) and 912 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) were used. PCR was performed in a 25-μL reaction volume. The amplification profile consisted of an initial step of 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 50 s, and finally an 8-min extension step at 72 °C, a final elongation at 72 °C (8 min), and final storage at 4 °C. The resulting PCR products were purified by precipitation in a 0.15 M CH_3COONa solution in 90% ethanol and then rinsed with 70% ethanol. The results were visualized by 1.5% agarose gel electrophoresis with ethidium bromide.

Purified PCR products were sequenced in both directions. DNA sequencing of the COI gene was performed according to Sanger using the BigDye Terminator v3.1 commercial kit (ThermoFisher) and the ABI 3130×l genetic analyzer (ThermoFisher) at Syntol JSC (Moscow, Russia). The GenBank accession numbers of the three sequences obtained in this study are MZ014021, MZ014022, and MZ014023.

Phylogenetic analysis

For the phylogenetic comparison, we used DNA data (sixty-one COI gene sequences) from both the GenBank and BOLD (Ratnasingham and Hebert 2007) databases. Accession numbers of used sequences in GenBank and BOLD: *Chironomus dorsalis* Meigen, 1818 (JN887047.1); *Ch. balatonicus* (JN016826.1); *Ch. muratensis* (AF192194.1); *Chironomus* sp. prope *agilis* (AF192190.1); *Ch. borokensis* (AB740261.1); *Ch. usenicus* (JN016807.1, JN016809.1, JN016808.1); *Ch. entis* (KF278213.1, KF278212.1, KJ085531.1, KJ087284.1, KJ089893.1, GBDPC429-14, MGOCF102-16); *Ch. plumosus* (AB740263.1, AB740262.1, JN016830.1, JN016829.1, CHBAL014-20, CHIFI298-16, CHIFI299-16, LEFIJ3947-16, LEFIJ3948-16, PGBAL006-19, PGBAL007-19, PGBAL009-19, PGCBG089-20, BSCHI661-17, BSCHI063-11, BSCHI115-17, BSCHI219-17, BSCHI284-17, BSCHI350-17, BSCHI517-17, BSCHI644-17, GBDP44143-19, GBDP44180-19, LC050899.1, LC050900.1, JCDB364-15, JCDB363-15, GBDP11685-12, GBDP11686-12, GBDP11687-12, GBDP12282-12, XJDQD1039-18, XJDQD1037-18, XJDQD1038-18, XJDQD1036-18, MN750315.1, GBDPC430-14, SDP408034-15, GBDPC133-14, GBDPC138-14, GBDPC144-14, GBDPC166-14); and *Pagastiella orophila* (Edwards, 1929) (JN265047.1).

We found some COI gene data in both the GenBank and BOLD databases only for seven species of the *Ch. plumosus* group out of 14. We used in our study COI gene sequences from both the aforementioned databases for *Ch. balatonicus*, *Ch. muratensis*, *Chironomus* sp. prope *agilis*, *Ch. borokensis*, *Ch. usenicus*, *Ch. entis*, and *Ch. plumosus*, with available data for species with Holarctic and Nearctic distributions. The most abundant data on the COI gene are available for *Ch. plumosus* (GenBank and BOLD – 66 and 138 sequences, respectively) and *Ch. entis* (GenBank and BOLD – 339 and 13 sequences, respectively). DNA sequences of *Ch. plumosus* obtained from material collected from both Western and Eastern Europe, the Middle East, the Far East, and Northern America were included into the analysis. Concerning *Ch. entis*, available DNA sequences are more uniform and were obtained from material collected almost exclusively from Northern America (Canada). In cases when a large number of sequences were available from the same region, we used no more than 5–6 sequences with different haplotypes to avoid overloading the phylogenetic tree.

We conducted the alignment of COI sequences with MUSCLE with a genetic code of "invertebrate mitochondrial" packaged in MEGA 6 (Tamura et al. 2013). The pairwise sequence distances (Tables 1–4) consisting of the estimated number of base substitutions per site using MEGA 6 and the K2P model (Kimura 1980) were calculated. The analysis involved 61 nucleotide sequences. The codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 579 positions in the final data set.

We conducted the estimation of phylogenetic relationships in BEAST V1.10.4 (Suchard et al. 2018) by the Bayesian Markov-chain Monte-Carlo (MCMC) method, using the HKY+G substitution model as selected in MEGA 6. The determination of

Table 1. Estimates of evolutionary divergence between sequences of Palearctic *Ch. plumosus* cluster. The number of base substitutions per site (%) from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

№	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	JN016807.1 *Ch. usenicus* Russia, Saratov terr.	0																								
2	JN016809.1 *Ch. usenicus* Russia, Saratov terr.	0.364	0																							
3	JN016808.1 *Ch. usenicus* Russia, Saratov terr.	0.182	0.182	0																						
4	MZ014023 *Ch. bonus* Georgia, S. Caucasus	0.547	0.547	0.364	0																					
5	MZ014022 *Ch. bonus* Georgia, S. Caucasus	0.364	0.364	0.182	0.182	0																				
6	MZ014021 *Ch. bonus* Georgia, S. Caucasus	0.547	0.547	0.364	0.364	0.182	0																			
7	JN016830.1 *Ch. plumosus* Russia, Saratov Terr.	0.547	0.547	0.364	0.730	0.547	0.731	0																		
8	JN016829.1 *Ch. plumosus* Russia Saratov Terr.	0.182	0.182	0	0.364	0.182	0.364	0.364	0																	
9	AB740263.1 *Ch. plumosus* Russia	0.547	0.547	0.364	0.730	0.547	0.730	0.730	0.364	0																
10	AB740262.1 *Ch. plumosus* Russia	0.182	0.182	0	0.364	0.182	0.364	0.364	0	0.364	0															
11	CHBAL014-20 *Ch. plumosus* Montenegro	0.364	0.364	0.182	0.547	0.364	0.547	0.182	0.182	0.547	0.182	0														
12	PGBAL006-19 *Ch. plumosus* Montenegro	0.364	0.364	0.182	0.547	0.364	0.547	0.182	0.182	0.547	0.182	0	0													
13	PGBAL007-19 *Ch. plumosus* Montenegro	0.547	0.547	0.364	0.730	0.547	0.731	0.364	0.364	0.730	0.364	0.182	0.182	0												
14	PGBAL009-19 *Ch. plumosus* Montenegro	0.547	0.547	0.364	0.730	0.547	0.731	0.364	0.364	0.730	0.364	0.182	0.182	0	0											
15	PGCBG089-20 *Ch. plumosus* Montenegro	0.364	0.364	0.182	0.547	0.364	0.547	0.182	0.182	0.547	0.182	0	0	0.182	0.182	0										
16	BSCHI661-17 *Ch. plumosus* Poland	0.547	0.547	0.364	0.730	0.547	0.731	0.731	0.364	0.730	0.364	0.547	0.547	0.731	0.731	0.547	0									
17	BSCHI063-11 *Ch. plumosus* Sweden	0.731	0.731	0.547	0.914	0.731	0.916	0.916	0.547	0.914	0.547	0.731	0.731	0.916	0.916	0.731	0.182	0								
18	BSCHI115-17 *Ch. plumosus* Sweden	0.364	0.364	0.182	0.547	0.364	0.547	0.547	0.182	0.547	0.182	0.364	0.364	0.547	0.547	0.364	0.547	0.731	0							
19	BSCHI219-17 *Ch. plumosus* Sweden	0.182	0.182	0	0.364	0.182	0.364	0.364	0	0.364	0	0.182	0.182	0.364	0.364	0.182	0.364	0.547	0.182	0						
20	BSCHI284-17 *Ch. plumosus* Sweden	0.182	0.182	0	0.364	0.182	0.364	0.364	0	0.364	0	0.182	0.182	0.364	0.364	0.182	0.364	0.547	0.182	0	0					
21	BSCHI350-17 *Ch. plumosus* Sweden	0.364	0.364	0.182	0.547	0.364	0.547	0.547	0.182	0.547	0.182	0.364	0.364	0.547	0.547	0.364	0.547	0.731	0	0.182	0.182	0				
22	BSCHI517-17 *Ch. plumosus* Sweden	0.547	0.547	0.364	0.730	0.547	0.731	0.731	0.364	0.730	0.364	0.547	0.547	0.731	0.731	0.547	0	0.182	0.547	0.364	0.364	0.547	0			
23	BSCHI644-17 *Ch. plumosus* Sweden	0.182	0.182	0	0.364	0.182	0.364	0.364	0	0.364	0	0.182	0.182	0.364	0.364	0.182	0.364	0.547	0.182	0	0	0.182	0.364	0		
24	GBDP44143-19 *Ch. plumosus* UK	0.547	0.547	0.364	0.730	0.547	0.731	0.731	0.364	0.730	0.364	0.547	0.547	0.731	0.731	0.547	0	0.182	0.547	0.364	0.364	0.547	0	0.364	0	
25	GBDP44180-19 *Ch. plumosus* Iran	2.028	2.406	2.217	2.592	2.406	2.595	2.217	2.217	2.219	2.217	2.028	2.028	2.217	2.217	2.028	2.217	2.406	2.406	2.217	2.217	2.406	2.217	2.217	2.217	0

the appropriate model in MEGA 6 (Tamura et al. 2013) was performed. The strict clock as a clock model and the Yule process as a speciation model were used. We run MCMC for 10.000.000 iterations and 1000 iterations of burn in. Our analysis involved 61 nucleotide sequences, and we eliminated all positions with less than 95% site coverage. There were 579 positions in the final data set. We used the COI sequence of *Pagastiella orophila* (Genbank accession number JN265047.1) as an outgroup.

We also tried to get average estimates of divergence time between different branches and clusters that appear on the obtained phylogenetic tree (Figs 3, 4). The age of the most recent common ancestors (TMRCAs) for DNA clades was estimated in BEAST V1.10.4 (Suchard et al. 2018) by the MCMC method, using the HKY+G substitution model as selected in MEGA 6. We used a strict clock as a clock model and a constant size as a coalescent model, with the same calibration point assumed by Cranston et al. (2012). The time estimate of 36 million years ago (Mya) for the root node of the divergence between

Table 2. Estimates of evolutionary divergence between sequences of Nearctic *Ch. entis-Ch. plumosus* cluster. The number of base substitutions per site (%) from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

№	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13
1	GBDPC429-14 *Ch. entis* US, Michigan	0												
2	KF278213.1 *Ch. entis* Canada, Quebec	0.914	0											
3	KF278212.1 *Ch. entis* Canada, Quebec	1.099	0.182	0										
4	MGOCF102-16 *Ch. entis* US, New York	0.914	0	0.182	0									
5	KJ085531.1 *Ch. entis* Canada, Ontario	0.547	0.730	0.914	0.730	0								
6	KJ087284.1 *Ch. entis* Canada, Ontario	0.547	0.730	0.914	0.730	0	0							
7	KJ089893.1 *Ch. entis* Canada, Ontario	0.547	0.730	0.914	0.730	0	0	0						
8	GBDPC430-14 *Ch. plumosus* US, Michigan	0.730	0.730	0.913	0.730	0.547	0.547	0.547	0					
9	SDP408034-15 *Ch. plumosus* US, Minnisota	0.730	0.730	0.913	0.730	0.547	0.547	0.547	0	0				
10	GBDPC133-14 *Ch. plumosus* Canada	1.285	0.364	0.547	0.364	1.099	1.099	1.099	1.098	1.098	0			
11	GBDPC138-14 *Ch. plumosus* Canada	1.285	0.364	0.547	0.364	1.099	1.099	1.099	1.098	1.098	0	0		
12	GBDPC144-14 *Ch. plumosus* Canada	0.730	0.730	0.913	0.730	0.547	0.547	0.547	0	0	1.098	1.098	0	
13	GBDPC166-14 *Ch. plumosus* Canada	1.099	0.547	0.731	0.547	0.914	0.914	0.914	1.098	1.098	0.916	0.916	1.098	0

Table 3. Estimates of evolutionary divergence between sequences of Far Eastern *Ch. borokensis-Ch. suwai* cluster. The number of base substitutions per site (%) from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

№	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	AB740261.1 *Ch. borokensis* Russia	0													
2	GBDP17582-15 *Ch. plumosus* Japan	1.283	0												
3	GBDP17583-15 *Ch. plumosus* Japan	1.468	0.547	0											
4	JCDB364-15 *Ch. plumosus* Japan	1.469	0.182	0.364	0										
5	JCDB363-15 *Ch. plumosus* Japan	1.469	0.182	0.364	0.000	0									
6	GBDP11685-12 *Ch. plumosus* South Korea	0.364	1.654	1.839	1.841	1.841	0								
7	GBDP11686-12 *Ch. plumosus* South Korea	0.182	1.469	1.654	1.656	1.656	0.182	0							
8	GBDP11687-12 *Ch. plumosus* South Korea	0.182	1.469	1.654	1.656	1.656	0.182	0	0						
9	GBDP12282-12 *Ch. plumosus* South Korea	0	1.283	1.468	1.469	1.469	0.364	0.182	0.182	0					
10	XJDQD1039-18 *Ch. plumosus* China	1.468	0.913	1.467	1.097	1.097	1.839	1.654	1.654	1.468	0				
11	XJDQD1037-18 *Ch. plumosus* China	1.468	0.913	1.467	1.097	1.097	1.839	1.654	1.654	1.468	0	0			
12	XJDQD1038-18 *Ch. plumosus* China	1.468	0.913	1.467	1.097	1.097	1.839	1.654	1.654	1.468	0	0	0		
13	XJDQD1036-18 *Ch. plumosus* China	1.468	0.913	1.467	1.097	1.097	1.839	1.654	1.654	1.468	0	0	0	0	
14	MN750315.1 *Ch. plumosus* China	1.845	2.028	2.592	2.217	2.217	2.219	2.034	2.034	1.845	2.214	2.214	2.214	2.214	0

Pagastiella orophila and all *Chironomus* species was used as a calibration point. We ran MCMC for 10.000.000 iterations and 1000 iterations of burn in. Tracer v1.7.1 was used to examine the BEAST log file and ESSs for each parameter, which were all > 200.

Recent research demonstrates that the range of divergence rates of the COI gene sequence in insects varies from 1.5% to 2.3% per 1 Mya (Jamnongluk et al. 2003; Stevens et al. 2006 etc.). In the study of tenebrionid beetles, Papadopoulou et al. (2010) obtained

Table 4. Estimates of evolutionary divergence between sequences of *Ch. plumosus* from Finland and sequences of *Ch. balatonicus*, *Ch. muratensis* and *Chironomus* sp. prope *agilis*. The number of base substitutions per site (%) from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

№	Sequences	1	2	3	4	5	6	7
1	CHIFI299-16 *Ch. plumosus* Finland, Satakunta	0						
2	CHIFI298-16 *Ch. plumosus* Finland, Satakunta	1.099	0					
3	LEFIJ3947-16 *Ch. plumosus* Finland, Regio aboensis	3.939	3.555	0				
4	LEFIJ3948-16 *Ch. plumosus* Finland, Regio aboensis	3.566	3.573	3.362	0			
5	AF192190.1 *Chironomus* sp. prope *agilis* Russia	6.306	6.315	8.357	6.930	0		
6	JN016826.1 *Ch. balatonicus* Russia, Saratov_terr.	3.372	3.378	3.555	0.547	6.315	0	
7	AF192194.1 *Ch. muratensis* Russia	4.119	4.123	3.156	4.123	8.115	3.929	0

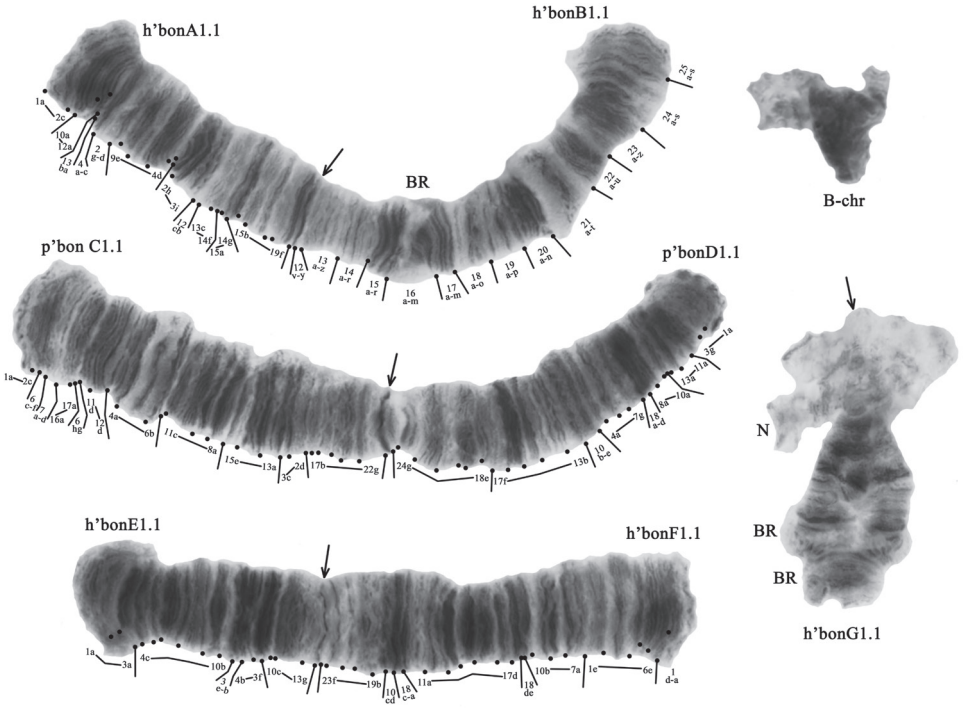

Figure 2. Karyotype of *Ch. bonus* from the South Caucasus; h'bonA1.1, h'bonB1.1 etc. – genotypic combinations of banding sequences; BR – Balbiani rings, N – nucleolus. Arrows indicate centromeric bands.

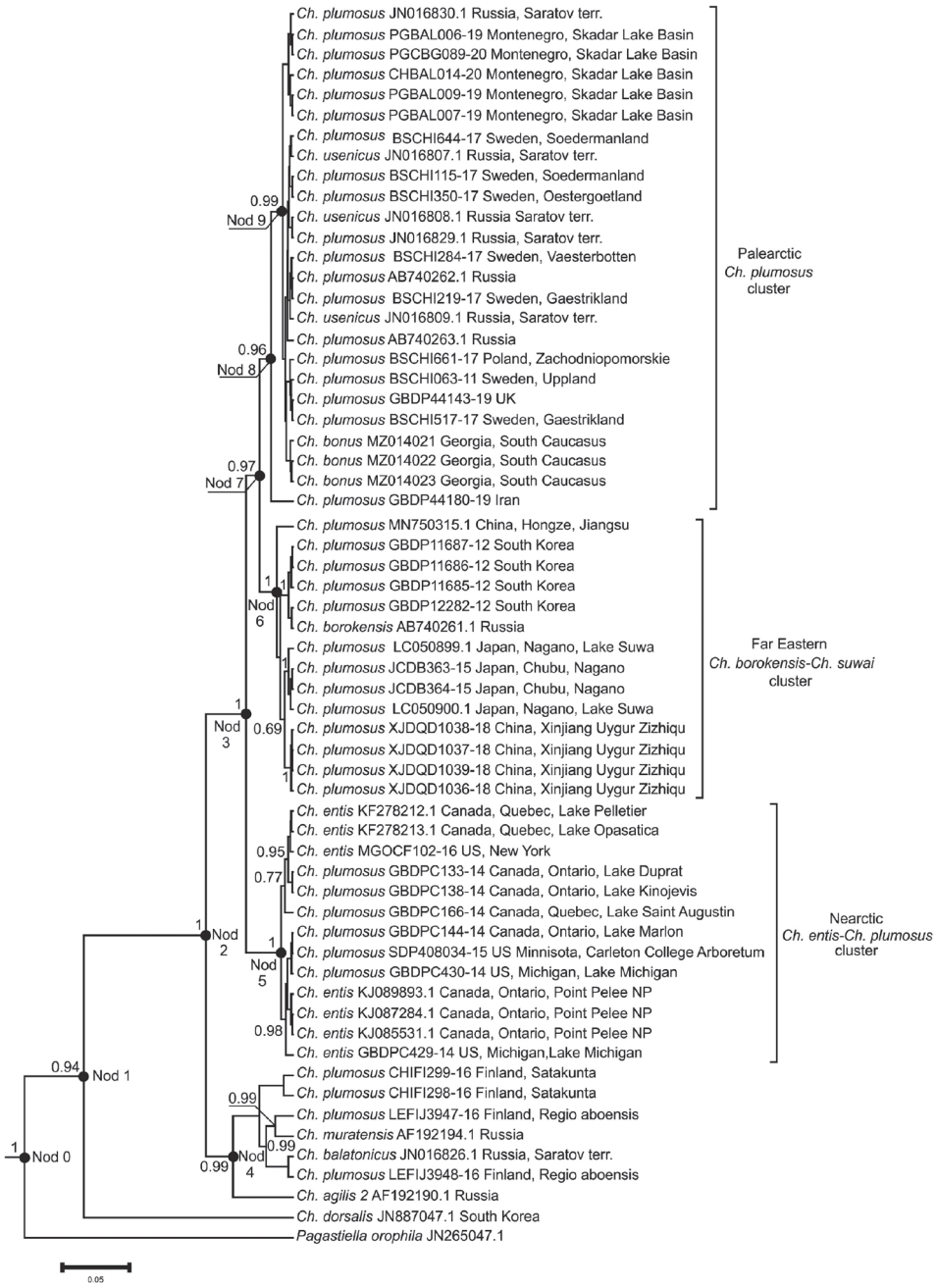

Figure 3. Phylogenetic tree of *Chironomus* species estimated by the Bayesian inference (BA). Support values are given if they exceed 0.5. The numbers at the nodes indicate posterior probabilities; Node 1, Node 2 etc. – nodes of the tree for which TMRCAs were calculated.

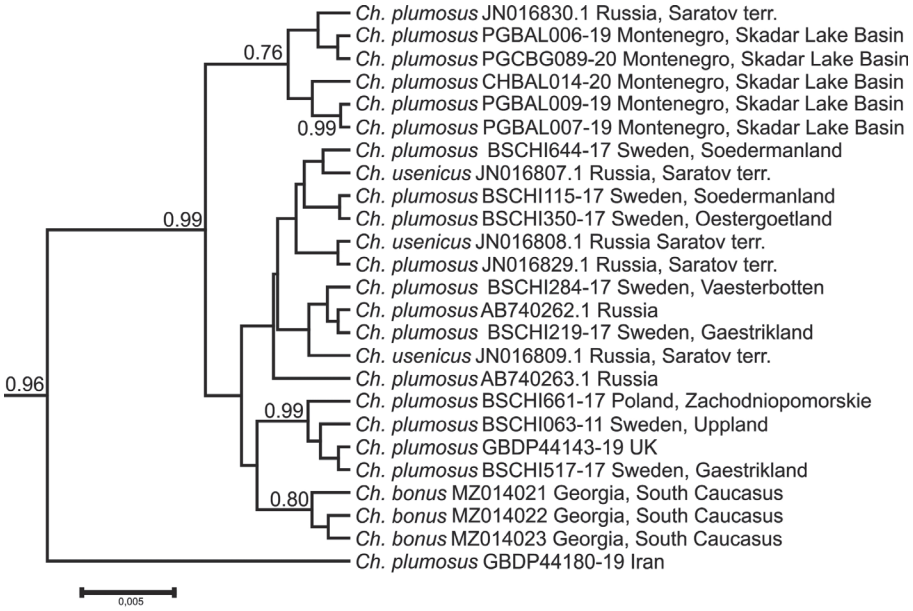

Figure 4. Phylogenetic tree of Palearctic *Ch. plumosus* cluster estimated by the Bayesian inference (BA). Support values are given if they exceed 0.5. The numbers at the nodes indicate posterior probabilities.

Table 5. Substitutions that distinguish *Ch. bonus* sequence from sequences in the Palearctic *Ch. plumosus* cluster; nonsyn. and syn. - nonsynonymous and synonymous substitutions respectively.

№	Substitution type	Position in the sequence	Codon	Position in codon	Ch. bonus sequence
1	nonsyn.	2	1	1st	MZ014021.1
2	syn.	212	71	1st	MZ014021.1
3	syn.	340	113	3rd	MZ014021.1 MZ014022.1 MZ014023.1
4	nonsyn.	609	203	2nd	MZ014023.1
5	nonsyn.	642	214	2nd	MZ014023.1
6	nonsyn.	644	215	1st	MZ014023.1

a divergence rate of 3.54% per 1 Mya for the COI gene (2.69% when combined with the 16S rRNA gene) under the preferred partitioning scheme and substitution model selected using Bayes factors. In our study, we used for calculations of divergence time these three commonly assumed mutation rates: 1.5%, 2.3%, and 3.54%. We calculated TMRCAs for the nodes 1–9 of the phylogenetic tree (Fig. 3). The obtained values are given in Table 5.

Results

Based on morphological and chromosomal characters, we identified the larvae belonging to the genus *Chironomus* at the studied site as *Ch. bonus*. The morphology of *Ch. bonus* larvae from the South Caucasus is similar to that previously described for this species by Kiknadze et al. (1991b).

Karyotype of *Ch. bonus* from the South Caucasus

The diploid number of chromosomes in the *Ch. bonus* karyotype is 2n = 8 plus the B-chromosome. Such a picture for the *C. bonus* karyotype is based on the almost constant presence of an additional B-chromosome in the karyotype of each larva. The chromosome arm combinations are AB, CD, EF, and G (the "thummi" cytocomplex) (Fig. 2). The chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. Arm G homologues are paired in the nucleolus and Balbiani rings (BRs) regions. The centromeric bands are easily identifiable. There is one nucleolus and two BRs on the arm G, and one BR is present on the arm B.

Banding sequences and chromosomal polymorphism of *Ch. bonus* from the South Caucasus

The karyotype of *Ch. bonus* from the South Caucasus is monomorphic. The banding sequences of all the chromosome arms of *Ch. bonus* are identical to those of *Ch. plumosus*. The difference between the karyotypes of both species is the presence of one additional B-chromosome in almost all studied *Ch. bonus* larvae. In total, there are 7 banding sequences in the *Ch. bonus* banding sequences pool (Fig. 2):

h'bonA1 1a-2c 10a-12a 13ba 4a-c 2g-d 9e-4d 2h-3i 12cb 13c-14f 15a-14g 15b-19f C*
h'bonB1 25s-a 24s-a 23z-a 22u-a 21t-a 20n-a 19p-a 18o-a 17m-a 16m-a 15r-a 14r-a 13z-a 12y-v C**
p'bonC1 1a-2c 6c-f 7a-d 16a-17a 6hg 11d-12d 4a-6b 11c-8a 15e-13a 3c-2d 17b-22g C
p'bonD1 1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C
h'bonE1 1a-3e 5a-10b 4h-3f 10c-13g C***
h'bonE1 1a-3a 4c-10b 3e-b 4b-3f 10c-13g C*
h'bonF1 1a-d 6e-1e 7a-10b 18ed 17d-11a 18a-c 10dc 19a-23f C
h'bonG1 not mapped

* revised mapping by Golygina and Kiknadze (2008, 2012)
** mapped according to system of Maximova-Shobanov (Maximova 1976; Shobanov 1994), mapping revised by Golygina and Kiknadze (2008).
*** mapped according to Keyl (1962).

Results of phylogenetic analysis of COI gene sequences of *Ch. bonus* and estimated ages of the most recent common ancestors (TMRCAs) for DNA clades

Overall, we successfully obtained three complete COI gene sequences of *Ch. bonus* from six larvae from the South Caucasus. (MZ014021.1: 627 bp, base composition is 25.99% A, 36.84% T, 16.91% G, and 20.26% C; MZ014022.1: 658 bp, base composition is 26.59% A, 36.17% T, 16.57% G, and 20.67% C; MZ014023.1: 650 bp, base composition is 27.08% A, 35.38% T, 16.77% G, and 20.77% C). Each of the three sequences had a different haplotype. This is the first DNA data obtained for *Ch. bonus*.

The resulting phylogenetic tree (Fig. 3) represents a very complex pattern, with several obvious clusters with rather high probabilities. We conditionally named them the Palearctic *Ch. plumosus* cluster, the Far Eastern *Ch. borokensis-Ch. suwai* cluster, and the Nearctic *Ch. entis-Ch. plumosus* cluster.

The Palearctic *Ch. plumosus* cluster (Figs 3, 4), is formed mostly by *Ch. plumosus* sequences from Western and Eastern Europe (UK, Sweden, Poland, Montenegro, and the European part of Russia). The only available sequences of *Ch. usenicus* from Russia (Saratov Terr.) and, surprisingly, sequences of *Ch. bonus* obtained in this study, are also included in this cluster. It is formed by sequences obtained from material identified through both karyological and morphological analyses (all *Ch. usenicus* and *Ch. bonus* sequences, together with a few *Ch. plumosus* ones, i.e., JN016830.1, JN016829.1, AB740262.1, AB740263.1), and we therefore named it the Palearctic *Ch. plumosus* cluster.

The Far Eastern *Ch. borokensis-Ch. suwai* cluster mostly formed by *Ch. plumosus* sequences from the Far East (China, South Korea, and Japan) and a sequence of *Ch. borokensis* from Russia. We named this branch as the Far Eastern *Ch. borokensis-Ch. suwai* cluster because this particular *Ch. borokensis* sequence (AB740261.1) was obtained from the material identified through karyological analysis (Kondo et al. 2016). According to the BOLD database, *Ch. plumosus* sequences from the Far East were obtained from specimens identified only through morphological analysis. Perhaps the observed picture is an error in species identification, which can happen quite often when chromosomal analysis is not involved, and at least some of these *Ch. plumosus* specimens from the Far East could actually be *Ch. borokensis*. On the other hand, at least two Japanese sequences that we used in our study from Lake Suwa could be *Ch. suwai*. We assume this because Lake Suwa is the type locality for the species. According to Golygina et al. (2003), the karyotype of *Ch. suwai* is closely related to that of *Ch. borokensis* as indicated by many common banding patterns, but it differs by the much smaller size of the centromeric bands. Also, Golygina et al. (2003) suppose that the true *Ch. plumosus* does not occur in Japan.

Almost the same pattern is observed in the Nearctic *Ch. entis-Ch. plumosus* cluster, consisting equally of *Ch. plumosus* and *Ch. entis* sequences. According to the data from Proulx et al. (2013), just two sequences of *Ch. entis* (KF278213.1 and KF278212.1) and three sequences of *Ch. plumosus* (GBDPC133-14/KF278209.1, GBDPC138-14/KF278210.1 and GBDPC144-14/KF278216.1) from this cluster were obtained from material identified through karyological analysis. Except for these sequences, it is most likely an error in species identification as well, and at least some of the *Ch. plumosus* sequences presented in BOLD from Northern America could actually be *Ch. entis* and vice versa. Also, in this cluster, there are no *Ch. plumosus* or *Ch. entis* sequences from the Palearctic region. Given all this data, we named this cluster the Nearctic *Ch. entis-Ch. plumosus* cluster.

In addition to the above-mentioned obvious clusters of the tree (Fig. 3), there is a fourth ambiguous cluster formed by *Ch. balatonicus*, *Ch. muratensis*, *Chironomus* sp. prope *agilis*, and all *Ch. plumosus* sequences from Finland. We know from the BOLD database that these *Ch. plumosus* sequences were obtained from adults identified just through morphological analysis. Perhaps this pattern is an error in species identification, and these Finnish specimens could actually belong to other already known or even previously undescribed species.

Genetic distances

Calculated pairwise sequence distances (Tables 1–4) consisting of the estimated number of base substitutions per site using the K2P model (Kimura 1980) show an interesting pattern. Proulx et al. (2013), who used genetic, morphological, and karyological information to discriminate *Chironomus* species from Canada, showed that intraspecific K2P distances for *Chironomus* species characterized by the COI gene ranged from zero to 3%. These values also could be used as a reference for distinguishing *Chironomus* species in the present work, but data on the COI gene should be complemented with other methods. In our study, the distances between the Palearctic *Ch. plumosus* cluster's sequences are less than 3% and range from 0 to 2.595% (Table 1). If data on the Iranian *Ch. plumosus* are removed, the distances between all other sequences in this cluster are less than 1%, ranging from 0 to 0.914%. Distances between the sequences of *Ch. bonus* obtained in this study are very small, varying from 0.182% to 0.364%. The sequences of *Ch. usenicus* and of several individuals of *Ch. plumosus* from Russia (Saratov Terr.), Sweden, and Montenegro are closest to those of *Ch. bonus* in terms of distances.

Almost the same pattern is observed in the Nearctic *Ch. entis-Ch. plumosus* cluster, where the distances between the sequences are also lower than the 3% range, varying from 0 to 1.285% (Table 2).

In the Far Eastern *Ch. borokensis-Ch. suwai* cluster, the distances between the sequences are also lower than the 3% range, varying from 0 to 2.217% (Table 3). If we disregard *Ch. plumosus* sequence MN750315.1 from China, Hongze, Jiangsu, the distances between all other sequences in this cluster are significantly less, reaching only 1.839%.

At the same time, the average distances between the various clusters exceed the 3% threshold. The distance between Palearctic *Ch. plumosus* and Far Eastern *Ch. borokensis-Ch. suwai* clusters is 3.55%. The distance between Palearctic *Ch. plumosus* and Nearctic *Ch. entis-Ch. plumosus* clusters is 3.75%. Finally, the distance between Far Eastern *Ch. borokensis-Ch. suwai* and Nearctic *Ch. entis-Ch. plumosus* clusters is 5.98%.

In the fourth cluster, which contains *Ch. plumosus* sequences from Finland, the distances between the sequences are generally higher than the 3% range (Table 4). Interestingly, the distances between the sequence of *Chironomus* sp. prope *agilis* and all other sequences are pretty high, varying from 4.123 to 8.357%. On the other hand, analogous distances in the case of *Ch. muratensis* are also fairly high, varying from 4.119 to 8.115%. However, the distances between the sequence of *Ch. balatonicus* and most of the Finnish sequences of *Ch. plumosus* are also high enough, varying from 3.372 to 3.555%. At the same time, the distance between the sequence of *Ch. balatonicus* and one Finnish sequence of *Ch. plumosus* from Regio aboensis (LEFIJ3948-16) is just 0.547%, which is much lower than the 3% range, and we therefore can assume that this *Ch. plumosus* sequence could actually belong to *Ch. balatonicus*. Moreover, the distances between the three other *Ch. plumosus* sequences from Finland, i.e., that from Regio aboensis (LEFIJ3947-16) and two from Satakunta (CHIFI299-16 and CHIFI298-16), are relatively high, varying from 3.555 to 3.939%. At the same time, the distance between the two latter sequences is just 1.099%, which is lower than the 3% threshold. Considering the tree topology (Fig. 3) and genetic distances between the sequences, we can suggest that a particular sequence

from Regio aboensis, on the one hand, and another two sequences from Satakunta, on the other hand, belong to two different, possibly previously undescribed species. This assumption is quite possible because Michailova (2001) found in Finland (Lake Arima and Lokka Reservoir) two unknown karyotypes similar to those of the *Ch. plumosus* group. She proposed that at least one of these karyotypes could correspond to *Ch. coaetaneus* Hirvenoja, 1998 (Hirvenoja 1998), which may be related to *Ch. plumosus*.

Some sequences in the Palearctic *Ch. plumosus* cluster initially were not complete, and it was hard to make a good comparison. But still, we found a small number of substitutions that distinguish the sequences of *Ch. bonus* from other sequences in the Palearctic *Ch. plumosus* cluster. Overall, we found six substitutions of that kind (Table 5). Four of them are nonsynonymous substitutions, and the remaining two are synonymous ones. Among them, there is a single unique 340-position substitution that was found in all three sequences of *Ch. bonus*. All other substitutions are also found in certain sequences from other clusters. Only this unique substitution clearly distinguishes *Ch. bonus* from other species in our entire data set.

Tempo of diversification

According to the obtained results, the earliest split of the *Ch. plumosus* group of sibling species occurred during the Late Miocene (7,3–5,3 Mya) and early Pliocene (5,3–2,58 Mya) epoch (Fig. 3; Table 6, node 2), dating back to 5.75–3.43 Mya (substitution rates for all earliest and latest estimates in this chapter are 1.5% and 3.54%, respectively). The most recent common ancestor of all Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch. suwai*, and Nearctic *Ch. entis-Ch. plumosus* clusters lived 2.88–1.72 Mya (Fig. 3; Table 6, node 3). This split occurred in the Early Pleistocene. The most recent common ancestor of all members of the Nearctic *Ch. entis-Ch. plumosus* cluster lived 0.638–0.378 Mya (Fig. 3; Table 6, node 5). The split between Palearctic *Ch. plumosus* and Far Eastern *Ch. borokensis-Ch. suwai* clusters dates back to 1.97–1.17 Mya (Fig. 3; Table 6, node 7). The most recent common ancestor of all members of the Far Eastern *Ch. borokensis-Ch. suwai* cluster lived 0.906–0.539 Mya (Fig. 3; Table 6, node 6). The most recent common ancestor of all members of the Palearctic *Ch. plumosus* cluster lived 1.288–0.759 Mya (Fig. 3; Table 6, node 6). If we disregard the Iranian *Ch. plumosus* sequence, the most recent common ancestor of all other members of the Palearctic *Ch. plumosus* cluster dates back even later, 0.517–0.307 Mya (Fig. 3; Table 6, node 6).

Discussion

Studied larvae of *Ch. bonus* have a monomorphic karyotype, with its details similar to those previously described for this species by Kiknadze et al. (1991a). Following Proulx et al. (2013), we can conclude that the genetic distances between observed Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch. suwai*, and Nearctic *Ch. entis-Ch. plumosus* clusters exceed the 3% range. This result leads us to some interesting conclusions about the level of divergence between the Palearctic and Nearctic populations of *Ch. plumosus*.

Table 6. Estimations of the age of the most recent common ancestors (TMRCAs) for DNA clades.

Node number	Mean value (Mya)	Stdev.	95% HPD interval	ESS
		Divergence rate 1.5%		
Node 0	29.177	5.499	19.403, 40.569	6368
Node 1	17.288	3.874	10.391, 25.051	5221
Node 2	5.746	1.293	3.321, 8.280	4724
Node 3	2.883	0.698	1.689, 4.333	4562
Node 4	3.895	0.995	2.147, 5.844	5323
Node 5	0.638	0.212	0.284, 1.057	3230
Node 6	0.906	0.26	0.447, 1.427	3866
Node 7	1.971	0.505	1.027, 2.927	4836
Node 8	1.288	0.395	0.612, 2.077	5470
Node 9	0.517	0.169	0.229, 0.852	3570
		Divergence rate 2.3%		
Node 0	24.538	4.519	15.765, 33.035	7072
Node 1	13.716	2.992	8.518, 20.073	5990
Node 2	4.380	0.933	2.639, 6.220	5168
Node 3	2.204	0.511	1.288, 3.228	5429
Node 4	2.962	0.727	1.683, 4.467	5722
Node 5	0.481	0.155	0.217, 1.378	3759
Node 6	0.692	0.197	0.335, 1.071	4840
Node 7	1.503	0.375	0.820, 2.230	5210
Node 8	0.979	0.296	0.466, 1.570	5899
Node 9	0.395	0.129	0.177, 0.649	3576
		Divergence rate 3.54%		
Node 0	21.017	3.923	13.900, 28.841	6753
Node 1	11.123	2.492	6.596, 16.182	5914
Node 2	3.431	0.763	2.043, 4.944	4731
Node 3	1.715	0.410	1.013, 2.548	4676
Node 4	2.317	0.586	1.257, 3.468	5414
Node 5	0.378	0.124	0.164, 0.624	3784
Node 6	0.539	0.151	0.271, 0.835	4660
Node 7	1.170	0.295	0.634, 1.743	4902
Node 8	0.759	0.229	0.349, 1.206	6108
Node 9	0.307	0.101	0.137, 0.509	3832

Our calculations show that the distance (3.75%) between the Palearctic and Nearctic sequences of *Ch. plumosus* exceeds the 3.0% range for *Chironomus* species. One can say that since the divergence time of 2.88–1.72 Mya (Fig. 3; Table 6, node 3), the Nearctic populations of *Ch. plumosus* have already become a separate species.

We can propose two possible explanations for the observed pattern within the Palearctic *Ch. plumosus* cluster (Fig. 4), which also included the *Ch. bonus* sequences obtained during this study. The first explanation is similar to that earlier suggested by Guryev and Blinov (2002), who found that populations of *Ch. entis* and *Ch. plumosus* did not group together on the trees based on the mitochondrial cytb gene according to their species affiliation. They suggested that it could result from interspecific hybridization followed by recurrent crosses. Consequently, the offspring inherited mtDNA of one of the parental species. In this case, even an insignificant selective advantage of this mtDNA is able to lead to the rapid fixation of the new haplotype in the population. Later, Polukonova et al. (2009) in the work where they studied the COI sequences of cytologically identified *Ch. usenicus*, also inclined to this explanation when some *Ch. usenicus* and *Ch. plumosus* COI gene sequences were almost identical. In addition, Proulx et al. (2013) reported that the COI

sequences of cytologically identified *Ch. plumosus* and *Ch. entis* larvae collected from Canada cluster together, and some of these sequences are identical. We can therefore assume that the separation of *Ch. bonus* and *Ch. plumosus* from a common ancestor could occur long ago. During this time, in the gene pool of *Ch. bonus*, a unique, separate line of COI gene emerged, but then, an interspecific hybridization between a male of *Ch. bonus* and a female of *Ch. plumosus* occurred. In the hybrid offspring, the *Ch. plumosus* COI sequence gradually replaced that of *Ch. bonus*.

The observed pattern also can be explained by a relatively recent separation of the two species, with *Ch. plumosus* being a parental species to *Ch. bonus*. The COI gene sequences of these species are therefore very similar, with a very low number of new substitutions in the *Ch. bonus* lineage. However, we discovered a number of substitutions that clearly distinguish *Ch. bonus* from *Ch. usenicus* and *Ch. plumosus* from European populations (Table 5).

We can assume that the *Ch. plumosus* group originated from the common ancestor during the Pliocene of 5.75–3.43 Mya. However, since we have certain DNA data only for seven species of the *Ch. plumosus* group out of 14, this temporary estimate could change in the future in favor of the older age. At the same time, the obtained age of the most recent common ancestor of the *Ch. plumosus* group corresponds rather well to the estimations by Demin and Polukonova (2008) (5.8–3.7 Mya), despite the substantially lower amount of data available for those authors.

We can be more confident about the age of the most recent common ancestors of species constituting the Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch. suwai*, and Nearctic *Ch. entis-Ch. plumosus* clusters. It is possible that the age of the Palearctic *Ch. plumosus*, Far Eastern *Ch. borokensis-Ch. suwai*, and Nearctic *Ch. entis-Ch. plumosus* clusters is 0.638–0.378, 0.906–0.539 and 1.288–0.759 million years (Myr) respectively. The age of European populations of *Ch. plumosus* is approximately 0.517–0.307 Myr. We therefore suggest that observed clusters have arisen relatively recently in the Middle Pleistocene sub-epoch.

We concluded that the most recent common ancestor of the *Ch. plumosus* group originated in the Pliocene epoch (5.3–2.58 Mya). It is known that this epoch is characterized by the appearance of a new type of biome, the first true grasslands, due to the retreat of the forests associated with the gradual cooling of the climate that began in the previous epochs. True grasslands and Serengeti-like communities of grazing animals probably did not appear until the Late Miocene in the New World and the Pliocene in the Old World (ca. 5 Mya) (Pärtel 2005).

Due to the heterogeneity of the landscapes, new stagnant water bodies became increasingly abundant. In contrast to lowland rivers, which usually have similar environmental parameters, each of these stagnant water bodies was often characterized by a unique combination of size, shape, depth, temperature profile, mineralization level etc. This variation in environmental parameters could easily lead to differences in breeding time between various populations or individuals that can potentially lead to reproductive isolation and the emergence of new species. We suggest that the species divergence in this group could have been caused by invasion of their common ancestor into newly originated water bodies.

Data availability statement

The data (Figs and Tables) that support this study are available in FigShare at https://doi.org/10.6084/m9.figshare.17060912.v1 and https://doi.org/10.6084/m9.figshare.17060666.v1.

Acknowledgements

We are sincerely grateful to E.A. Kuchinova, a researcher of the Laboratory of Ecology and Evolution of Vertebrates, Tembotov Institute of Ecology of Mountain territories RAS, for her big help in lab work.

This study was partially supported by a grant No. 18-04-00961 from the Russian Foundation for Basic Research (RFBR).

References

Belyanina SI (1983) Karyotypical analysis of the chironomid (Diptera, Chironomidae) of the USSR fauna. Doctor of Science Dissertation, Saratov, Russian Federation: Medical Institute, 455 pp. [In Russian]

Butler MG, Kiknadze II, Golygina VV, Martin J, Istomina AG, Wülker W, Sublette JE, Sublette MF (1999) Cytogenetic differentiation between Palearctic and Nearctic populations of *Chironomus plumosus* L. (Diptera, Chironomidae). Genome 42: 797–815. https://doi.org/10.1139/g99-014

Cranston PS, Hardy NB, Morse GE (2012) A dated molecular phylogeny for the Chironomidae (Diptera). Systematic Entomology 37(1): 172–188. https://doi.org/10.1111/j.1365-3113.2011.00603.x

Demin AG, Polukonova NV (2008) Divergence time estimation of chironomids of genus *Chironomus* (Diptera) using the «molecular clock» hypothesis. Entomological and parasitological research in the Volga region 7: 8–14. [In Russian]

Dévai G, Miskolczi M, Wülker W (1989) Standardization of chromosome arms B, C and D in *Chironomus* (Diptera, Chironomidae). Acta Biologica Debricina. Supplementum Oecologica Hungarica 2(1): 79–92.

Dyomin SYu, Iliynskaya NB (1988) Change of the density of polytene chromosomes from different organs of *Chironomus plumosus* larvae. Tsitologiya 30(4): 407–415. [In Russian]

Dyomin SYu, Shobanov NA (1990) Karyotype of *Chironomus entis* from the plumosus group (Diptera, Chironomidae) living in the European part of the Soviet Union. Tsitologiya 32(10): 1046–1054. [In Russian]

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Golygina VV, Kiknadze II (2001) Karyopool of *Chironomus plumosus* L. (Diptera, Chironomidae) in Palearctic. Tsitologiya 43: 507–519. [In Russian] https://doi.org/10.1139/g00-052

Golygina VV, Kiknadze II (2008) The revision of chromosome I (AB) mapping in *Chironomus plumosus* group (Diptera, Chironomidae). Comparative Cytogenetics 2: 37–55.

Golygina VV, Kiknadze II (2012) A revision of chromosome II (CD) mapping in *Chironomus plumosus* group (Diptera, Chironomidae). Comparative Cytogenetics 6: 249–266. https://doi.org/10.3897/compcytogen.v6i3.2831

Golygina VV, Martin J, Kiknadze II, Siirin MT, Ivanchenko OV, Makarchenko EA (2003) *Chironomus suwai*, a new species of the *plumosus* group (Diptera, Chironomidae) from Japan. Aquatic Insects 25: 177–189. https://doi.org/10.1076/aqin.25.3.177.15258

Guryev VP, Blinov AG (2002) Phylogenetic relationships among holarctic populations of *Chironomus entis* and *Chironomus plumosus* in view of possible horizontal transfer of mitochondrial genes. Russian Journal of Genetics 38(3): 310–315.

Gvozdetskii NA (1963) The Caucasus. A Description of Its Nature. Moscow: State Publishing House of Geographic Literature, 261 pp. [In Russian]

Hirvenoja M (1998) *Chironomus coaetaneus* sp.n. (Diptera, Chironomidae) from the Lokka reservoir, northern Finland. Oulanka Reports 18: 53–62.

Jamnongluk W, Baimai V, Kittayapong P (2003) Molecular phylogeny of tephritid fruit flies in the *Bactrocera tau* complex using the mitochondrial COI sequences. Genome 46: 112–118. https://doi.org/10.1139/g02-113

Kerkis IE, Kiknadze II, Istomina AG (1989) Comparative analysis of karyotypes of three sibling-species in *plumosus* group (Diptera. Chironomidae). Tsitologiya 31: 713–720. [In Russian]

Keyl H–G (1962) Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. Chromosoma 13(4): 464–514. https://doi.org/10.1007/BF00327342

Kiknadze II, Shilova AI, Kerkis IE, Shobanov NA, Zelentsov NI, Grebenyuk LP, Istomina AG, Prasolov VA (1991a) Karyoypes and larval morphology in tribe Chironomini. Atlas. Nauka Publish, Novosibirsk, 115 pp. [In Russian]

Kiknadze II, Filippova MA, Siirin MT, Kerkis IE (1991b) Chromosomal polymorphism in *Chironomus entis* (Diptera, Chironomidae). Zoologicheskii Zhournal 70: 106–120. [In Russian]

Kiknadze II, Istomina AG, Gunderina LI, Aimanova KG, Salova TA, Savvinov DD (1996) Banding sequence pools of chironomid of Yakutian Permafrost. Tribe Chironomini. Novosibirsk, 166 pp. [In Russian with English summary]

Kiknadze II, Butler MG, Golygina VV, Wülker WF, Martin J, Sublette JE, Sublette MF (2000) Intercontinental cytogenetic differentiation in *Chironomus entis* Shobanov, a Holarctic species in the *plumosus* group (Diptera, Chironomidae). Genome 43: 857–873. https://doi.org/10.1139/g00-052

Kiknadze II, Istomina AV, Golygina VV, Gunderina LI (2016) Karyotypes of Palearctic and Holarctic species of the genus *Chironomus* [Electronic resource]. Novosibirsk, 489 pp.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120. https://doi.org/10.1007/BF01731581

Kondo NI, Ueno R, Ohbayashi K, Golygina VV, Takamura K (2016) DNA barcoding supports reclassification of Japanese *Chironomus* species (Diptera: Chironomidae). Entomological Science 19: 337–350. https://doi.org/10.1111/ens.12212

Linevich AA, Sokolova NYu (1983) Geographical distribution. In: Sokolova NYu et al. (Eds) *Chironomus plumosus* L. (Diptera, Chironomidae). Systematics, Morphology, Ecology, Production. Nauka, Moscow, 9–22. [In Russian]

Maximova FL (1976) The karyotype of *Chironomus plumosus* from Ust'-Izhora wild population of Leningrad region. Tsitologiya 18: 1264–1268. [In Russian]

Michailova P (1994) Karyological and external morphological characteristics of *Chironomus bonus* Shilova et Dyvarsheishvili (Chironomidae, Diptera) from Bulgaria. Studia Dipterologica 1(2): 187–194.

Michailova P (2001) Cytogenetic characteristics of species of the *Chironomus plumosus* group (Chironomidae, Diptera) in Finland. Cytobios 105(409): 99–114.

Ney G, Frederick K, Schul J (2018) A Post-pleistocene Calibrated Mutation Rate from Insect Museum Specimens. PLoS Currents-Tree of Life. 1st edn. [PMID: 30116653] https://doi.org/10.1371/currents.tol.aba557de56be881793261f7e1565cf35

Pape P, Beuk T (2016) Diptera, Chironomidae. Fauna Europaea version 2017.06. https://fauna-eu.org [accessed 1 December 2021]

Papadopoulou A, Anastasiou I, Vogler AP (2010) Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. Molecular Biology and Evolution 27(7): 1659–1672. https://doi.org/10.1093/molbev/msq051

Pärtel M, Bruun HH, Sammul M (2005) Biodiversity in temperate European grasslands: origin and conservation. Grassland Science in Europe 10: 1–14.

Polukonova NV, Djomin AG, Mugue NS, Shaikevich EV (2009) Comparison of *Chironomus usenicus* and *Chironomus curabilis* with species of the group plumosus (Diptera) inferred from the mitochondrial DNA gene COI and by the polytene chromosomes banding pattern. Russian Journal of Genetics 45(8): 899–905. https://doi.org/10.1134/S102279540908002X

Proulx I, Martin J, Carew M, Hare L (2013) Using various lines of evidence to identify *Chironomus* species (Diptera: Chironomidae) in eastern Canadian lakes. Zootaxa 3741(4): 401–458. https://doi.org/10.11646/zootaxa.3741.4.1

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System. Molecular Ecology Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Shobanov NA (1989) Morphological differentiation of species of the genus *Chironomus* of plumosus group (Diptera, Chironomidae). In: Biology, systematic and functional morphology of invertebrates. Nauka, Leningrad, 250–279.

Shobanov NA (1994) Karyofund of *Chironomus plumosus* L. (Diptera, Chironomidae). 2. Inversion variants of chromosomal arms. Tsitologiya 36: 123–128. [In Russian]

Shobanov NA (2000) The genus *Chironomus* Meigen (Diptera, Chironomidae). Taxonomy, Biology, and Evolution. Doctor of Science Dissertation, St. Petersburg, Russian Federation: Zoological Institute, Russian Academy of Sciences, 464 pp. [In Russian]

Shobanov NA (2001) Function of Ventral and Lateral Processes in Larvae of *Chironomus* (Diptera, Chironomidae). Journal of Evolutionary Biochemistry and Physiology 37: 384–387. https://doi.org/10.1023/A:1012970711312

Shilova AI, Dzhvarsheishvili BA (1974) A new species of the genus *Chironomus* from East Georgia. Informatsionnyi Bulleten "Biologiya Vnutrennich Vod" 24: 37–42. [In Russian]

Stevens MI, Greenslade P, Hogg ID, Sunnucks P (2006) Southern Hemisphere Springtails: Could Any Have Survived Glaciation of Antarctica? Molecular Biology and Evolution 23(5): 874–882. https://doi.org/10.1093/molbev/msj073

Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A (2018) Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evolution 4(1): vey016. https://doi.org/10.1093/ve/vey016

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729. https://doi.org/10.1093/molbev/mst197

Wülker W, Klötzli AM (1973) Revision der Gattung *Chironomus* Meig. IV. Arten des lacunarius- (commutatus-) complexes. Archiv für Hydrobiologie 72(2): 474–489.

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CompCytogen 16(1): 39–54 (2022)
doi: 10.3897/compcytogen.v16.i1.79056
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Distribution patterns of rDNA loci in the *Schedonorus-Lolium* complex (Poaceae)

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Academic editor: Ekaterina Badaeva | Received 9 December 2021 | Accepted 29 January 2022 | Published 24 March 2022

http://zoobank.org/EB6F2AC0-A868-4D61-9701-7DC4E2D0581B

Citation: Ansari HA, Ellison N, Stewart AV, Williams WM (2022) Distribution patterns of rDNA loci in the *Schedonorus-Lolium* complex (Poaceae). Comparative Cytogenetics 16(1): 39–54. https://doi.org/10.3897/compcytogen.v16.i1.79056

Abstract

The *Schedonorus-Lolium* complex of the subtribe Loliinae (Poaceae) includes several economically important forage and turf grasses. This complex encompasses *Lolium* Linnaeus, 1753, *Festuca* Linnaeus, 1753 subgenus *Schedonorus* (P. Beauvois, 1824) Petermann, 1849 and *Micropyropsis* Romero Zarco et Cabezudo, 1983. New FISH results of 5S and 18S–26S rDNA sequences are presented for three species and the results are interpreted in a review of distribution patterns of 5S and 18S–26S rDNA sequences among other species in the complex. *Micropyropsis tuberosa* Romero Zarco et Cabezudo, 1983 ($2n = 2x = 14$) displayed a distribution pattern of rDNA sequences identical to that of *F. pratensis* Hudson, 1762, supporting a close phylogenetic relationship at the bottom of the phylogenetic tree. "*Lolium multiflorum*" Lamarck, 1779 accessions sourced from Morocco showed a different pattern from European *L. multiflorum* and could be a unique and previously uncharacterised taxon. North African *Festuca simensis* Hochstetter ex A. Richard, 1851 had a marker pattern consistent with allotetraploidy and uniparental loss of one 18S–26S rDNA locus. This allotetraploid has previously been suggested to have originated from a hybrid with *Festuca glaucescens* (*Festuca arundinacea* var. *glaucescens* Boissier, 1844). However, the distribution patterns of the two rDNA sequences in this allotetraploid do not align with *F. glaucescens*, suggesting that its origin from this species is unlikely. Furthermore, comparisons with other higher alloploids in the complex indicate that *F. simensis* was a potential donor of two sub-genomes of allohexaploid *Festuca gigantea* (Linnaeus) Villars, 1787. In the overall complex, the proximal locations of both rDNA markers were conserved among the diploid species. Two types of synteny of the two markers could, to a considerable extent, distinguish allo- and autogamous *Lolium* species. The ancestral parentage of the three *Festuca* allotetraploids has not yet been determined, but all three appear to have been sub-genome donors to the higher allopolypoids of sub-genus *Schedonorus*. Terminal locations of both the markers were absent from the diploids but were very frequently observed in the polyploids.

Keywords
Festuca, FISH, karyotype evolution, *Lolium*, rDNA locus evolution, species diversification

Introduction

Ryegrasses of the genus *Lolium* Linnaeus, 1753 with ten diploid species and fescues of the genus *Festuca* Linnaeus, 1753 subgenus *Schedonorus* (P. Beauvois, 1824) Petermann, 1849 are closely related and, together with *Micropyropsis* Romero Zarco et Cabezudo, 1983, form the "*Schedonorus-Lolium* complex", belonging to the family Poaceae Barnhart, 1895, subtribe Loliinae Dumortier, 1829 (Inda et al. 2013; Cheng et al. 2016). Several of these *Lolium* and *Festuca* species, which are native to temperate regions of Europe, Asia and Africa, are widely used for forage and turf purposes in all major temperate regions of the planet. *Micropyropsis tuberosa* Romero Zarco et Cabezudo, 1983 (Romero Zarco and Cabezudo 1983) is the sole species of the genus and is diploid (Romero Zarco 1988).

Since the last major taxonomic revision of the genus *Lolium* by Terrell (1968), new species have been discovered and named, notably *Lolium saxatile* H. Scholz et S. Scholz, 2005 (Scholz and Scholz 2005) and *Lolium edwardii* H. Scholz, Stierstorfer et van Gaisberg, 2000 (Scholz et al. 2000). Although *Festuca* has over 500 diploid to dodecaploid species, subgenus *Schedonorus* is limited to approximately 20 species, most from Europe, W Asia or N Africa. However, the broad-leaved *Festuca* species from highland tropical Africa, including *Festuca simensis* Hochstetter ex A. Richard, 1851 have also been shown to be part of the *Schedonorus-Lolium* complex (Namaganda et al. 2006; Inda et al. 2014; Minaya et al. 2015).

Several molecular genetic analyses involving DNA markers have been successfully carried out for the phylogenetic reconstruction of subtribe Loliinae. It has been shown that the *Schedonorus-Lolium* complex represents a monophyletic group, with *Lolium* clearly differentiated from *Festuca* (Charmet et al. 1997; Gaut et al. 2000; Catalán et al. 2004; Namaganda et al. 2006; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Cheng et al. 2016). Fertile hybrids formed between *Lolium* and *Festuca* species show chromosome pairing and recombination but the chromosomes can be distinguished using genomic *in situ* hybridization (Humphreys et al. 1995).

Karyological differences featuring chromosome number, structure and morphology have long been used to infer the systematic status and the evolutionary history of species divergence. However, in some groups of species conventionally stained chromosome preparations do not clearly delineate structural differences among chromosomes or species karyotypes. Molecular cytogenetic mapping of specific DNA sequences through fluorescence *in situ* hybridization (FISH) can overcome such problems, and provide enhanced pictures of chromosome architecture, leading to clear karyotype and genome discrimination (Albert et al. 2010; Chester et al. 2010; Xiong and Pires

2011). Two different families of multicopy and highly conserved ribosomal RNA genes (rDNA), one coding for 5S and the other for 35S rRNA arrays are universally present in plants. Tandemly repeated blocks of these genes are located independently at particular chromosomal sites and provide species-specific markers (Roa and Guerra 2015). Each 35S rDNA unit carries 18S, 5.8S and 26S RNA genes along with two internal transcribed spacers (ITSs) and tandemly repeated blocks of these units form the nucleolar organizer regions (NORs) or secondary constrictions on chromosomes. FISH mapping of 5S and 35S rDNA sequences is widely used to compare the chromosomal structural changes of related species and to infer the karyoevolutionary variations that accompany species diversification (Fukushima et al. 2011; Lan and Albert 2011; Roa and Guerra 2012, 2015; Jang et al. 2013).

Species of the *Schedonorus-Lolium* complex all share $x = 7$ as the base chromosome number and all have very similar biarmed chromosome morphologies and symmetrical karyotypes. Therefore, conventional karyological information is of little value for evaluating evolutionary changes (Malik and Thomas 1966; Namaganda et al. 2006; Kopecký et al. 2010). Molecular cytogenetic mapping of 5S and 35S rDNA has detected variations in the distributional patterns of the two rDNA markers among diploids and polyploids in this complex (Thomas et al. 1996, 1997; Książczyk et al. 2010; Inda and Wolny 2013; Ansari et al. 2016; Ezquerro-López et al. 2017; Shafiee et al. 2020). Based on their report, Ezquerro-López et al. (2017) made a preliminary attempt to decipher the evolutionary relationships among *Festuca* species belonging to this complex.

In this study, we have mapped the chromosomal dispositions of 5S and 18S rDNA loci in five taxa, three of which were previously unmapped, and have discussed the evolutionary implications of the new results. Following this we have drawn together all the available information from disparate sources and have framed a more complete picture of rDNA chromosome patterns within the whole of this economically important complex. This is the first time such information has been integrated across numerous studies.

Methods

Plant materials and chromosome preparations

Seeds from five populations (Table 1) belonging to the *Schedonorus-Lolium* complex were accessed from the Margot Forde Forage Germplasm Centre at AgResearch Grasslands, Palmerston North and PGG Wrightson Seeds, Christchurch, New Zealand. *Lolium multiflorum* Lamarck, 1779 of Moroccan origin was designated MRCN to distinguish it from *L. multiflorum* material of European origin. Seeds were germinated and grown in a glasshouse. Somatic chromosome preparations were obtained from the meristematic tissue of actively growing root tips according to the flame-drying technique described earlier (Ansari et al. 1999, 2016). Good quality cytological preparations were selected after screening using phase contrast optics.

Table 1. List of *Schedonorus-Lolium* complex taxa used in this study.

Taxon	Identity and source of seed
Festuca simensis Hochstetter ex A. Richard, 1851	BL 2043, Margot Forde Forage Germplasm Centre
Lolium perenne Linnaeus, 1753	Cv Impact, Margot Forde Forage Germplasm Centre
Lolium multiflorum Lamarck, 1779	B 3380, Margot Forde Forage Germplasm Centre
Lolium multiflorum MRCN	Cv. Barberia, PGG Wrightson Seeds
Micropyropsis tuberosa Romero Zarco et Cabezudo, 1983	BZ 8319, Margot Forde Forage Germplasm Centre

Fluorescence *in situ* hybridization (FISH)

The DNA probes used for FISH were pTr18S (GenBank accession number AF071069), a 1.8 kb fragment from *Trifolium repens* Linnaeus, 1753 containing almost the entire 18S rDNA sequence representing the 35S rDNA and pTr5S (GenBank accession number AF072692), a 596 bp DNA fragment encoding the *T. repens* 5S rRNA. 35S and 5S rDNA probes were directly labelled with fluorochromes Fluor-X-dCTP and Cy-3-dCTP (GE Healthcare, NZ), respectively by nick translation according to manufacturer's specifications. Double target FISH using the above DNA probes, post-hybridisation washing and counterstaining of somatic chromosomes with DAPI were carried out as described earlier (Ansari et al. 1999). Chromosome preparations were mounted in Vectashield (Vector Laboratories). Fluorescence images were acquired using a Zeiss monochrome MRm CCD camera on a Nikon epifluorescence microscope Microphot-SA and were processed with an ISIS FISH Imaging System (MetaSystems, Germany). At least five good quality early to late metaphase cells from each plant were used for analysing hybridization signals.

Results

Results of double colour FISH mapping using 35S and 5S rDNA sequences as probes on pro-metaphase or metaphase chromosomes of *Lolium perenne* Linnaeus, 1753 ($2n = 2x = 14$) are given in Fig. 1. Six 35S rDNA signals representing three loci were located proximally on three pairs of chromosomes (Fig. 1a, b). One locus was on the short arm of one chromosome pair, and the other two displayed hybridization on the long arms of two pairs of chromosomes. One of the chromosome pairs with 35S on the long arm displayed co-localization of the single 5S rDNA locus proximally on the short arm. The chromatin housing 35S rDNA regions, representing GC-rich nucleolus organizer regions (NORs) or secondary constrictions, were frequently decondensed and sometimes stretched in our flame-dried somatic chromosome preparations. These loci are positioned pericentromerically, and the cloudy decondensed and stretched 35S rDNA FISH signals could be observed joining the two condensed parts of NOR-bearing chromosomes (Fig. 1a, b). *L. multiflorum* ($2n = 2x = 14$) of north European/Mediterranean origin produced rDNA FISH signals identical to the pattern observed for *L. perenne* (Fig. 1c, d).

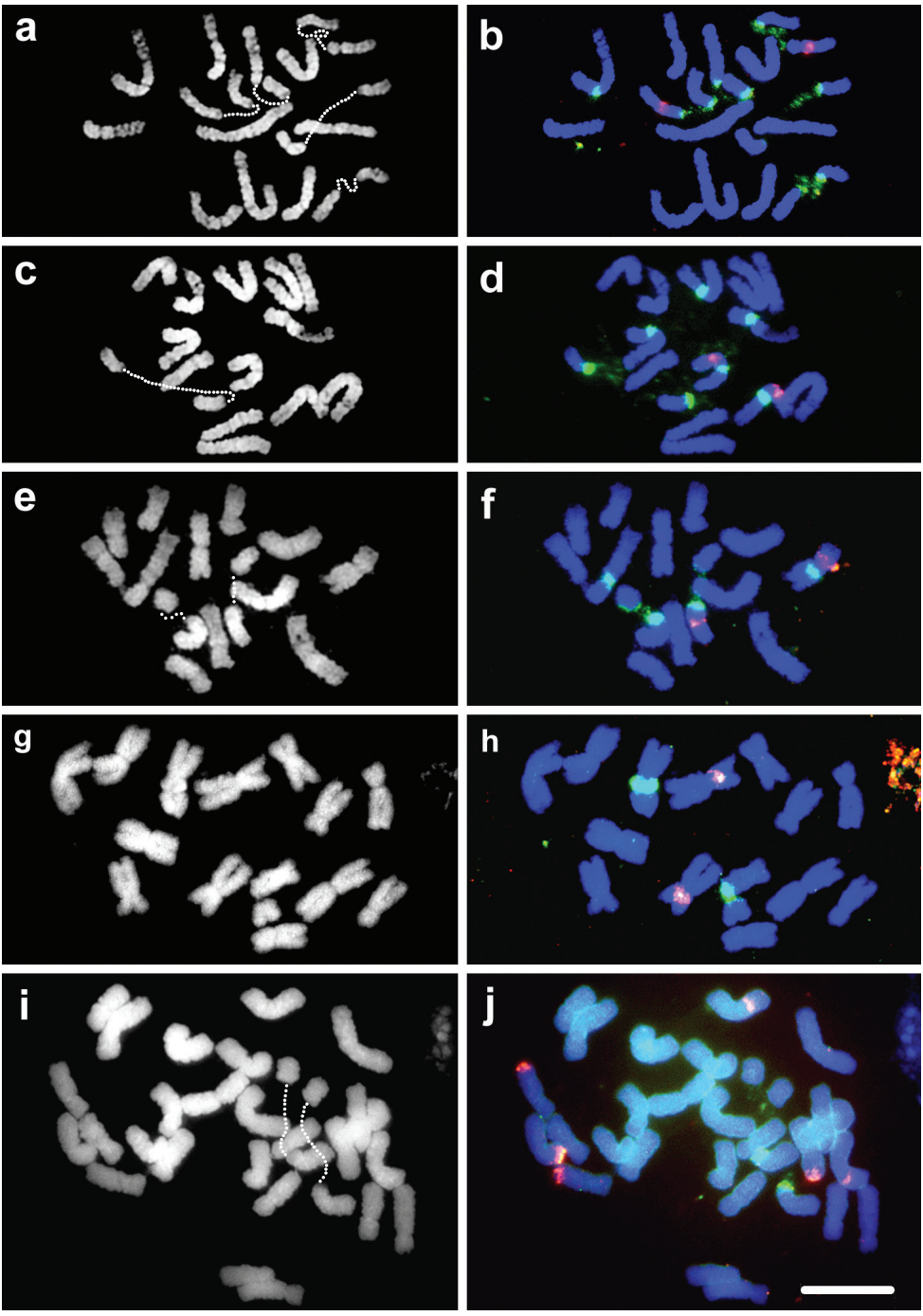

Figure 1. DAPI stained (grey scale) metaphase cells in the left column and the same cells in the right column displaying FISH mapping of 5S (red signals) and 35S rDNA sequences (green signals) in **a, b** *L. perenne* **c, d** *L. multiflorum*, European origin **e, f** *L. multiflorum* MRCN Moroccan origin **g, h** *M. tuberosa* **i, j** *F. simensis*. Dotted lines in **a, c, e, g**, and **i** denote decondensed 35S rDNA chromatin.

In contrast to *L. perenne* and *L. multiflorum* of north European origin, *L. multiflorum* ($2n = 2x = 14$) of Moroccan origin displayed only two pairs of NORs (Fig. 1e, f), each pair located proximally on the long arm. One of these NOR-bearing chromosome pairs co-localised 5S sequences proximally on the short arm.

Micropyropsis tuberosa, $2n = 2x = 14$, with a symmetrical karyotype, displayed one 5S and one 35S rDNA locus, each on separate chromosome pairs, and located proximally on the short arms (Fig. 1g, h). Co-localization of the two rDNA sequences on the same chromosome was not observed in *M. tuberosa*.

Festuca simensis, $2n = 4x = 28$, displayed all biarmed chromosomes and a symmetrical karyotype. The eight FISH signals were distributed on separate chromosomes (Fig. 1i, j). One of the three pairs of 5S rDNA signals hybridized interstitially on the short arms. Each of the remaining two pairs of 5S signals were located distally in terminal regions, one in the short arm and the other in the long arm of two pairs of chromosomes. The only pair of 35S signals was located proximally on the short arms of a chromosome pair. Again, *F. simensis* did not show co-localization of the two rDNA sequences.

Discussion

We have mapped the diversity in the chromosomal locations of the two rDNA sequences for five taxa of the *Schedonorus-Lolium* complex. Three of these, *M. tuberosa*, *L. multiflorum* MRCN and *F. simensis*, were previously unmapped. The results for *L. perenne* and N European *L. multiflorum* agree with previous studies (Thomas et al. 1996; Ansari et al. 2016). The new results are discussed first and then rDNA chromosomal patterns across the complex are reviewed.

Micropyropsis tuberosa exhibited single 5S and 35S rDNA loci positioned proximally on separate chromosomes as was also the case for *F. pratensis* (Thomas et al. 1997). In phylogenetic reconstructions within the *Schedonorus-Lolium* complex based on ITS and plastid DNA sequences, the divergence of *M. tuberosa* preceded the basal split between the diploid lineages of *Festuca* and *Lolium* (Torrecilla and Catalán 2002; Catalán et al. 2004; Inda et al. 2008, 2014; Šmarda et al. 2008). The similar arrangement of single 5S and 35S rDNA loci in *M. tuberosa* and *F. pratensis* is consistent with the interpretation that this was the ancestral diploid *Schedonorus* arrangement before the *Lolium* split.

The "*L. multiflorum*" of Moroccan origin is typical of the main *Lolium* lineage in having more than one 35S rDNA locus. One of these 35S loci has a syntenic 5S locus on the opposite chromosome arm, in common with *L. perenne* and *L. multiflorum* of Eurasian origin. However, compared with Eurasian *L. multiflorum* the Moroccan taxon has one fewer 35S locus. The Moroccan "*L. multiflorum*" could be a new and unique N African taxon that has chromosomal affinities with the allogamous Eurasian *Lolium* species.

A previous cytological analysis of the tropical African broad-leaved fescue, *F. simensis*, showed it to be tetraploid ($2n = 4x = 28$) and AFLP fingerprinting revealed a close

phylogenetic relationship with European broad-leaved fescues, especially with hexaploid *F. gigantea*, (Namaganda et al. 2006). Nuclear and plastid DNA sequence studies also placed *F. simensis* in the *Schedonorus-Lolium* complex, close to *Lolium* (Inda et al. 2014). In this first molecular cytogenetics analysis of *F. simensis*, we have confirmed the tetraploidy, revealed a symmetrical biarmed karyotype and a distributional pattern of the two rDNA sequences consistent with allopolyploidy (Figs 1 and 2). In addition to two terminal 5S loci, on separate chromosomes, an interstitial 5S locus was observed on the short arm of a separate chromosome, a new location for this group of fescues. None of these 5S positions was consistent with the suggested close relationship with *Lolium*. On the other hand, the 35S rDNA locus was positioned proximally and could represent a link with a common ancestor to *Lolium*. Only one 35S locus was encountered in this allotetraploid, indicating uniparental loss during diploidisation. There are numerous examples of uniparental loss of 35S loci occurring in other allopolyploids (Ansari et al. 1999; Kotseruba et al. 2003, 2010; Williams et al. 2012; Kolano et al. 2016).

Based on a low-copy nuclear gene analysis, Minaya et al. (2015) suggested a Mediterranean origin of Afromontane *F. simensis* through hybridization between a diploid *F. glaucescens* and a *Lolium*-like diploid species. However, none of the distribution patterns of the two rDNA sequences in this allotetraploid align with *F. glaucescens* (*Festuca arundinacea* var. *glaucescens* Boissier, 1844). Instead, the distribution patterns are consistent with the possible involvement of *F. simensis* in the formation of 6x *F. gigantea* (Linnaeus) Villars, 1787. *Festuca pratensis* Hudson, 1762 is a putative diploid subgenome donor of allohexaploid *F. gigantea* (Hand et al. 2010), but the sources of the other subgenomes remain unknown. We have noted a close similarity between the 5S and 35S patterns of allotetraploid *F. simensis* (present results) and *F. gigantea* (Thomas et al. 1997, Fig. 2). These species also show a close phylogenetic proximity based on DNA sequences (Namaganda et al. 2006; Inda et al. 2014). Hence, we infer that allotetraploid *F. simensis* could be a potential donor of the remaining two sub-genomes of allohexaploid *F. gigantea* (Fig. 2).

rDNA locus patterns across the diploid *Schedonorus-Lolium* taxa

All *Lolium* species, along with *M. tuberosa* and *F. pratensis* are natural diploids. The *Lolium* species, are evolutionarily more recent than the *Festuca* species based on DNA sequence phylogenies (Gaut et al. 2000; Catalan et al. 2004; Inda et al. 2014). All *Lolium* taxa studied so far, comprising eight of the ten extant species, displayed exclusively proximal chromosomal locations of both 5S and 35S rDNA sequences (Fig. 2). After the divergence from *Festuca*, the *Lolium* lineage invariably conserved the proximal locations of both the rDNA loci, but changes in the numbers and syntenic status of these loci apparently occurred later. The proximal localization of 5S rDNA in these diploids matches well with the general distribution pattern of this locus among angiosperms but contrasts with most Poaceae (Roa and Guerra 2015). The proximal mapping of 35S loci contrasts with more terminal localizations in the majority of angiosperms, including Poaceae (Roa and Guerra 2012; Garcia et al. 2017).

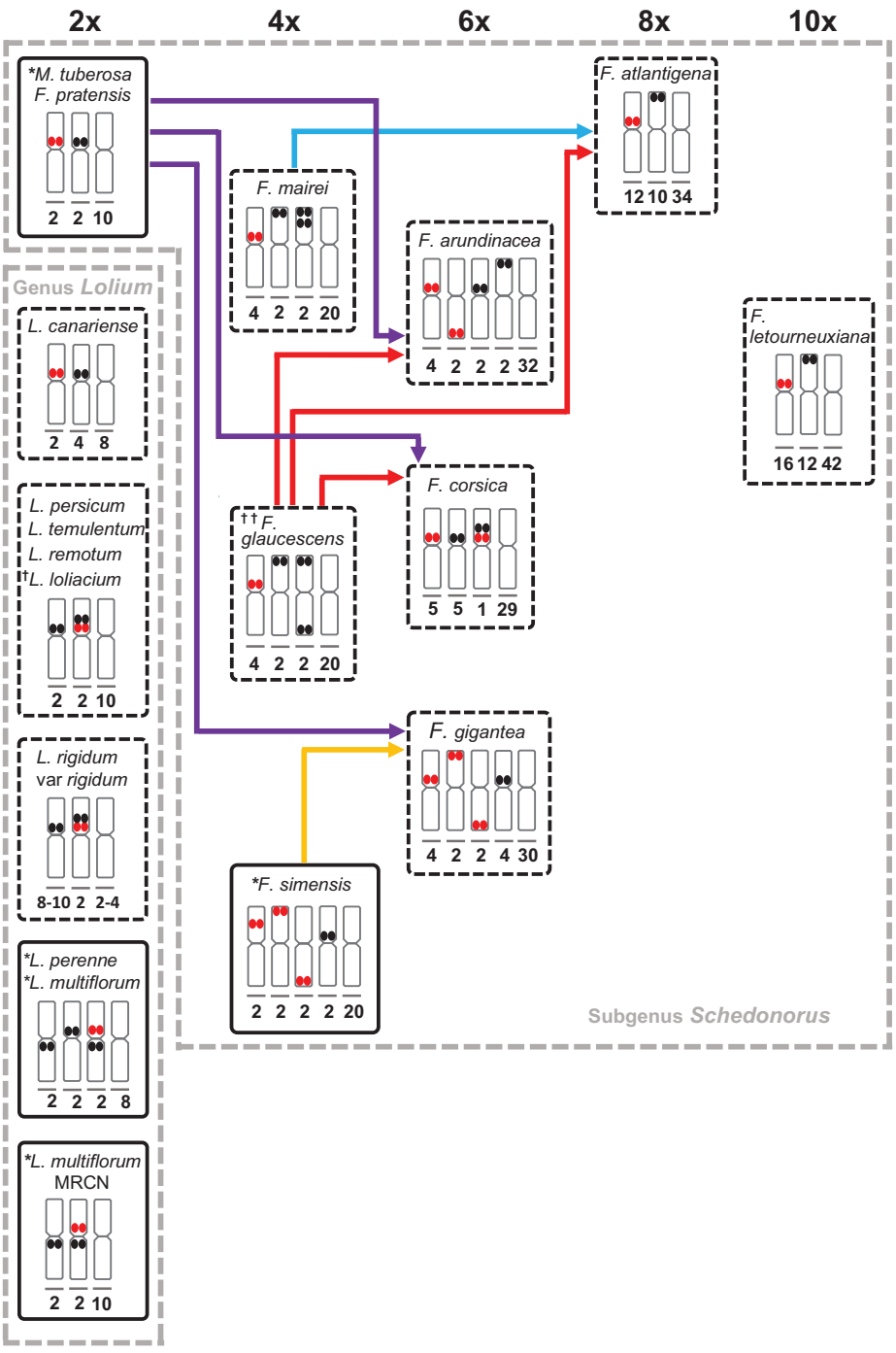

Figure 2. Schematic representation of the putative evolutionary lineages for chromosomes carrying 5S and 35S rDNA loci in the *Schedonorus-Lolium* complex. The numbers of marker and non-marker chromosomes are given inside the boxes. Red and black double circles represent 5S and 35S rDNA loci, respectively. *species in solid boxes were investigated during the present study; †synonym for *L. rigidum* var *rottbollioides*; ††synonym for *F. arundinacea* subsp. *fenas* (Lagasca y Segura) Bornmüller, 1928 (Ezquerro-López et al. 2017).

A single 5S rDNA locus (two FISH signals per cell) consistently occurred in all *Lolium* species. The number of 35S loci displayed has previously been noted as a distinguishing feature between *F. pratensis* (one locus) and *Lolium* species (more than one locus) (Thomas et al. 1996; Inda and Wolny 2013). All the *Lolium* taxa displayed increases in the number of 35S loci ranging from 2 to 5 (Fig. 2). Accordingly, there are two loci in *L. multiflorum* (Moroccan origin), *L. persicum* Boissier et Hohenacker, 1854, *L. temulentum* Linnaeus, 1753, *L. remotum* Schrank, 1789, *L. rigidum* var. *rottbollioides* Heldreich ex Boissier, 1884 and *L. canariense* Steudel, 1855, three in *L. perenne* and *L. multiflorum* (European origin) to four or five in *L. rigidum* var. *rigidum* Gaudin, 1811. These results were consistent with those of angiosperms in general, where numbers of 5S sites vary considerably less than 35S sites (Lan and Albert 2011; Garcia et al. 2017).

The two types of rDNA loci can be located on the same chromosome (syntenic) or on separate chromosomes (non-syntenic) (Morales et al. 2012; Barros e Silva et al. 2013; Olanj et al. 2015). The Macaronesian *Lolium* species, *L. canariense*, has no synteny of 5S and 35S loci (Inda and Wolny 2013). However, the remaining *Lolium* taxa (including both geographical races of *L. multiflorum*) have synteny (Fig. 2). The syntenic patterns can be differentiated into two groups. In one (allogamous) group, the two types of rDNA sequences were located proximally on either side of the centromere of the same chromosome, as represented by *L. perenne* and both geographical forms of *L. multiflorum*. In the other (largely autogamous) group, represented by *L. persicum*, *L. temulentum*, *L. remotum*, and subspecies and races of *L. rigidum*, both types of rDNA sequences were adjacent on the same chromosome arm, with 35S always distal to 5S. *L. canariense* shows the diploid *Micropyropsis-F. pratensis* arrangement with proximally located 5S and 35S rDNA loci on separate chromosomes as well as an additional pair of 35S loci (a *Lolium* characteristic, Fig. 2). On this basis, Inda and Wolny (2013) have suggested that *L. canariense* could be the link between the *Festuca* and *Lolium* lineages.

rDNA locus patterns among the polyploid *Festuca* species

The data presented in Fig. 2, based on the present investigation as well as earlier reports and analyses of DNA sequences (Thomas et al. 1997; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Ezquerro-López et al. 2017), summarise the patterns among polyploid species in subgenus *Schedonorus*. All the species are allopolyploid (Cao et al. 2000; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Ezquerro-López et al. 2017) and show no changes in the basic chromosome number ($x = 7$) and no apparent changes in the ancestral karyotype.

The numbers of 5S loci range from two in the tetraploids, *F. mairei* St. Yves, 1922 and *F. glaucescens* to eight in decaploid *F. letourneuxiana* (*Festuca arundinacea* var. *letourneuxiana* (St. Yves) Torrecilla et Catalán, 2002) while 35S numbers ranged from one in tetraploid *F. simensis* to six in *F. letourneuxiana* (Fig. 2). Localisation of two 35S loci on the same chromosome, as in the tetraploids *F. mairei* and *F. glaucescens* (Thomas et al. 1997) is not frequently encountered in plants.

Seven of the eight *Festuca* polyploids had the 5S rDNA loci in the proximal region, either exclusively or in addition to other regions (Fig. 2). Terminal 5S loci were encountered in only three polyploid species and an interstitial 5S locus was found only in *F. simensis* (present study). In contrast, terminal 35S loci were more frequent. Five species mapped at least one 35S locus in the terminal region while four displayed exclusively terminal 35S loci (Fig. 2). Among these were tetraploids either with terminal 35S loci on each arm of one chromosome (*F. glaucescens*) or two 35S loci adjacent to each other on the same arm (*F. mairei*) (Fig. 2; Thomas et al. 1997). Three polyploids displayed exclusively proximal 35S hybridization signals including tetraploid *F. simensis* with only one 35S locus. The higher frequency of terminal 35S loci among the *Festuca* polyploids aligns well with the majority of angiosperms (Roa and Guerra 2012; Garcia et al. 2017). None of the *Festuca* species in the *Schedonorus-Lolium* complex studied so far have a syntenic arrangement of 5S and 35S rDNA loci, except for hexaploid *F. corsica* Salm-Reifferscheid-Dyck, 1840 which displayed synteny only in heteromorphic form (Ezquerro-López et al. 2017).

Two allotetraploids, *F. mairei* and *F. glaucescens* have been suggested as the ancestral parents of allo-octoploid *F. atlantigena* (*Festuca arundinacea* subsp. *atlantigena* (St. Yves) Auquier, 1976) based on the formation of fertile interspecific hybrids between the two suggested ancestral parental species (Chandrasekharan and Thomas 1971) and FISH mapping of the two marker loci (Ezquerro-López et al. 2017). Six proximal 5S loci in the octoploid would reflect locus additivity from the ancestral parents while the elimination of one 35S locus may reflect genomic diploidisation. The ancestral parents of decaploid *F. letourneuxiana* could not be narrowed down by FISH mapping (Ezquerro-López et al. 2017). The allohexaploid species continental *F. arundinacea* Schreber, 1771 and *F. corsica* are hypothesised to share the same ancestral parents, *viz.*, diploid *F. pratensis* and allotetraploid *F. glaucescens* (Humphreys et al. 1995; Thomas et al. 1997; Ezquerro-López et al. 2017; Fig. 2). Two distribution patterns of 5S and 35S rDNA sequences were observed in these allohexaploids, with differential losses of 35S loci and transpositions of both 5S and 35S loci. The display of two different trajectories of speciation in allopolyploids sharing the same lower-ploid ancestors has been proposed in other angiosperms (Bao et al. 2010; Weiss-Schneeweiss et al. 2012).

All four *Festuca* higher polyploids with putative parents reveal additivity of numbers of 5S loci, but, in three cases, losses of 35S loci, (Fig. 2). Diploidisation of polyploids may lead to the evolutionary loss of repetitive sequences and duplicate copies of genes (Renny-Byfield et al. 2013). Older polyploids often, but not always, show losses of copies of 35S rDNA genes and, in allotetraploids, uniparental losses of 35S loci are common (Leitch et al. 2008; Pellicer et al. 2010; Roa and Guerra 2012; Weiss-Schneeweiss et al. 2013; Garcia et al. 2017). Although there were positional shifts involving both 5S and 35S types, the results were consistent with the general observation for angiosperms that 5S loci are less variable than 35S loci (Lan and Albert 2011; Garcia et al. 2017).

The three allotetraploids (*F. simensis*, *F. mairei* and *F. glaucescens*), as the putative sub-genome donors to the allohexaploid and octoploid species, provide a novel exam-

ple of sequential allopolyploidisation. The putative progenitors of all three allotetraploids remain unknown. However, nuclear and chloroplast DNA sequence analyses (Hand et al. 2010), supported by FISH mapping (Thomas et al. 1997) indicate that a diploid sub-genome is shared between *F. mairei* and *F. glaucescens*. The tetraploid species that became the sub-genome donors for higher ploidy fescues had terminal 5S and 35S loci that were largely conserved in the derivative species (Fig. 2). Among the *Schedonorus-Lolium* complex diploids studied so far, none have shown terminal localization of either marker, and neither were their DNA sequences consistent with them having been progenitors of these tetraploids (Hand et al. 2010). Harper et al. (2004) speculated on the basis of molecular cytogenetic findings, that diploid *F. scariosa* Lagasca y Segura ex Willkomm, 1861, belonging to the sub-genus *Scariosae* outside the *Schedonorus-Lolium* complex, was a potential ancestral parent for allotetraploid *F. mairei*. The likelihood of involvement of diploid sub-genome donor species from outside the *Schedonorus-Lolium* complex should be further explored using molecular and cytogenetic methods, including genomic *in situ* hybridization.

The variations in numbers of 35S sites in *Lolium* and the post-polyploidisation changes in the *Festuca* species have apparently occurred without any obvious changes in the symmetrical bi-armed karyotype that is a consistent feature of the *Schedonorus-Lolium* complex. Such lability in the absence of obvious structural changes might be attributable to paracentric chromosome rearrangements and/or the activity of transposable elements (Datson and Murray 2006; Raskina et al. 2008; Lan and Albert 2011; Barros e Silva et al. 2013; Weiss-Schneeweiss et al. 2013; Kolano et al. 2015).

Conclusion

This report has extended the distributional data on the rDNA sequences to seven of the ten known *Lolium* species and has added *F. simensis* to the list of seven polyploid fescue species already characterised. It has also explored the distribution patterns of rDNA loci within the *Schedonorus-Lolium* complex and considers some possible evolutionary trends. While these patterns can be used to deduce relationships among the higher polyploid *Festuca* species, the diploid progenitors of the allotetraploid species remain unidentified and enigmatic.

Author contributions

HAA designed the study with AVS and WMW. HAA performed the experiment, analysed the data and wrote the manuscript with co-writing from WMW. NWE isolated the DNA and labelled all the probes for FISH. AVS and NWE provided significant help in improving the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Joy Dick and Jaspreet Singh Sidhu for their help in formatting the manuscript for this journal. The work was supported by the New Zealand Ministry of Business, Innovation and Employment (contract no. C10X1202).

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Albert PS, Gao Z, Danilova TV, Birchler JA (2010) Diversity of chromosomal karyotypes in maize and its relatives. Cytogenetic and Genome Research 129: 6–16. https://doi.org/10.1159/000314342

Ansari HA, Ellison NW, Bassett SA, Hussain SW, Bryan GT, Williams WM (2016) Fluorescence chromosome banding and FISH mapping in perennial ryegrass, *Lolium perenne* L. BMC Genomics 17: e977. https://doi.org/10.1186/s12864-016-3231-z

Ansari HA, Ellison NW, Reader SM, Badaeva ED, Friebe B, Miller TE, Williams WM (1999) Molecular cytogenetic organization of 5S and 18S–26S rDNA loci in white clover (*Trifolium repens* L.) and related species. Annals of Botany 83: 199–206. https://doi.org/10.1006/anbo.1998.0806

Bao Y, Wendel JF, Ge S (2010) Multiple patterns of rDNA evolution following polyploidy in *Oryza*. Molecular Phylogenetics and Evolution 55: 136–142. https://doi.org/10.1016/j.ympev.2009.10.023

Barros e Silva AE, dos Santos Soares Filho W, Guerra M (2013) Linked 5S and 45S rDNA Sites are highly conserved through the subfamily Aurantioideae (Rutaceae). Cytogenetic and Genome Research 140: 62–69. https://doi.org/10.1159/000350695

Cao M, Sleper, DA, Dong F, Jiang J (2000) Genomic *in situ* hybridization (GISH) reveals high chromosome pairing affinity between *Lolium perenne* and *Festuca mairei*. Genome 43: 398–403. https://doi.org/10.1139/g99-129

Catalán P, Torrecilla P, López Rodríguez JÁ, Olmstead RG (2004) Phylogeny of the festucoid grasses of subtribe Loliinae and allies (Poeae, Pooideae) inferred from ITS and trnL-F sequences. Molecular Phylogenetics and Evolution 31: 517–541. https://doi.org/10.1016/j.ympev.2003.08.025

Chandrasekharan P, Thomas H (1971) Studies in *Festuca*. 5. Cytogenetic relationships between species of *Bovinae* and *Scariosae*. Zeitschrift für Pflanzenzüchtung 65: 345–354.

Charmet G, Ravel C, Balfourier F (1997) Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA. Theoretical and Applied Genetics 94: 1038–1046. https://doi.org/10.1007/s001220050512

Cheng Y, Ma X, Zhou K, Humphreys MW, Zhang XQ (2016) Phylogenetic analysis of *Festuca-Lolium* complex using SRAP markers. Genetic Resources and Crop Evolution 63: 7–18. https://doi.org/10.1007/s10722-015-0324-5

Chester M, Leitch AR, Soltis PS, Soltis DE (2010) Review of the application of modern cytogenetic methods (FISH/GISH) to the study of reticulation (polyploidy/hybridisation). Genes 1: 166–192. https://doi.org/10.3390/genes1020166

Datson PM, Murray BG (2006) Ribosomal DNA locus evolution in *Nemesia*: transposition rather than structural rearrangement as the key mechanism? Chromosome Research 14: 845–857. https://doi.org/10.1007/s10577-006-1092-z

Ezquerro-López D, Kopecký D, Inda LA (2017) Cytogenetic relationships within the Maghrebian clade of *Festuca* subgen. *Schedonorus* (Poaceae), using flow cytometry and FISH. Anales Jardin Botanico de Madrid 74: 1–9. https://doi.org/10.3989/ajbm.2455

Fukushima K, Imamura K, Nagano K, Hoshi Y (2011) Contrasting patterns of the 5S and 45S rDNA evolutions in the *Byblis liniflora* complex (Byblidaceae). Journal of Plant Research 124: 231–244. https://doi.org/10.1007/s10265-010-0366-x

Garcia S, Kovařík A, Leitch AR, Garnatje T (2017) Cytogenetic features of rRNA genes across land plants: analysis of the Plant rDNA database. Plant Journal 89: 1020–1030. https://doi.org/10.1111/tpj.13442

Gaut BS, Tredway LP, Kubik C, Gaut RL, Meyer W (2000) Phylogenetic relationships and genetic diversity among members of the *Festuca-Lolium* complex (Poaceae) based on ITS sequence data. Plant Systematics and Evolution 224: 33–53. https://doi.org/10.1007/BF00985265

Hand ML, Cogan NO, Stewart AV, Forster JW (2010) Evolutionary history of tall fescue morphotypes inferred from molecular phylogenetics of the *Lolium-Festuca* species complex. BMC Evolution Biology 10: e303. https://doi.org/10.1186/1471-2148-10-303

Harper JA, Thomas ID, Lovatt JA, Thomas HM (2004) Physical mapping of rDNA sites in possible diploid progenitors of polyploid *Festuca* species. Plant Systymatics and Evolution 245: 163–168. https://doi.org/10.1007/s00606-003-0110-2

Humphreys MW, Thomas HM, Morgan WG, Meredith MR, Harper JA, Thomas H, Zwierzykowski Z, Ghesquiere M (1995). Discriminating the ancestral progenitors of hexaploid *Festuca arundinacea* using genomic *in situ* hybridization. Heredity 75: 171–174. https://doi.org/10.1038/hdy.1995.120

Inda LA, Wolny E (2013) Fluorescent *in situ* hybridization of the ribosomal RNA genes (5S and 35S) in the genus *Lolium*: *Lolium canariense*, the missing link with *Festuca*? Anales Jardin Botanico de Madrid 70: 97–102. https://doi.org/10.3989/ajbm.2329

Inda LA, Sanmartín I, Buerki S, Catalán P (2014) Mediterranean origin and Miocene-Holocene Old World diversification of meadow fescues and ryegrasses (*Festuca* subgenus *Schedonorus* and *Lolium*). Journal of Biogeography 41: 600–614. https://doi.org/10.1111/jbi.12211

Inda LA, Segarra-Moragues JG, Müller J, Peterson PM, Catalán P (2008) Dated historical biogeography of the temperate Loliinae (Poaceae, Pooideae) grasses in the northern and southern hemispheres. Molecular Phylogenetics and Evolution 46: 932–957. https://doi.org/10.1016/j.ympev.2007.11.022

Jang TS, Emadzade K, Parker J, Temsch EM, Leitch AR, Speta F, Weiss-Schneeweiss H (2013) Chromosomal diversification and karyotype evolution of diploids in the cytologically diverse genus *Prospero* (Hyacinthaceae). BMC Evolutionary Biology 13: e136. https://doi.org/10.1186/1471-2148-13-136

Kolano B, Siwinska D, McCann J, Weiss-Schneeweiss H (2015) The evolution of genome size and rDNA in diploid species of *Chenopodium s.l.* (Amaranthaceae). Botanical Journal of the Linnean Society 179: 218–235. https://doi.org/10.1111/boj.12321

Kolano B, McCann J, Orzechowska M, Siwinska D, Temsch E, Weiss-Schneeweiss H (2016) Molecular and cytogenetic evidence for an allotetraploid origin of *Chenopodium quinoa* and *C. berlandieri* (Amaranthaceae). Molecular Phylogenetics and Evolution 100: 109–123. https://doi.org/10.1016/j.ympev.2016.04.009

Kopecký D, Havránková M, Loureiro J, Castro S, Lukaszewski AJ, Bartoš J, Kopecka J, Dolezel J (2010) Physical distribution of homoeologous recombination in individual chromosomes of *Festuca pratensis* in *Lolium multiflorum*. Cytogenetics and Genome Research 129: 162–172. https://doi.org/10.1159/000313379

Kotseruba V, Gernand D, Meister A, Houben A (2003) Uniparental loss of ribosomal DNA in the allotetraploid grass *Zingeria trichopoda* (2n = 8). Genome 46: 156–163. https://doi.org/10.1139/g02-104

Kotseruba V, Pistrick K, Blattner FR, Kumke K, Weiss O, Rutten T, Fuchs J, Endo T, Nasuda S, Ghukasyan A, Houben A (2010) The evolution of the hexaploid grass *Zingeria kochii* (Mez) Tzvel. (2n = 12) was accompanied by complex hybridization and uniparental loss of ribosomal DNA. Molecular Phylogenetics and Evolution 56: 146–155. https://doi.org/10.1016/j.ympev.2010.01.003

Ksia¸zczyk T, Taciak M, Zwierzykowski Z (2010) Variability of ribosomal DNA sites in *Festuca pratensis*, *Lolium perenne*, and their intergeneric hybrids, revealed by FISH and GISH. Japanese Journal of Genetics 51: 449–460. https://doi.org/10.1007/BF03208874

Lan T, Albert VA (2011) Dynamic distribution patterns of ribosomal DNA and chromosomal evolution in *Paphiopedilum*, a lady's slipper orchid. BMC Plant Biology 11: e126. https://doi.org/10.1186/1471-2229-11-126

Leitch IJ, Hanson L, Lim KY, Kovarik A, Chase MW, Clarkson JJ, Leitch AR (2008) The ups and downs of genome size evolution in polyploid species of *Nicotiana* (Solanaceae). Annals of Botany 101: 805–814. https://doi.org/10.1093/aob/mcm326

Malik CP, Thomas PT (1966) Karyotypic studies in some *Lolium* and *Festuca* species. Caryologia 19: 167–196. https://doi.org/10.1080/00087114.1966.10796216

Minaya M, Díaz-Pérez A, Mason-Gamer R, Pimentel M, Catalán P (2015) Evolution of the beta-amylase gene in the temperate grasses: non-purifying selection, recombination, semiparalogy, homeology and phylogenetic signal. Molecular Phylogenetics and Evolution 91: 68–85. https://doi.org/10.1016/j.ympev.2015.05.014

Morales AG, Aguiar-Perecin MLR, Mondin M (2012) Karyotype characterization reveals an up and down of 45S and 5S rDNA sites in *Crotalaria* (Leguminosae-Papilionoideae) species of the section Hedriocarpae subsection Macrostachyae. Genetic Resources and Crop Evolution 59: 277–288. https://doi.org/10.1007/s10722-011-9683-8

Namaganda M, Lye KA, Friebe B, Heun M (2006) AFLP-based differentiation of tropical African *Festuca* species compared to the European *Festuca* complex. Theoretical and Applied Genetics 113: 1529–1538. https://doi.org/10.1007/s00122-006-0400-5

Olanj N, Garnatje T, Sonboli A, Vallès J, Garcia S (2015) The striking and unexpected cytogenetic diversity of genus *Tanacetum* L. (Asteraceae): A cytometric and fluorescent

in situ hybridisation study of Iranian taxa. BMC Plant Biology 15: e174. https://doi.org/10.1186/s12870-015-0564-8

Pellicer J, Garnatje T, Hidalgo O, Tagashira N, Vallès J, Kondo K (2010) Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from *Artemisia* subgenera *Absinthium* and *Artemisia* (Asteraceae, Anthemideae). Plant Biosystems 144: 841–848. https://doi.org/10.1080/11263504.2010.522783

Raskina O, Barber JC, Nevo E, Belyayev A (2008) Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenetics and Genome Research 120: 351–357. https://doi.org/10.1159/000121084

Renny-Byfield S, Kovarik A, Kelly LJ, MacAs J, Novak P, Chase MW, Nichols RA, Pancholi MR, Grandbastien M, Leitch AR (2013) Diploidization and genome size change in allopolyploids is associated with differential dynamics of low- and high-copy sequences. Plant Journal 74: 829–839. https://doi.org/10.1111/tpj.12168

Roa F, Guerra M (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evolutionary Biology 12: e225. https://doi.org/10.1186/1471-2148-12-225

Roa F, Guerra M (2015) Non-random distribution of 5S rDNA sites and its association with 45S rDNA in plant chromosomes. Cytogenetics and Genome Research 146: 243–249. https://doi.org/10.1159/000440930

Romero Zarco C (1988) Números cromosomáticos de plantas occidentales, 472–486. Anales del Jardin Botanico de Madrid 45: 273–279.

Romero Zarco C, Cabezudo B (1983) *Micropyropsis*, género nuevo de Gramineae. Lagascalia 11: 94–99.

Scholz H, Stierstorfer C, Gaisberg MV (2000) *Lolium edwardii* sp. nova (Gramineae) and its relationship with *Schedonorus* sect. *Plantynia* Dumort. Feddes Repertorium 111(7–8): 561–565. https://doi.org/10.1002/fedr.20001110722

Scholz S, Scholz H (2005) A new species of *Lolium* (Gramineae) from Fuerteventura and Lanzarote (Canary Islands, Spain). Willdenowia 35: 281–286. https://doi.org/10.3372/wi.35.35208

Shafiee P, Amini F, Mirzaghaderi G, Mortazavian SMM, Noori SAS (2020) Karyotype analysis in six species of *Lolium* and *Festuca* (Poaceae). Cytologia 85: 281–288. https://doi.org/10.1508/cytologia.85.281

Šmarda P, Bureš P, Horová L, Foggi B, Rossi G (2008) Genome size and GC content evolution of *Festuca*: ancestral expansion and subsequent reduction. Annals of Botany 101: 421–433. https://doi.org/10.1093/aob/mcm307

Terrell EE (1968) A taxonomic revision of the genus *Lolium*. Technical Bulletin 1392. United States Department of Agriculture, Washington.

Thomas HM, Harper JA, Meredith MR, Morgan WG, King IP (1997) Physical mapping of ribosomal DNA sites in *Festuca arundinacea* and related species by *in situ* hybridization. Genome 40: 406–410. https://doi.org/10.1139/g97-054

Thomas HM, Harper JA, Meredith MR, Morgan WG, Thomas ID, Timms E, King IP (1996) Comparison of ribosomal DNA sites in *Lolium* species by fluorescence *in situ* hybridization. Chromosome Research 4: 486–490. https://doi.org/10.1007/BF02261775

Torrecilla P, Catalán P (2002) Phylogeny of broad-leaved and fine-leaved *Festuca* lineages (Poaceae) based on nuclear ITS sequences. Systematic Botany 27: 241–251.

Weiss-Schneeweiss H, Emadzade K, Jang TS, Schneeweiss GM (2013) Evolutionary consequences, constraints and potential of polyploidy in plants. Cytogenetic and Genome Research 140: 137–150. https://doi.org/10.1159/000351727

Weiss-Schneeweiss H, Blöch C, Turner B, Villaseñor JL, Stuessy TF, Schneeweiss GM (2012) The promiscuous and the chaste: frequent allopolyploid speciation and its genomic consequences in American daisies (*Melampodium* sect. *Melampodium*; Asteraceae). Evolution 66: 211–228. https://doi.org/10.1111/j.1558-5646.2011.01424.x

Williams WM, Ellison NW, Ansari HA, Verry IM, Hussain SW (2012) Experimental evidence for the ancestry of allotetraploid *Trifolium repens* and creation of synthetic forms with value for plant breeding. BMC Plant Biology 12: e55. https://doi.org/10.1186/1471-2229-12-55

Xiong Z, Pires JC (2011) Karyotype and identification of all homoeologous chromosomes of allopolyploid *Brassica napus* and its diploid progenitors. Genetics 187: 37–49. https://doi.org/10.1534/genetics.110.122473

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CompCytogen 16(1): 55–76 (2022)
doi: 10.3897/compcytogen.v16.i1.78852
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytotaxonomic investigations on species of genus *Narcissus* (Amaryllidaceae) from Algeria

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Academic editor: Alexander Belyayev | Received 9 December 2021 | Accepted 28 February 2022 | Published 5 April 2022

http://zoobank.org/E12C45F8-3DA6-4340-B684-2EC5D1A115C2

Citation: Boukhebache NC, Amirouche N, Amirouche R (2022) Cytotaxonomic investigations on species of genus *Narcissus* (Amaryllidaceae) from Algeria. Comparative Cytogenetics 16(1): 55–76. https://doi.org/10.3897/compcytogen.v16.i1.78852

Abstract

This paper provides new cytotaxonomic data on the genus *Narcissus* Linnaeus, 1753, in Algeria. Populations of seven taxa, *N. tazetta* Linnaeus, 1753, *N. pachybolbus* Durieu, 1847, *N. papyraceus* Ker Gawler, 1806, *N. elegans* (Haworth) Spach, 1846, *N. serotinus* sensu lato Linnaeus, 1753, including *N. obsoletus* (Haworth) Steudel, 1841, and *N. cantabricus* De Candolle, 1815, were karyologically investigated through chromosome counting and karyotype parameters. *N. tazetta* and *N. elegans* have the same number of chromosomes $2n = 2x = 20$ with different karyotype formulas. Karyological and morphological characteristics, confirm the specific status of *N. pachybolbus* and *N. papyraceus*, both are diploids with $2n = 22$ but differing in asymmetry indices. The morphotypes corresponding to *N. serotinus* sensu lato show two ploidy levels $2n = 4x = 20$ and $2n = 6x = 30$ characterized by a yellow corona. Some hexaploid cytotypes have more asymmetric karyotype with predominance of subtelocentric chromosomes. They are distinguished by orange corona and may correspond to *N. obsoletus*. Other cytotype $2n = 28$ of *N. serotinus* was observed in the North Western biogeographic sectors. *N. cantabricus* was found to be diploid with $2n = 2x = 14$, which is a new diploid report in the southernmost geographic range of this polyploid complex.

Keywords

Amaryllidaceae, chromosomes, karyotype, *Narcissus*, North-Africa, polyploidy

Introduction

The extended family of the Amaryllidaceae J. S. Hilaire, 1805, is one of the largest families of Asparagales. Among the subfamily Amaryllidoideae Burnett, 1835, species of tribe Narcisseae H.C. Lam et De Candolle, 1806, distributed in about 11 sections (Zonneveld 2008; Marquez et al. 2017), constitute the most attractive group of plants due to their botanical characteristics, evolutionary trends, biochemical properties and ornamental interests. Despite the well-known phylogenetic relationships at the generic level (Santos-Gally et al. 2012; Marques et al. 2017), many questions remain still unclear at the specific level. This is probably due to the lack of unequivocal diagnostic characters, a likely consequence of a variation driven by a deeply reticulated evolutionary history with their high ability to hybridize (Rønsted et al. 2008; Aedo et al. 2013; García et al. 2014; López-Tirado 2018; González et al. 2019). Moreover, species of tribe Narcisseae, constitute an enigmatic model of karyotype evolution in terms of chromosome numbers, base number and origin of the polyploids. This is particularly true for species of genus *Narcissus* Linnaeus, 1753, which with about fifty species, exhibit a high variation in chromosome numbers ranging from $2n = 10$ to 72 with occurrence of aneuploidy and polyploidy (Fernandes 1975; Brandham and Kirton 1987; Zonneveld 2008; Díaz Lifante et al. 2009; Sun et al. 2015). Many chromosome numbers have been reported and different basic numbers assumed but still unclarified. The most reported basic chromosome numbers in the literature were $x = 5$, $x = 7$, $x = 10$ and $x = 11$. In Algeria, species of genus *Narcissus* belong to three sections: Tazetteae De Candolle, 1806, Serotini Parlatore and Bulbocodii DC.

In the section Tazetteae, four species were recognized in the Algerian flora (Maire 1959). For this section, the common cited chromosome number was $2n = 2x = 20$ (Fernandes 1975; Brandham and Kirton 1987) especially for *Narcissus tazetta* Linnaeus, 1753, the most karyologically studied species. This species is widely distributed in the Mediterranean region, with the South Iberian Peninsula and Morocco as the center of diversity (Santos-Gally et al. 2012), and could reach the southern-west Asia, China and Japan (Hong 1982). These plants are characterized by a striking morphological variability expressed at the shape and color of corona and perianth divisions (Jones et al. 2008; Mifsud and Caruana 2010; Koopowitz et al. 2017). Comparison of the genome size by flow cytometry within *N. tazetta* had led Zonneveld (2008) to assume that this species is tetraploid with base number $x = 5$. In this same section, *Narcissus elegans* (Haworth) Spach, 1846, is also considered as tetraploid with $2n = 4x = 20$ according to studies on genome size (Zonneveld 2008), *in situ* hybridization (Díaz Lifante et al. 2009) and phylogenetic analyzis (Marques et al. 2017). In section Serotini, the base number is also $x = 5$ and concerns *Narcissus serotinus* Linnaeus, 1753, sensu lato, in which three cytotypes have been observed: diploid ($2n = 10$), tetraploid ($2n = 20$) and hexaploid ($2n = 30$). These cytotypes were observed in populations respectively from the Iberian Peninsula and Morocco (Fernandes 1968; Aedo 2013), Sicily (Garbari et al. 1973; Phitos and Kamari 1974) and Central Italy (D'Amato 2004). The geographic range of the type *N. serotinus* would cover the Iberian Peninsula and northern Morocco. The presence of this taxon in Algeria, was recorded by all the

botanists in XIX and XX centuries (Munby 1847; Battandier and Trabut 1895; Maire 1959; Quézel and Santa 1962) but remains doubtful and raises controversy as underlined in the Red List of IUCN (Juan Vicedo et al. 2018).

Although belonging to two different sections, *N. elegans* and *N. serotinus* would be involved as parents in the origin of natural hybrids such as *N. obsoletus* (Haworth) Steudel, 1841, and *N. miniatus* Donnison-Morgan, Koopowitz, Zonneveld, 2005, this latter species was discovered in Southern Spain (Donnison-Morgan et al. 2005). Both *N. miniatus* and *N. obsoletus* would be allohexaploid with $2n = 6x = 30$ as highlighted by flow cytometry (Donnison-Morgan et al. 2005; Zonneveld 2008), and molecular cytogenetics (Diaz Lifante et al. 2009; Marques et al. 2010). In the district of Algiers, Quézel and Santa (1962) following Maire (1959), referred to a hybrid × *obsoletus* (= *N. elegans* var. *intermedius* J. Gay). Two other daffodils of the flora of Algeria, *N. pachybolbus* Durieu, 1847, and *N. papyraceus* Ker Gawler,1806, were often confused. Regarding their inflorescence and flowers, these species share many similarities with *N. tazetta*, that led Maire (1959) to consider them under *N. tazetta* subsp. *pachybolbus* (Durieu) Baker, 1888, and *N. tazetta* subsp. *papyraceus* (Ker Gawler) Baker, 1888. Yet, *N. pachybolbus* was discovered in 1846 by Durieu de Maisonneuve in the NW Algeria near Oran (Battandier and Trabut 1895), and was first considered as endemic to this region (Munby 1847; Battandier and Trabut 1895). *N. papyraceus* would be introduced from Europe, cultivated and then locally naturalized (Maire 1959). Phylogenetic analyses highlighted their very close relationships in the same clade (Jiménez et al. 2015; Marques et al. 2017) but were recognized today as distinct species by most nomenclatural databases.

Similar ambiguity arose in Algeria for *Narcissus cantabricus* De Candolle, 1815, of the section Bulbocodii. This species has been considered first under *N. bulbocodium* subsp. *monophyllus* (Durieu) Maire, 1931, then later, as a distinct species (Quézel and Santa 1962). *N. bulbocodium* is distinguished by a large polyploid series ranging from diploid $2n = 14$ to octaploid $2n = 72$ (Fernandes 1963, 1968; Zonneveld 2008; Marques et al. 2017) while *N. cantabricus* was known as diploid and tetraploid in Spain and Morocco.

Despite its central biogeographic position in the southwestern Mediterranean region, Algeria is characterized by an obvious lack of cytotaxonomic data leading to controversies about status and circumscription of many taxonomic units particularly within the Asparagales (Hamouche et al. 2010; Azizi et al. 2016; Khedim et al. 2016; Boubetra et al. 2017). Unfortunately, genus *Narcissus* is little known and poorly studied in our country.

The aim of this study is to fill the gap in the karyological data that links between the floras of the western Mediterranean region. It focuses on the main taxa of genus *Narcissus* recognized in the flora of Algeria, namely *N. tazetta*, *N. elegans*, *N. serotinus* sensu lato, *N. pachybolbus*, *N. papyraceus* and *N. cantabricus*. Chromosomal counting, structural parameters of the karyotype and the geographical distribution of the polyploidy have been done for each species. Karyological data were linked to morphological and chorological criteria in order to improve taxonomic and nomenclatural knowledge on the genus *Narcissus* in Algeria.

Materials and methods

Sampling and plant identification

Plant material used in this study consists of 32 natural populations of genus *Narcissus* sampled in contrasting ecological conditions along the east-west biogeographic gradient of the northern Algeria (Table 1). Systematic determinations were made using the main Algerian floras (Munby 1847; Battandier and Trabut 1895, 1902; Maire 1959; Quézel and Santa 1962) as well as floras from the Iberian Peninsula (Aedo 2013), from Morocco (Fennane et al. 2014), and from Tunisia (Le Floc'h et al. 2010). Status of the species and synonyms have been checked on the two main specialized websites, World Check List of Selected Plant Families (Govaerts 2015) and African plant data-

Table 1. Coordinates, altitude and bioclimate of the collecting sites in northern Algeria.

Locality	Altitude (m)	Geographic coordinates	Bioclimate†	Collected species‡
Beni Bahdel	760	34°42'30.49"N, 01°31'08.33"W	Subhumid	*N. cantabricus*
Ain Ftouh	831	34°43'23.00"N, 01°27'13.00"W	Subhumid	*N. elegans* / *N. serotinus* s.l.*
Ahfir	1202	34°46'56.40"N, 01°24'54.70"W	Subhumid	*N. serotinus* s.l.
Mansourah	1160	34°50'12.60"N, 01°02'20.90"W	Subhumid	*N. cantabricus*
El-Ourit	739	34°25'00.00"N, 01°16'00.00"W	Subhumid	*N. pachybolbus*
Emir Abdelkader	460	35°13'34.50"N, 01°23'33.50"W	Subhumid	*N. pachybolbus*
Tessala	801	35°16'09.90"N, 00°46'16.80"W	Subhumid	*N. elegans*
Boutlélis	291	35°34'11.40"N, 00°54'00.00"W	Semi arid	*N. elegans* / *N. serotinus* s.l.
Santa Cruz	319	35°42'36.40"N, 00°39'51.10"W	Semi arid	*N. elegans*
Miliana	570	36°18'45.60"N, 02°16'22.06"E	Subhumid	*N. tazetta*
Mouzaïa	110	36°28'00.00"N, 02°41'00.00"E	Subhumid	*N. tazetta*
Chréa	1000	36°28'16.50"N, 02°55'37.40"E	Humid	*N. tazetta*
Chenoua	15	36°36'23.00"N, 02°22'21.00"E	Subhumid	*N. elegans*
Sainte Salsa	20	36°35'31.00"N, 02°26'58.00"E	Subhumid	*N. elegans* / *N. serotinus* s.l.
Hammam Mélouane	142	36°29'51.70"N, 03°03'29.60"E	Humid	*N. tazetta*
Ain Tagourait	219	36°35'00.00"N, 02°37'00.00"E	Subhumid	*N. elegans* / *N. serotinus* s.l.
Béni Messous	50	36°46'44.00"N, 02°58'30.10"E	Subhumid	*N. elegans*
Baraki	22	36°39'58.00"N, 03°05'30.00"E	Subhumid	*N. tazetta*
Baïnem	248	36°48'00.00"N, 02°58'00.00"E	Subhumid	*N. serotinus* s.l.
Bologhine	25	36°48'24.20"N, 03°02'24.50"E	Subhumid	*N. papyraceus*
El Alia	30	36°43'12.00"N, 03°10'00.00"E	Subhumid	*N. papyraceus*
Yakouren	700	36°43'49.90"N, 04°27'51.00"E	Humid	*N. tazetta*
Tizi Tghidet	750	36°44'48.00"N, 04°26'55.00"E	Humid	*N. tazetta*
Adekar	500	36°41'00.00"N, 04°40'00.00"E	Humid	*N. elegans*
Mont Gouraya	540	36°46'07.20"N, 04°49'50.00"E	Subhumid	*N. elegans*
El Aouana	74	36°46'00.00"N, 06°33'00.00"E	Humid	*N. elegans*
Aït Ali (Ziama)	970	36°37'04.40"N, 05°28'44.10"E	Humid	*N. serotinus* s.l.
Djebel Ouahch	983	36°24'24.50"N, 06°40'32.50"E	Subhumid	*N. tazetta*
Sidi Khélifa	864	36°21'08.90"N, 06°17'01.40"E	Subhumid	*N. tazetta*
Oued Djenane	302	36°49'17.10"N, 08°37'30.10"E	Humid	*N. tazetta*
El Aïoun	282	36°49'04.80"N, 08°37'29.40"E	Humid	*N. tazetta*
Tabarka (Tunisia)	80	36°52'21.70"N, 08°43'53.70"E	Humid	*N. tazetta*

† Bioclimate from Quézel and Santa (1962).

‡ Nomenclature from Maire (1959), Quézel and Santa (1962) and Dobignard and Chatelain (2010–2013).

* *N. serotinus* sensu lato includes also *N. obsoletus*.

base (Dobignard and Chatelain 2013). The studied taxa are presented in Table 2 and Fig. 1: *N. tazetta* and *N. elegans* are represented by several populations. Two natural populations of *N. pachybolbus* were narrowly located in the north-west of Algeria on the Mounts of Tlemcen, while those belonging to *N. papyraceus* are naturalized relics of cultivated plants. *N. serotinus* sensu lato is represented by populations collected over all the sampling area, some of which belong to *N. obsoletus*. *N. cantabricus* is narrowly located in the NW of Algeria at Tlemcen and near the Algerian-Moroccan border. From each site, 3–10 plants per taxon, with bulb, leaves and flowers, were collected. Voucher specimens were deposited at the Official Herbarium of ENSA (Algiers, Algeria) under numbers: ENSA13367-68 (*N. cantabricus*), ENSA13369-73 (*N. elegans*), ENSA13374-75 (*N. pachybolbus*), ENSA13376-77 (*N. papyraceus*), ENSA13378-81 (*N. serotinus*), ENSA13386-93 (*N. tazetta*).

Table 2. Comparison of the studied species of *Narcissus* based on the main diagnostic criteria.

Section	**Tazetteae**				**Serotini**	**Bulbocodii**
Species	***N. tazetta***	***N. pachybolbus***	***N. papyraceus***	***N. elegans***	***N. serotinus* sensu lato**	***N. cantabricus***
Bulb length (mm)	28–58	39–77	37–62	15–38	13–22	19–21
Bulb width (mm)	15–58	37–68	30–55	12–34	7–20	10–15
Color of the tunic	black brown	black	black brown	black	black	black
Leaf number at flowering	2–8	3–5	3–6	1	0	1–5
Synanthous *versus* hysteranthous	synanthous	synanthous	synanthous	synanthous	hysteranthous	synanthous
Length of scape (mm)	80–510	204–496	370–672	102–523	85–240	104–137
Length of spathe (mm)	32–70	30–50	35–50	17–44	15–30	18–25
Number of flowers per scape	3–12	9–15	6–13	1–5	1 rarely 2	1
Hypanthial tube length (mm)	23–44	19–39	14–36	14–30	13–24	23–47
Hypanthial tube shape	cylindric	cylindric	cylindric	subcylindric narrow	subcylindric	obconic–funnel
Corona color	yellow–orange	white	white	olive yellow / greenish orange	variable yellow to orange	White rarely white–yellowish
Corona size	medium	medium	medium	small	small	very large
Color of tepals	white yellow	white	white	greenish white	greenish white	white
Pedicel length (mm)	18–52	19–40	27–62	9–40	11–25	3–4
Stamen position	emergent / not emergent	emergent	not emergent	not emergent	not emergent	emergent

Note: Diagnostic criteria from the main floras of Algeria: Battandier and Trabut (1902), Maire (1959), Quézel and Santa (1962).

Chromosome preparations

Chromosomal analysis was based on metaphase plates of root-tip cells from cultivated bulbs. Young roots (6–10 mm long) were pre-treated with 1% colchicine for 5–6 hours at room temperature, then fixed in ethanol-acetic acid (3:1) for 48 hours and conserved at 4 °C in ethanol 70°.The protocol was adapted from the Feulgen method (Jahier et al. 1992). The root-tips were hydrolysed in 1N hydrochloric acid for 7–12 min at 60 °C before stained with Schiff's reagent in darkness for 1–2 hours. The squash was made in a drop of 45% acetic acid or carmine acetic. Metaphase plates were examined with a Zeiss Axiostar-Plus Microscope. Cells with good spreading of chromosomes were photographed.

Figure 1. Habits and flowers of species of genus *Narcissus* from Algeria. *N. tazetta*: **A, B** Sidi Khélifa **C, D** Hammam Mélouane **E** Yakouren **F–G** Tizi Tghidet. N. pachybolbus: **H–K** El-Ourit. *N. papyraceus*: **L–M** Bologhine. *N. elegans*: **N–Q**. *N. serotinus*: **R–S** Ain Ftouh. *N. obsoletus*: **T–U** Sainte Salsa. *N. cantabricus*: **V–X** Mansourah. Photos by Rachid Amirouche.

Karyotype analysis

Karyomorphometric measurements and the homologous chromosome ordering were made using the KaryoType Software 2.0 (Altınordu et al. 2016). Chromosomes are described according to the nomenclature of Levan et al. (1964) based on the arm

ratio (r = long arm / short arm) and the centromeric index (CI% = short arm / long arm + short arm × 100): metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t). Ideograms were drawn from at least 5 well-spread metaphase plates of different individuals. Karyotype asymmetry indices were estimated following the proposal of Peruzzi and Eroğlu (2013). The intrachromosomal asymmetry index is represented by the mean centromeric asymmetry MCA = A × 100, where A is the average ratio of long arm-short arm/long arm + short arm (Watanabe et al. 1999). The interchromosomal asymmetry index is the coefficient of variation of chromosome length CV_{CL} = A2 × 100 (Paszko, 2006) where A2 is the standard deviation of chromosome length/mean chromosome length (Romero Zarco 1986). The coefficient of variation of the centromeric index CV_{CI} = SCI / $\overline{X}$ CI × 100 is the ratio between the standard deviation SCI and the mean centromeric index $\overline{X}$ CI (Paszko 2006).

Morphological analysis

In order to link karyological data to morphological relationships between the studied species, multivariate analyses were carried out using the diagnostic descriptors of vegetative and reproductive parts, some from personal observations (Table 3). Principal Component Analysis (PCA) were performed using the program R Software 4.1.0 (2021).

Table 3. List and abbreviations of the morphological characters used in the multivariate analysis.

Quantitative characters		**Shape of the scape**	
Bl	Bulb length (mm)	SScyl	cylindrical slightly ridged
Bw	Bulb width (mm)	SSang	angular ribbed
Ln	Leaf number	**Section of the scape**	
Scl	Scape length (mm)	SSfill	filled
Spl	Spathe length (mm)	SSfist	fistilous
Nf	Number of flowers by scape	**Shape of pedicel**	
Pl	Pedicel length (mm)	SPs	smooth
Hl	Hypanthial tube length (mm)	SPa	angular
Ns	Number of scape sheath/ scape	**Color of tunic bulb**	
Ow	Ovary width (mm)	TBcol1	black
Ol	Ovary length (mm)	TBcol2	brown
Tl	Outer Tepal length (mm)	**Color of corona**	
Tl/w	Ratio tepal length / width (mm)	Corcol1	orange bright
Tel	Tunic extension wrapping the scape (mm)	Corcol2	yellow-orange
Ch	Corona height (mm)	Corcol3	Yellow-lemon
		Corcol4	white
Qualitative characters		Corcol5	orange / orange greenish
Leaves at flowering		Corcol6	yellow / yellow greenish
Syn	Synanthous	**Shape of hypanthial tube**	
Hyst	Hysteranthous	Hysh1	subcylindric large
Color of the tepals		Hysh2	subcylindric narrow
Tc1	White	Hysh3	cylindric
Tc2	yellow	Hysh4	obconic funnel

Results

Chromosome numbers, ploidy level and characteristics of the karyotypes of the examined populations are summarized in Table 4. Comparisons of chromosome numbers from this study with those reported in the literature are summarized in Table 5. Representative metaphases and ideograms are shown in Figs 2, 3 respectively. Following the karyological data, we carried out morphological analysis for the studied taxa i.e., *N. tazetta*, *N. elegans*, *N. pachybolbus*, *N. papyraceus*, *N. serotinus* and *N. cantabricus*. Morphological analyses aim to highlight on interspecific variability in relation to karyological characteristics of the species.

Table 4. Chromosome number, ploidy level and karyotype characteristics of the examined populations of genus *Narcissus* in Algeria.

Species/ Populations	Ind/ cells	*x*	*2n*	Pl	Karyotype formula	THL	Asymmetry indices					
							Stebbins	A1	A2	MCA	CV_{CL}	CV_{CI}
***Narcissus tazetta* L.**												
Tabarka (Tunisia)	4/16	10	20	2x	4m + 10sm (2sat) + 6st	114.75	3B	0.54	0.36	38.85	35.83	27.56
Oued Djenane	5/20	10	20	2x	2m + 8sm + 10st	126.12	3B	0.62	0.40	46.68	39.93	29.23
Sidi Khélifa	3/8	10	20	2x	10sm + 10st	126.17	3B	0.66	0.38	50.27	38.45	20.54
El Aïoun	4/12											
Hammam Mélouane	7/31											
Yakouren	3/15											
Baraki	5/38											
Mouzaïa	5/15											
***Narcissus pachybolbus* Dur.**												
Emir Abdelkader	5/21	11	22	2x	6m (2sat) + 6sm (2sat) + 8st + 2t	151.92	3B	0.53	0.43	40.18	43.06	40.73
El-Ourit	3/10											
***Narcissus papyraceus* Ker Gawl.**												
Bologhine	6/36	11	22	2x	6m (2sat) + 12sm + 4st	115.50	3B	0.55	0.38	39.86	37.62	29.57
El Alia	3/10											
***Narcissus elegans* (Haw.) Spach**												
Ain Tagourait	4/8	10	20	2x	2m + 2sm + 14st + 2t (2sat)	145.23	3B	0.72	0.29	58.73	29.00	46.78
Boutlélis	3/14	10	20	2x	2m + 4sm + 14st	125.32	2B	0.69	0.32	54.15	31.94	32.92
Santa Cruz	4/9											
Sainte Salsa	7/28											
Béni Messous	3/9											
Chenoua	3/20											
Tessala	4/8											
***Narcissus serotinus* L.**												
Aït Ali	2/30	5	20	4x	2m + 6sm + 12st	66.01	3B	0.69	0.33	55.29	34.40	39.40
Ain Ftouh	6/10	-	28	-	-	-	-	-	-	-	-	-
Boutlélis	4/10											
Sainte Salsa	6/10	5	30	6x	6m + 6sm + 18st	78.89	3C	0.58	0.39	43.53	39.34	35.20
Ain Tagourait	4/12	5	30	6x	1M + 11m + 6sm + 12st	101.89	3B	0.47	0.37	34.86	37.15	38.07
***N. obsoletus* (Haw.) Steud**												
Ain Ftouh	4/10	-	30	-	-	-	-	-	-	-	-	-
Boutlélis	5/15											
Sainte Salsa	3/10											
***Narcissus cantabricus* DC.**												
Mansourah	5/15	7	14	2x	6m + 4sm + 4st	67.80	3A	0.45	0.27	31.33	26.91	29.16

Abbreviations: Ind/cells numbers of individuals/metaphase plates used for ideogram construction, **Pl** ploidy, **THL** Total Haploid Length, m, sm, st, t: type of chromosome according to Levan et al. (1964), sat: satellite, **MCA** Mean Centromeric Asymmetry, $\mathbf{CV_{CL}}$ Coefficient of Variation of Chromosome Length, $\mathbf{CV_{CI}}$ Coefficient of Variation of Centromeric Index, **A1**, **A2** intra and inter chromosomal asymmetry index, **Stebbins** Karyotype asymmetry degree.

Narcissus tazetta **Linnaeus, 1753, sensu lato**

≡ *Narcissus tazetta* subsp. *tazetta*

This species has many heterotypic synonyms. It is widespread in the north of Algeria and shows a high polymorphism with regard to the color of the perianth and corona (Fig. 1). The somatic chromosome number is generally $2n = 20$ (Fig. 2A) and constant in all the karyologically examined populations (Table 4). Sometimes 1 to 2 supernumerary chromosomes have been observed such as in populations of Sidi Khélifa (Fig. 2B, C), and Oued Djenane (Fig. 2D). Three different cytotypes were observed (Table 3). The karyotypic formula 10sm + 10st was found in most of populations. Two other cytotypes concern populations located towards the east, Oued Djenane and Tabarka, with 2m + 8sm + 10st and 4m + 10sm (2sat) + 6st, respectively. The last two karyotypes are distinguished by a lower asymmetry indices MCA, 38.85 and 46.68 respectively, *versus* 50.27 for the remain populations. Satellites were observed in population of Tabarka only (Fig. 2E), which is characterized by a relative smaller total haploid length (THL = 114.75 µm).

Narcissus pachybolbus **Durieu, 1847**

≡ *Narcissus tazetta* subsp. *pachybolbus* (Durieu) Baker, 1888
≡ *Narcissus papyraceus* subsp. *pachybolbus* (Durieu) D.A. Webb, 1978

Narcissus pachybolbus is narrowly distributed in NW Algeria mainly in the region of Tlemcen. Two populations were sampled at Emir Abdelkader and El Ourit. Both are diploids with $2n = 2x = 22$ and share the same karyotype formula 6m (2sat) + 6sm (2sat) + 8st + 2t (Table 3). This species has the highest total haploid length THL = 151.92 µm. The karyotype is distinguished by terminal satellites on the second and third largest submetacentric and subtelocentric pairs (Figs 2F, 3D).

Narcissus papyraceus **Ker Gawler, 1806**

≡ *Narcissus tazetta* subsp. *papyraceus* (Ker Gawler) Baker, 1888

This species has long been confused with the spontaneous *N. pachybolbus* due to strong similarities in the flower. *N. papyraceus* is an ancient cultivated species locally naturalized in Algeria. Two populations were found in the cemeteries of Algiers at Bologhine (ex. Saint Eugène) (Fig. 2G) and El Alia. Both populations show $2n = 2x = 22$ chromosomes with the same karyotype formula 6m (2sat) + 12sm + 4st (Table 3). The karyotype of this species differs from that of *N. pachybolbus* by the presence of satellites on the 3rd metacentric pair. For this taxon, the coefficients of variation of the length of the chromosomes (CV_{CL} = 37.62) as well as the centromeric index (CV_{CI} = 29.57) are lower. Despite their morphological similarity, the THL of *N. papyraceus* is closer to that of *N. tazetta* than that of *N. pachybolbus* (Table 3, Fig. 3E).

Table 5. Chromosome numbers of the studied species of genus *Narcissus* from Algeria compared to reports from the literature.

Species	This study	Reports from the literature	
N. tazetta L.	$2n = 20$	$2n = 14, 20, 22, 24, 28, 30, 32$	Sharma and Sharma (1961), Brandham and Kirton (1987)
	$2n = 20 + 1$	$2n = 20$	Hong (1982), Garbari et al. (1988), Baldini (1990), Dominicis et al. (2002), Aquaro et al. (2007), Zonneveld (2008), Diaz Lifante et al. (2009), Marques et al. (2010), Boukhenane et al. (2015)
		$2n = 10, 20, 21, 22, 30, 31, 32$	Aedo (2013)
N. pachybolbus Dur.	$2n = 22$	$2n = 22$	Maugini (1953), Brandham and Kirton (1987)
		$2n = 36$	Aedo (2013)
N. papyraceus Ker Gawl.	$2n = 22$	$2n = 22$	Brandham (1942), D'Amato (2004), Aedo (2013), Samaropoulou et al. (2013), Marques et al. (2017)
N. elegans (Haw.) Spach	$2n = 20$	$2n = 20$	Fernandes (1966), Brandham and Kirton 1987, D'Amato (2004), Donnison-Morgan et al. (2005), Zonneveld (2008), Díaz Lifante et al. (2009), Marques et al. (2012), Aedo (2013), Troia et al. (2013)
		$2n = 30$	Brandham and Kirton (1987)
N. serotinus L.	$2n = 20$	$2n = 10$	Fernandes (1968, 1975), Brandham and Kirton (1987), Zonneveld (2008)
	$2n = 28$		
	$2n = 30$	$2n = 10$ (15)	Aedo (2013)
		$2n = 20$	Garbari et al. (1973), Phitos and Kamari (1974)
		$2n = 30$	D'Amato (2004), Zonneveld (2008)
N. obsoletus (Haw.) Steud.	$2n = 30$	$2n = 30$ (20, 29, 31, 45)	Aedo 2013
		$2n = 30$	Díaz Lifante et al. (2009), Troia et al. (2013)
N. cantabricus DC.	$2n = 14$	$2n = 14$	Zonneveld (2008), Aedo (2013)
	$2n = 14 + 1$	$2n = 28$	Zonneveld (2008)

N. elegans (Haworth) Spach, 1846

≡ *Hermione elegans* Haworth, 1831

N. elegans is encountered mainly in the Tell of the biogeographical sectors of Oranie, Algiers and the Kabylies. Seven representative populations were karyologically examined. The same diploid somatic chromosome number $2n = 20$ are observed in all the samples with $x = 10$ (Table 4). However, two slightly different karyotypes were observed (Table 4, Fig. 2H, I). The most frequent concerns populations from the western region (Boutlélis, Santa Cruz, Tessala) and the center region (Chenoua, Sainte Salsa, Béni Messous) (Fig. 2H). The karyotype formula is 2m + 4sm + 14st. The second karyotype with formula 2m + 2sm + 14st + 2t (2sat) was observed only in the population of Ain Tagourait (Fig. 2J). It is distinguished by a coefficient of variation of centromeric index CV_{CI} (46.78 *vs* 32.92) and total haploid length THL (145.23 µm *vs* 125.32 µm).

N. serotinus Linnaeus, 1753, sensu lato

= *Narcissus serotinus* var. *emarginatus* Chabert, 1889
Including *N. obsoletus* (Haworth) Steudel, 1841
≡ *Hermione obsoleta* Haworth, 1819

N. serotinus sensu lato is found in the same biogeographical areas than *N. elegans*, however with a much smaller occurrence. Sometimes, the two species grow in sympatry as

in Ain Ftouh, Boutlélis, Ain Tagourait and Sainte Salsa. Five populations belonging to *N. serotinus* s.l. were examined and three chromosome numbers were observed, $2n = 20$, $2n = 28$ and $2n = 30$ (Table 4). Most of the individuals of these populations from the central region, share the same chromosome number $2n = 30$ corresponding to hexaploid

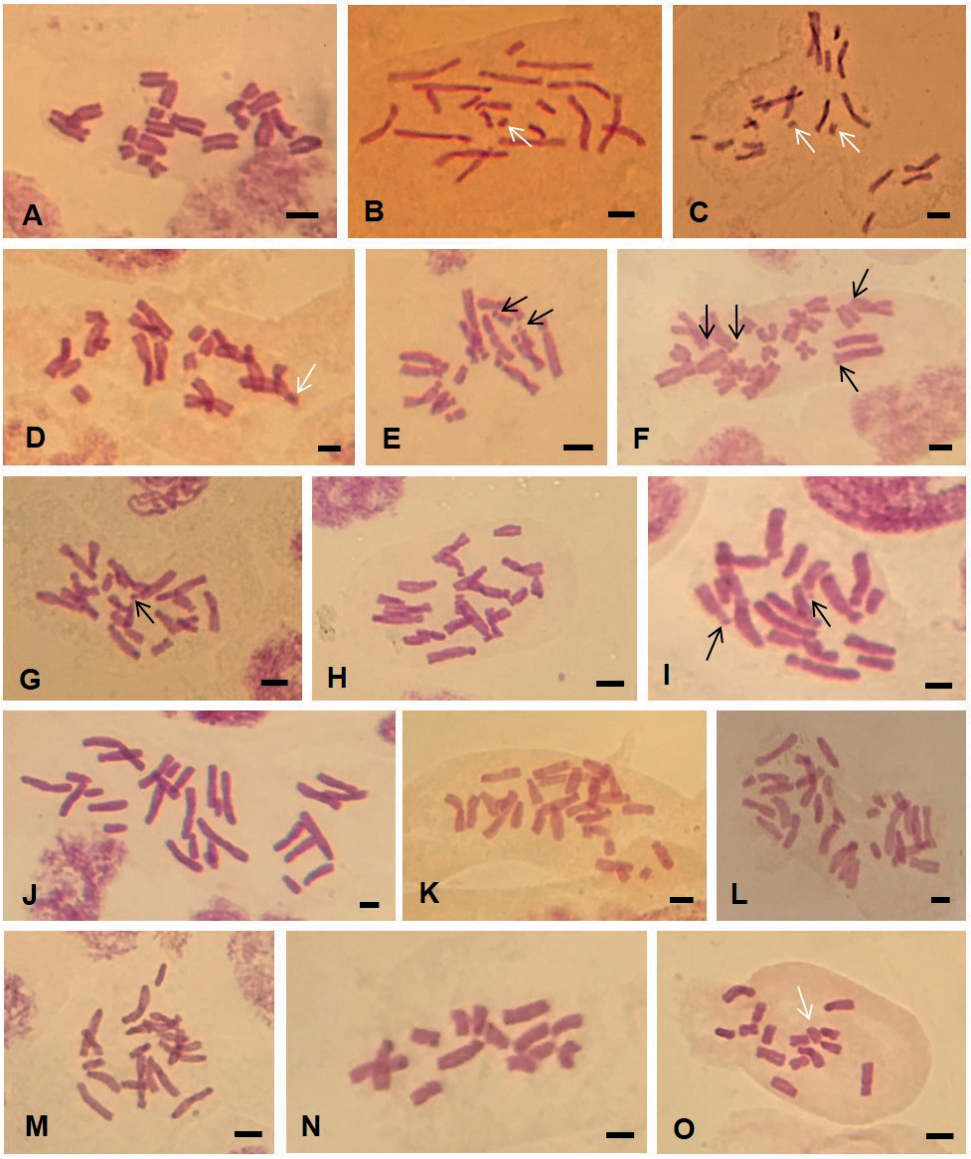

Figure 2. Somatic metaphases of some species of genus *Narcissus* from Algeria. **A–D** *N. tazetta*: **A** $2n = 20$ El Aïoun **B** $2n = 20 + 1$ Sidi Khélifa **C** $2n = 20 + 2$ Sidi Khélifa **D** $2n = 20 + 1$ Oued Djenane E $2n = 20$ Tabarka **F** *N. pachybolbus* $2n = 22$ Emir Abdelkader **G** *N. papyraceus* $2n = 22$ Bologhine **H–I** *N. elegans*: **H** $2n = 20$ Sainte Salsa **I** $2n = 20$ Ain Tagourait **J–M** *N. serotinus* s.l. : **J–K** $2n = 30$ Ain Tagourait, Sainte Salsa **L** $2n = 28$ Ain Ftouh **M** $2n = 20$ Aït Ali. **N–O** *N. cantabricus*: **N** $2n = 14$ **O** $2n = 14 + 1$ Mansourah. Black arrows indicate satellites. White arrows indicate supernumerary chromosomes. Scale bar: 10 µm.

level with base number $x = 5$. The karyotype formulas were slightly different particularly for THL and asymmetry indices A1 and MCA (Table 4, Fig. 2J, K, Fig. 3H, I). The cytotypes with $2n = 28$ are unusual and concern individuals of two populations from the far west at Ain Ftouh and Boutlélis (Fig. 2L, Fig. 3J). The chromosome number $2n = 20$ is observed for one population only of Aït Ali located toward east of the sampling area (Table 4, Fig. 2M, Fig. 3K). This tetraploid karyotype is moderately asymmetric and distinguished by a small total haploid length (THL = 66.01 µm).

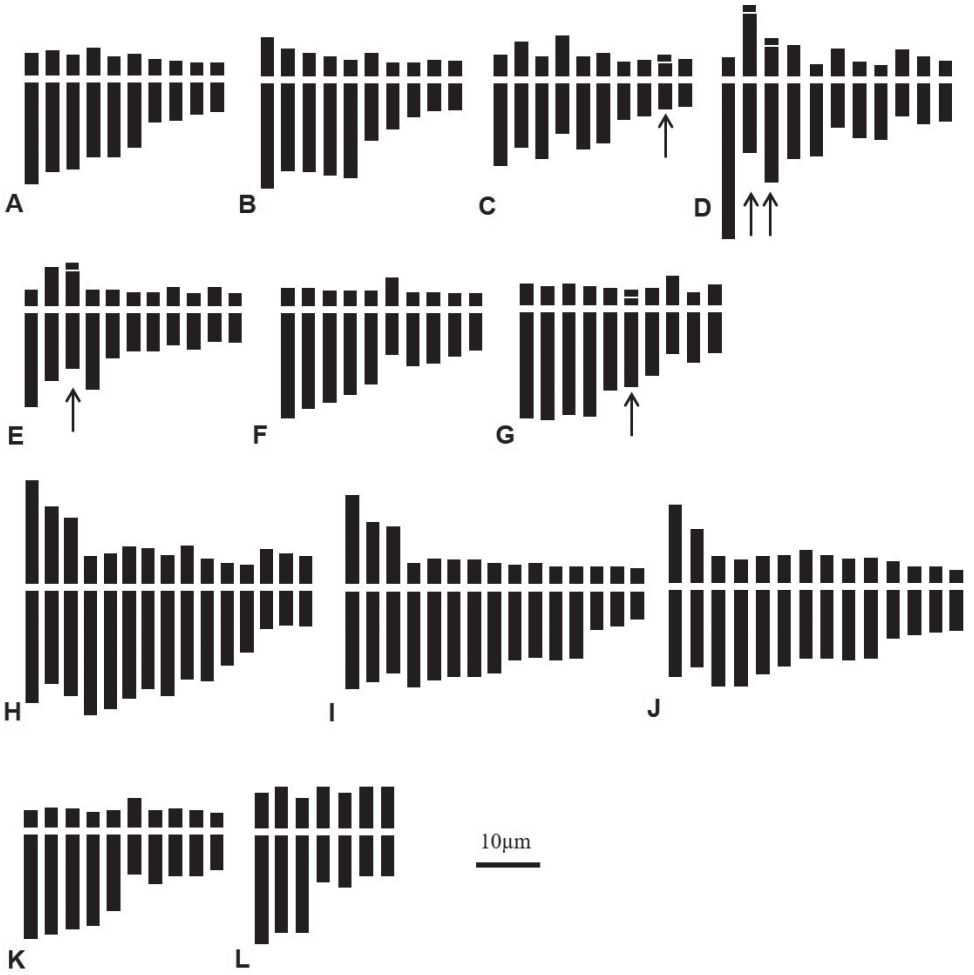

Figure 3. Ideograms of the studied species of genus *Narcissus* in Algeria **A** *N. tazetta* 2*x* (El Aïoun, Yakouren, Hammam Mélouane, Baraki, Sidi Khélifa, Mouzaïa) **B** *N. tazetta* 2*x* Oued Djenane **C** *N. tazetta* 2*x* Tabarka **D** *N. pachybolbus* (Emir Abdelkader, El Ourit) 2*x*. **E** *N. papyraceus* (Bologhine, El Alia) **F** *N. elegans* 2*x* (Boutlélis, Tessala, Béni Messous, Chenoua, Sainte Salsa, Santa Cruz) **G** *N. elegans* 2*x* Ain Tagourait **H** *N. serotinus* s.l. 6*x* Ain Tagourait **I** *N. serotinus* s.l. 6*x* Sainte Salsa **J** *N. serotinus* s.l. $2n = 28$ Ain Ftouh **K** *N. serotinus* s.l. 4*x*. Aït Ali **L** *N. cantabricus* 2*x* Mansourah. Arrows indicate satellites. Scale bar: 10 µm.

***Narcissus cantabricus* De Candolle, 1815**

= *Narcissus bulbocodium* subsp. *monophyllus* (Durieu) Maire, 1931

For this baetico-rifan species, two populations were sampled in NW Algeria, on clayey-marly slope in Mansourah forest near Tlemcen and on the edge of Lake Beni Bahdel towards the Algerian-Moroccan border. A diploid chromosome number was established $2n = 2x = 14$ (Table 3, Fig. 2N, Fig. 3L). The karyotypic formula is 6m + 4sm + 4st with respectively intra and inter chromosomal asymmetry indices, A1 = 0.45 and A2 = 0.27 (Table 4). The total haploid length THL is 67.80 µm. One supernumerary chromosome was sometimes observed $2n = 14 + 1$ (Fig. 2O).

Discussion

In order to link karyological and morphological data of the Algerian species, Principal Components Analysis (PCA) were performed on the basis of the main taxonomic criteria (see Table 3). Figure 4 underline strong interspecific differentiation between the studied taxa. Compared to PC1, the *N. tazetta-pachybolbus-papyraceus* species constitute a group clearly opposed to *N. cantabricus*, *N. serotinus* s.l. and *N. elegans*. The last two species *N. serotinus* s.l. and *N. elegans* show morphological affinities. This distribution is in full correlation with the chromosome numbers.

The *N. tazetta-pachybolbus-papyraceus* group

All of the ten Algerian populations belonging to *N. tazetta* share the same chromosome number $2n = 20$ with sometimes one or two B chromosomes. This somatic number was previously reported by Boukhenane et al. (2015) in the district of Constantine. This number is the most commonly observed in the Mediterranean region such as in Greece, Cyprus, Italy, and Southern France (Hong 1982; Garbari et al. 1988; Baldini 1990; Dominicis et al. 2002; Aquaro et al. 2007). Other chromosome numbers have been reported e.g., $2n = 14$, 20, 22, 24, 28, 30 and 32 (Sharma and Sharma 1961; Brandham and Kirton 1987). The occurrence of one or two B chromosomes makes uncertain the base number (Baldini 1995; Dominicis et al. 2002; Zonneveld 2008). Indeed, most of the studies mention only the somatic chromosomal numbers ($2n$) without indication on the base number. Hong (1982) refer to $x = 10$ following the pioneering work of Fernandes (1951, 1966) who had already suggested three base numbers $x = 7$, $x = 10$ and $x = 11$ withing genus *Narcissus*. While, Brandham and Kirton (1987) have assumed a tetraploid ($2n = 4x = 20$) and hexaploid ($2n = 6x = 30$) levels for *N. tazetta*. On the basis of an exhaustive study on genome size measured by flow cytometry, Zonneveld (2008) has also assumed $x = 5$ as common base number for *N. tazetta*, *N. elegans* and *N. serotinus*. Most of the Algerian populations of *N. tazetta* show karyotypes expressing roughly similar formula. However, two populations collected in the

eastern part near the Tunisian border (Oued Djenane, Tabarka), are distinguished by a less asymmetric karyotype. That of Tabarka, in Tunisia, was singularized by satellites on the 9th submetacentric chromosome pairs contrary to those observed on the 6th and 7th subtelocentric chromosome pairs for some *tazetta* taxonomic units (Maugini1953; Hong 1982; Dominicis et al. 2002; Boukhenane et al. 2015).

Due to their morphological similarities, Maire (1959) had considered *N. pachybolbus* and *N. papyraceus* as subspecies of *N. tazetta*. Although *N. papyraceus* has never been reported in the ancient flora of Algeria (Munby 1847; Battandier and Trabut 1895, 1902). *N. pachybolbus* first described in NW of Algeria by Durieu (1846), is currently considered as an Ibero-Mauritanian species quoted in Morocco (Fennane et al. 2014) and Spain (Aedo 2010). For the Algerian populations of *N. pachybolbus* we have counted a diploid number of $2n = 2x = 22$ consistent with previous studies (Maugini 1953; Brandham and Kirton 1987). However, in Flora Iberica, Aedo (2013) mentions 2n = 36. These two different chromosome numbers in two distinct territories suggest the need for a revision of this taxon. In our knowledge, the karyotypic formula is here provided for the first time: 6m (2sat) + 6sm (2sat) + 8st + 2t. A few karyological studies were devoted to this species. Brandham and Kirton (1987) have described just talk about a karyotype significantly different consisting of "…8 large acrocentric and 14 smaller acrocentric or submetacentric chromosomes". Our samples of *N. papyraceus* exhibit also $2n = 22$ chromosomes confirming previous reports (D'Amato 2004; Aedo 2013; Samaropoulou et al. 2013; Marques et al. 2017). The structure of the karyotype of *N. papyraceus* has been widely discussed by Brandham and Kirton (1987) and D'Amato (2004). Satellites have been observed on the 6th and 7th chromosomes pairs in contrast to Algerian samples which exhibit satellites on the 3rd pair only. Although the karyotypic structures of these two species were considered as similar by Brandham and Kirton (1987), the Algerian samples of *N. pachybolbus* and *N. papyraceus* differ notably in the asymmetry indices. Contrary to the karyological diversity observed between *N. pachybolbus* and *N. papyraceus*, trees resulting from molecular phylogenies reconstruction show a polytomy indicating a very close relationship between these two species (Santos-Gally et al. 2012; Marques et al. 2017).

Morphologically *N. tazetta*, *N. pachybolbus* and *N. papyraceus* constitute three distinct clusters (Fig. 4). In respect to PC2, *N. pachybolbus* and *N. papyraceus* ($2n = 22$) are clearly in opposition to *N. tazetta* ($2n = 20$). The main morphological characters involved in this differentiation, relate to the color of the corona, the size and color of the outer layers of the bulb as well as the number of flowers per scape. Although sharing the same chromosome number $2n = 22$, *N. pachybolbus* differs from *N. papyraceus* by higher values in the size of the bulb, the number of flowers per scape and emerging stamens from the corona (Fig. 1, Table 2). *N. papyraceus* is in intermediate position between *N. pachybolbus* and *N. tazetta*. The latter shows a high morphological variability expressed by small to medium bulb with rather brown outer tunics, a perianth white to yellow and a corona lemon to orange. These results agree with molecular phylogenies (Santos-Gally et al. 2012). The specific statute of *N. pachybolbus* and *N. papyraceus* agree with recent typification and taxonomic updating on daffodils (Aedo 2010; Koopowitz et al. 2017).

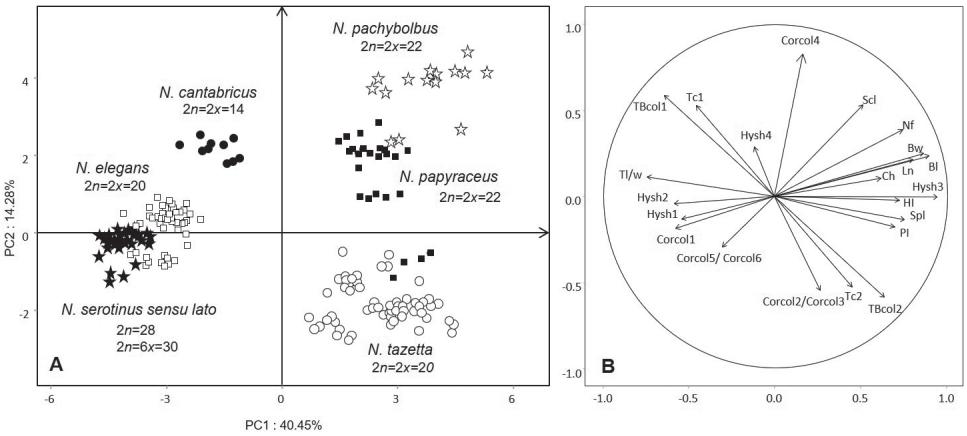

Figure 4. Principal Component Analysis of the main taxa of genus *Narcissus* in Algeria **A** overall scatter plot of 186 individuals representative of all the studied species **B** loading of the 24 quantitative and qualitative morphological and floral traits on the circle of correlations (see Table 3 for abbreviations). The distribution on PC1 and PC2 underlines the grouping of individuals belonging to *N. tazetta*, *N. pachybolbus* and *N. papyraceus* in opposition to *N. serotinus* sensu lato, *N. elegans* and *N. cantabricus*. The main discriminating criteria are relative to the length of the scape (Scl) and size of the bulb (Bl, Bw) as well as the number of flowers per inflorescence (Nf) and especially the height (Ch) and color of the corona (Corcol). This analysis highlights the strong relationships between the serotinus sensu lato type with the *elegans* type, likewise for *N. papyraceus* and *N. pachybolbus*.

Narcissus elegans, *N. serotinus* and *N. obsoletus*

Narcissus elegans and *N. serotinus* s.l. have been described in all ancient floras of Algeria (Desfontaines 1798; Munby 1847; Battandier and Trabut 1895, 1902; Maire 1959; Quézel and Santa 1962) and several intermediate forms and putative hybrids have been reported. In Zonneveld (2008) and Marques et al. (2017), these two taxa were placed in section Serotini and section Tazettae, respectively. Some authors have grouped them together in the section Tazetteae (Santos-Gally et al. 2012). Regarding the Algerian material, these two species show close morphological relationships (Fig. 5). *N. serotinus* sensu lato within the meaning of Maire (1959) and Quézel and Santa (1962), is distinguished from *N. elegans* by its hysteranthous and smaller habit, and by "stable" characters such as single, or rarely 2, flowers per scape, larger and obtuse outer tepals. The other diagnostic descriptors, in particular the color and the shape of the corona, are variable and therefore difficult to use in practice. The inconstancy of these characters was noted by Maire (1959) and Quézel and Santa (1962) who had described around Algiers, intermediate forms attributed to × *N. obsoletus* (= *Hermione obsoleta*), as a putative hybrid *N. elegans* × *serotinus*. These two species are also distinguished by their karyological characteristics. The natural hybrid × *N. obsoletus* was underlined by DNA content of specimens from Spain and Morocco (Donnison-Morgan et al. 2005).

In our study, *N. elegans* has a constant somatic chromosome number $2n = 20$ reported also in the literature but often without mention of the base number (D'Amato 2004; Díaz Lifante et al. 2009; Aedo 2013; Troia et al. 2013). The reconstructed ideo-

grams of *N. elegans* show groupings preferentially in pairs of homologous suggesting a diploid level with $x = 10$. This is inconsistent with Donnison-Morgan et al. (2005), Zonneveld (2008) and Marques et al. (2012) who have assumed that *N. elegans* is tetraploid with $x = 5$. The karyotypic structure of *N. elegans* compared to that of *N. tazetta* from Algeria, shows similarities in agreement with the first assumptions of Fernandes (1966). The values of THL and the asymmetry indices of these two species vary within the same interval, except for CV_{CI} and CV_{CL} which are different. These differences would be due to chromosome structural changes as suggested by D'Amato (2004).

The Algerian populations belonging to *N. serotinus* sensu lato, display three somatic chromosome numbers $2n = 20$, $2n = 28$ and $2n = 30$. The karyotype formula and the ideograms let suppose a base number $x = 5$ and consequently tetraploid and hexaploid levels. The tetraploids ($2n = 20$) were encountered in Sicily (Garbari et al. 1973) and in Greece (Phitos et Kamari, 1974), the hexaploids ($2n = 30$) were quoted in Italy (D'Amato 2004; Troia et al. 2013). Diploid forms $2n = 2x = 10$ were mentioned in Iberian Peninsula and Morocco by Fernandes (1968, 1975), Brandham and Kirton (1987) and Aedo (2013). This diploid cytotype ($2n = 10$) is considered very rare and would represent the *N. serotinus* type narrowly distributed in this region (Zonneveld 2008). In the literature, the most accepted and widespread ploidy level for *N. serotinus* remains the tetraploid $2n = 20$. The hexaploid would raise controversy over its systematic statute. Analysis of genome size by flow cytometry led Zonneveld (2008) to attribute the hexaploid cytotype to *N. miniatus* which would be also confused with *N. serotinus*. Subsequent studies (Díaz Lifante et al. 2009; Marques et al. 2010, 2012, 2017) support that *N. miniatus* is an allohexaploid from *N. serotinus* ($2n = 10$) × *N. elegans* ($2n = 20$). This hexaploid form, firstly located in Spain, have a geographic range through the northern Mediterranean edge from Italy toward Lebanon, Palestine until Syria (Zonneveld 2008). On the contrary, the hexaploid specimens found by Troia et al. (2013) in Mazara del vallo (Sicily, Italy) have been attributed to *N. obsoletus*, which would have a larger geographic distribution area, especially in North Africa. Díaz Lifante et al. (2009) confirmed that the hexaploid cytotype of Spain and Greece belong to *N. obsoletus*. In our study, the karyologically examined populations are all mixed and would include individuals belonging to *N. serotinus* and *N. obsoletus*. The PCA focused on specimens of *N. serotinus* sensu lato and *N. elegans* (Fig. 5) show that the cytotypes with 30 and 28 chromosomes are all distributed along PC2. This distribution is determined by the color of the corona. All individuals located in positive pole of PC2, have orange corona and would correspond to *N. obsoletus*. At the opposite, individuals with yellow corona correspond to *N. serotinus*. This differentiation is consistent with the observations of Díaz Lifante and Andrès Camacho (2007) and Koopowitz (2017). In Algeria, *N. obsoletus* was often misidentified and sometimes confused with *N. serotinus.* In our opinion, the two species *N. serotinus* (4*x*, 6*x*) and *N. obsoletus* (6*x*) are well present in Algeria in mixed populations. The hexaploid cytotypes are located mainly in the center region near Algiers (Ain Tagourait, Sainte Salsa). The unusual cytotypes $2n = 28$ were encountered in the northwest near Oran (Boutlélis) and Tlemcen (Ain Ftouh), could be due to aneuploidy event (Figs 4, 5). The tetraploid cytotypes ($2n = 20$) belongs to *N. serotinus* are rare in Algeria and its

encountered rather in pure populations, localized mostly in the eastern region. In the IUCN Red List of Threatened Species, *N. serotinus* was considered uncertain in our country (Juan Vicedo et al. 2018).

Narcissus cantabricus

The presence of *N. cantabricus* in Algeria, was subject to controversy with *N. bulbocodium*. *N. cantabricus* was not mentioned previously in the floras of North Africa. Maire (1959) had described this species under *N. bulbocodium* subsp. *monophyllus* var. *typicus* with *Corbularia monophylla* as synonym. *C. monophyla* was initially reported in Algeria by Battandier and Trabut (1895) and then considered as synonym of *N. monophyllus* before being accepted by Quézel and Santa (1962) under *N. cantabricus*. *N. cantabricus* is distinguished from *N. bulbocodium* by a "white or slightly yellowish flower" (Battandier and Trabut 1895). These two species are mentioned in *Flora Iberica* (Aedo 2013) and *Flore Pratique du Maroc* (Fennane et al. 2014). Phylogenetic analyzes carried out successively by Fonseca et al. (2016) and Marques et al. (2017) confirmed their separation. The Algerian populations of *N. cantabricus* is diploid ($2n = 14$) with sometimes one B chromosome. The karyotype established here for the first time for this species, is rather symmetrical comprising mostly meta and submetacentric chromosomes. In the literature, diploid cytotypes were reported on the Cantabrian

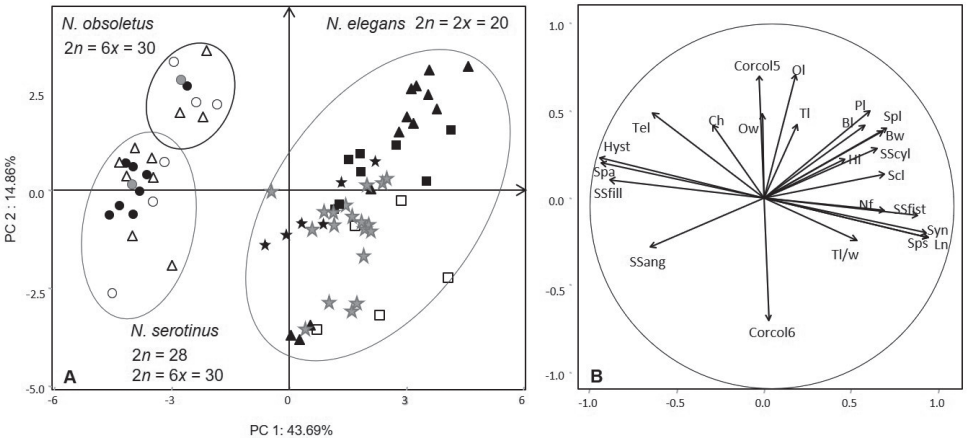

Figure 5. Principal components analysis focused on populations of *Narcissus elegans* and *N. serotinus* sensu lato **A** scatter plot on the first two PC of individuals of each taxon **B** loading of the morphological variables on the circle of correlations (see Table 3 for abbreviations). Morphologically *N. elegans* is well separated from *N. serotinus* sensu lato, by its synanthous habit (Syn), the number of flowers per scape (Nf), a full section of the scape (SSfill). With respect to PC2, individuals of *N. serotinus* s.l. are distributed in two opposed groups by the color of the corona. In the negative side individuals with yellow corona (Corcol6) correspond to *N. serotinus* type. Others individuals with orange corona (Corcol5) belong to *N.obsoletus* type. *N. serotinus* s.l.: black circle - St Salsa, white circle - Ahfir, white triangle - Ain Ftouh, gray circle - Boutlélis. *N. elegans*: gray star - Boutlélis, white square - Ain Tagourait, black triangle - Santa Cruz, black square - Chenoua, black star - St Salsa.

Mounts in the north, and in the center of Spain, while tetraploids are quite rare and found in Morocco on the Anti-Atlas (Zonneveld, 2008). Therefore, the Algerian diploids would be the southernmost within the geographic range of this species. Although the haploid amount of DNA is similar in the two species, it seems that *N. cantabricus* derived from *N. bulbocodium* following structural changes (Zonneveld, 2008). *N. bulbocodium* is distinguished by a high polyploid series from 2*x* to 8*x* with 2*n* = 72 as the highest chromosome number (Fernandes 1963; Fernandes and Franca 1974; Brandham and Kirton 1987; Marques et al. 2017). *N. bulbocodium* is an Ibero-Mauritanian whose polyploids propagate from North to South towards Morocco and from West to East through the Maghreb as already hypothesized by Fernandes (1951). This geographical distribution of the polyploidy is similar for the two species, and therefore the Algerian diploids of *N. cantabricus* constitute original and interesting material. The supernumerary chromosomes in the Algerian peripheral diploids, would express an adaptive response to aridity.

Conclusion

Overall, this work has contributed with new information supplementing our knowledge on chromosome numbers, karyotypes and ploidy levels of species of the genus *Narcissus*. The relationships between karyological and morphological characteristics made it possible to confirm and/or update the nomenclature and the taxonomy of species of genus *Narcissus* in Algeria. Therefore, seven main taxa have been recognized. Into the section Tazetteae, *N. tazetta* and *N. elegans* are diploids showing 2*n* = 2*x* = 20, while *N. pachybolbus* and *N. papyraceus* have 2*n* = 2*x* = 22 chromosomes. Section Serotini is represented by both tetraploid and hexaploid *N. serotinus* (2*n* = 20, 2*n* = 30) and also by the hexaploid *N. obsoletus* (2*n* = 30). These two species are very similar morphologically and have long been confused with each other in the field. Among *N. serotinus* type, tetraploids are rare comparatively to hexaploids. The distribution of *N. obsoletus* (6*x*) is widespread from west to east through various habitats. *N. cantabricus* show 2*n* = 2*x* = 14 and one recurrent B chromosome and constitute the southernmost diploids, providing new element for our understanding of the distribution of polyploidy within this species.

Acknowledgements

This work has received a financial assistance from the University of Sciences and Technology Houari Boumediene (USTHB, Algiers, Algeria). It was conducted in the framework of the Project on “Asparagales in Algeria” (PRFU No. D01N01UN160420180016) of the Team Biosystematics, Genetics and Evolution. We are grateful to S. Benhouhou, the manager of the Official Herbarium of ENSA (Algiers). The authors wish to thank also the two anonymous reviewers for their suggestions and comments that have improved our manuscript.

References

Aedo C (2010) Typifications of the names of Iberian accepted species of *Narcissus* L. (Amaryllidaceae). Acta Botanica Malacitana 35: 133–142. https://doi.org/10.24310/abm.v35i0.2860

Aedo C (2013) *Narcissus*. In: Castroviejo et al. (Eds) Flora Iberica. CSIC 20: 340–397.

Aedo C, Medina L, Fernández-Albert M (2013) Species richness and endemicity in the Spanish vascular flora. Nordic Journal of Botany 31(4): 478–488. https://doi.org/10.1111/j.1756-1051.2012.00009.x

Altınordu F, Peruzzi L, Yu Y, He X (2016) A tool for the analysis of chromosomes: KaryoType. Taxon 65(3): 586–592. https://doi.org/10.12705/653.9

Aquaro G, Peruzzi L, Cesca G (2007) Chromosome numbers of 20 flowering plants from ex-Yugoslav countries. Bocconea 21: 303–312.

Azizi N, Amirouche R, Amirouche N (2016) Karyological investigations and new chromosome number reports in *Bellevalia* Lapeyrouse, 1808 and *Muscari* Miller, 1758 (Asparagaceae) from Algeria. Comparative Cytogenetics 10: 171–187. https://doi.org/10.3897/CompCytogen.v10i1.6445

Baldini RM (1990) Chromosomal numbers for the Italian flora: 1231–1238. Informatore Botanico Italiano 22: 227–236.

Baldini RM (1995) *Narcissus tazetta*. In: Kamari G, Felber F, Garbari F (Eds) Mediterranean chromosome number reports. Flora Mediterranea 5: 346–350.

Battandier JA, Trabut L (1895) Flore de Algérie et catalogue des plantes du Maroc. Monocotylédones. Editeur Adolphe Jourdan, Alger, 46–48.

Battandier JA, Trabut L (1902) Flore analytique et synoptique de l'Algérie et de la Tunisie. Edition Vve Girault, Alger, 327 pp. https://doi.org/10.5962/bhl.title.10806

Boubetra K, Amirouche N, Amirouche R (2017) Comparative morphological and cytogenetic study of five *Asparagus* (Asparagaceae) species from Algeria including the endemic *A. altissimus* Munby. Turkish Journal of Botany 41: 588–599. https://doi:10.3906/bot-1612-63

Boukhenane M, Khalfallah N, Pustahija F, Siljak-Yakovlev S (2015) Cytogenetic characterization of six populations of *Narcissus tazetta* L. (Amaryllidaceae) from western Mediterranean. International Journal of Advanced Research 3(11): 1538–1546.

Brandham PE, Kirton PR (1987) The chromosomes of species, hybrids and cultivars of *Narcissus* L. (Amaryllidaceae). Kew Bulletin 42(1): e65. https://doi.org/10.2307/4109898

D'Amato G (2004) Karyotype and heterochromatin characterization in some species of *Narcissus* (Amaryllidaceae). Caryologia 57(1): 99–105. https://doi.org/10.1080/00087114.2004.10589377

Desfontaines R (1798) Flora Atlantica. I, sive Historia Plantarum quae in Atlante, agrotunetano et algeriensis crescent. Edition Desgranges. Paris, Vol. 1, 282–283. https://doi.org/10.5962/bhl.title.323

Díaz Lifante Z, Camacho CA (2007) Morphological variation of *Narcissus serotinus* L. s.l. (Amaryllidaceae) in the Iberian Peninsula. Botanical Journal of the Linnean Society 154: 237–257. https://doi.org/10.1111/j.1095-8339.2007.00653.x

Díaz Lifante Z, Camacho CA, Viruel J, Caballero AC (2009). The allopolyploid origin of *Narcissus obsoletus* (Alliaceae): Identification of parental genomes by karyotype characterization

and genomic in situ hybridization. Botanical Journal of the Linnean Society 159(3): 477–498. https://doi.org/10.1111/j.1095-8339.2009.00951.x

Dobignard A, Chatelain C (2010–2013) Index synonymique et bibliographique de la flore d'Afrique du Nord. Conservatoire et Jardin Botaniques de la Ville de Genève (CH). http://www.ville-ge.ch/musinfo/bd/cjb/africa/recherche.php [accessed 05 October 2021]

Dominicis RID, D'Amato G, Tucci GF (2002) On the hybrid origin of *Narcissus biflorus* (Amaryllidaceae): Analysis of C-banding and rDNA structure. Caryologia 55(2): 129–134. https://doi.org/10.1080/00087114.2002.10589268

Donnison-Morgan D, Koopowitz H, Zonneveld B, Howe M (2005–2006) *Narcissus miniatus* Donnison-Morgan, Koopowitz, Zonneveld sp. nov. a new species of *Narcissus* (Amaryllidaceae) from southern Spain. In: Daffodils, Snowdrops and Tulips Yearbook. Royal Horticultural Society, United Kingdom, 19–25.

Fennane M, Ibn tattou M, Mathez J, Ouyahya A, El Oualidi J (2014) Flore pratique du Maroc, Institut Scientifique de Rabat, Maroc. Vol. 3, 418 pp.

Fernandes A (1951) Sur la phylogénie des espèces du genre *Narcissus* L. Boletim da Sociedade Broteriana 25 : 113–195.

Fernandes A (1963) On the evolution of the subgenus "*Corbularia*" of the genus "*Narcissus*" L. Mem Acad Cienc Lisboa Cl Science 8: 363–381.

Fernandes A (1966) De nouvelles études caryologiques sur la section Jonquilla DC du genre *Narcissus* L. Boletim da Sociedade Broteriana 40: 207–261.

Fernandes A (1968) Keys to the identification of native and naturalized taxa of the genus *Narcissus* L. Royal Horticultural Society, United Kingdom, 37–66.

Fernandes A (1975) Évolution dans le genre *Narcissus* L. Anales del Instituto Botánico A.J. Cavanilles 32(2): 843–872.

Fernandes A, Franca F (1974) Sur le comportement des hétérochromatinosomes dans une population de *Narcissus hispanicus* Gouan. Boletim da Sociedade Broteriana 48: 30–35.

Fonseca JP, Levy A, Henriques R, Costa JC, Neto C, Robalo J (2016) Phylogenenetic approach of the section Bulbocodii D.C. of *Narcissus* based on cpDNA. A case of taxonomic inflation? Plant Biosystematics 150(4): 787–798. https://doi.org/10.1080/11263504.2014.1001460

Garbari F, Giordani A, Arnold N (1988) Chromosome numbers for the flora of Cyprus. Atti della Società Toscana di Scienze Naturali 95: 35–40.

Garbari F, Tornadore N, Pecori E (1973) Numeri cromosomici per la flora italiana. Inform Bot Ital. 5: 161–169.

García N, Meerow AW, Soltis DE, Soltis PS (2014) Testing Deep Reticulate Evolution in Amaryllidaceae Tribe Hippeastreae (Asparagales) with ITS and Chloroplast Sequence Data. Systematic Botany 39(1): 75–89. https://doi.org/10.1600/036364414X678099

González JFÁ, Prigent PC, Murillo PG, García S (2019) Dos nuevos híbridos de *Narcissus* L., (Amaryllidaceæ) en la Península Ibérica. Folia Geobotanica Exremadurensis 13(2): 33–38.

Govaerts R (2015) World Checklist of Selected Plant Families Asparagaceae. Royal Botanic Gardens, Kew. http://apps.kew.org/wcsp/ [accessed 05 October 2021]

Hamouche Y, Amirouche N, Misset MT, Amirouche R (2010) Cytotaxonomy of autumnal flowering species of Hyacinthaceae from Algeria. Plant Systematics and Evolution 285: 177–187. https://doi.org/10.1007/s00606-010-0275-4

Hong D (1982) A new Karyotype for *Narcissus tazetta* L. Hereditas 97: 29–31. https://doi.org/10.1111/j.1601-5223.1982.tb00707.x

Jahier J, Chèvre AM, Delourme R, Eber F, Tanguy AM (1992) Techniques de Cytogénétique Végétale. Edition de l'INRA, Paris, 202 pp.

Jiménez JF, López-Romero C, Rosselló JA, Sánchez-Gómez P (2017) Genetic diversity of *Narcissus tortifolius*, an endangered endemic species from Southeastern Spain. Plant Biosystematics 151: 117–125. https://doi.org/10.1080/11263504.2015.1108937

Jones RN, Viegas W, Houben A (2008) A Century of B Chromosomes in Plants: So, What? Annals of Botany 101(6): 767–775. https://doi.org/10.1093/aob/mcm167

Juan Vicedo J, Draper Munt D, Marques I, Vela E (2018) *Narcissus serotinus*. The IUCN Red List of Threatened Species 2018: e.T13147080A18613955. https://doi.org/10.2305/IUCN.UK.2018-1

Khedim T, Amirouche N, Amirouche R (2016) Morphological and cytotaxonomic data of *Allium trichocnemis* and *A. seirotrichum* (Amaryllidaceae) endemic to Northern Algeria, compared with *A. cupanii* group. Phytotaxa 243: 247–259. https://doi.org/10.11646/phytotaxa.243.3.3

Koopowitz H, Howe M, Christenhusz MJM (2017) Nomenclatural notes on some autumn flowering daffodils (*Narcissus*, Amaryllidaceae). Phytotaxa 297(2): e157. https://doi.org/10.11646/phytotaxa.297.2.3

Le Floc'h E, Boulos L, Véla E (2010) Catalogue synonymique commenté de la flore de Tunisie. Ministère de l'Environnement et du Développement durable et Banque nationale de Gènes, Tunis, Tunisie, 334–335.

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

López-Tirado J (2018) *Narcissus* × *munozii-alvarezii* (Amaryllidaceae): A new hybrid from southern Spain. Phytotaxa 364(3): 267–274. https://doi.org/10.11646/phytotaxa.364.3.6

Maire R (1959) Flore de l'Afrique du Nord. Édition Paul Lechevalier, Paris, Vol. 6, 51–76.

Marques I, Feliner GN, Munt DD, Aguilar JF (2010) Unraveling cryptic reticulate relationships and the origin of orphan hybrid disjunct populations in *Narcissus*. Evolution 64(8): 2353–2368. https://doi.org/10.1111/j.1558-5646.2010.00983.x

Marques I, Fuertes Aguilar J, Martins-Louçao MA, Moharrek F, Nieto Feliner G (2017) A three-genome five-gene comprehensive phylogeny of the bulbous genus *Narcissus* (Amaryllidaceae) challenges current classifications and reveals multiple hybridization events. Taxon 66(4): 832–854. https://doi.org/10.12705/664.3

Marques I, Nieto Feliner G, Martins-Loução MA, Fuertes Aguilar J (2012) Genome size and base composition variation in natural and experimental *Narcissus* (Amaryllidaceae) hybrids. Annals of Botany 109(1): 257–264. https://doi.org/10.1093/aob/mcr282

Maugini E (1953) Citosistematica di alcuni Narcisi della flora italiana appartenenti alla Sez. Hermione. Caryologia 5(3): 313–341. https://doi.org/10.1080/00087114.1953.10797449

Mifsud S, Caruana E (2010) Records of *Narcissus elegans* (Fam. Amaryllidaceae) and notes on the wild *Narcissus* in the maltese islands. The Central Mediterranean Naturalist 5(2): 19–29.

Munby G (1847) Flore d'Algérie ou Catalogue des plantes indigènes du Royaume d'Alger. Edition J. B. Baillière, Alger, Montpellier, 120 pp.

Paszko B (2006) A critical review and a new proposal of karyotype asymmetry indices. Plant Systematics and Evolution 258: 39–48. https://doi.org/10.1007/s00606-005-0389-2

Peruzzi L, Eroğlu HE (2013) Karyotype asymmetry: again, how to measure and what to measure? Comparative Cytogenetics 7: 1–9. https://doi.org/10.3897/compcytogen.v7i1.4431

Phitos D, Kamari G (1974) Cytotaxonomische Beiträge zur Flora von Kreta. Botaniska Notiser. 127: 302–308.

Quézel P, Santa S (1962) Nouvelle flore de l'Algérie et des régions désertiques méridionales. Edition du CNRS, Paris, Tome 1, 216–218.

Romero Zarco C (1986) A new method for estimating karyotype asymmetry. Taxon 35(3): 526–530. https://doi.org/10.2307/1221906

Rønsted N, Savolainen V, Mølgaard P, Jäger AK (2008) Phylogenetic selection of *Narcissus* species for drug discovery. Biochemical Systematics and Ecology. 36(5–6): 417–422. https://doi.org/10.1016/j.bse.2007.12.010

Samaropoulou S, Bareka P, Kamari G (2013) *Narcissus papyraceus*. In: Kamari G, Blanche C, Siljak-Yakovlev S (Eds) Mediterranean Chromosome Number Reports. Flora Mediterranea 23: e287. https://doi.org/10.7320/FlMedit23.255

Santos-Gally R, Vargas P, Arroyo J (2012) Insights into Neogene Mediterranean biogeography based on phylogenetic relationships of mountain and lowland lineages of *Narcissus* (Amaryllidaceae): Historical biogeography of *Narcissus*. Journal of Biogeography 39(4): 782–798. https://doi.org/10.1111/j.1365-2699.2011.02526.x

Sharma AK, Sharma A (1961) Chromosome studies of some varieties of *Narcissus tazetta* L. Caryologia 14(1): 97–106. https://doi.org/10.1080/00087114.1961.10796014

Stebbins GL (1971) Chromosomal evolution in higher plants. Edward Arnold, London, 216 pp.

Sun X, Sun Q, Yang H, Zhang L, Wang Y (2015) Karyotype analysis in seven cultivars of *Narcissus* spp. Caryologia, 68(1): 63–68. https://doi.org/10.1080/00087114.2015.1013338

Troia A, Orlando AM, Baldini MR (2013) *Narcissus obsoletus* and *N. elegans*. In: Kamari G, Blanche C, Siljak-Yakovlev S (Eds) Mediterranean Chromosome Number Reports. Flora Mediterranea 23: 282–284. https://doi.org/10.7320/FlMedit23.255

Watanabe K, Yahara T, Denda T, Kosuge K (1999) Chromosomal evolution in genus *Brachyscome* (Asteraceae, Astereae): statistical tests regarding correlation between changes in karyotype and habit using phylogenetic information. Journal of Plant Research 112: 145–161. https://doi.org/10.1007/PL00013869

Zonneveld BJM (2008) The systematic value of nuclear DNA content for all species of *Narcissus* L. (Amaryllidaceae). Plant Systematics and Evolution 275(1–2): 109–132. https://doi.org/10.1007/s00606-008-0015-1

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CompCytogen 16(1): 77–92 (2022)
doi: 10.3897/compcytogen.v16.i1.79953
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotypic analysis and isolation of four DNA markers of the scleractinian coral *Favites pentagona* (Esper, 1795) (Scleractinia, Anthozoa, Cnidaria)

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Academic editor: V. Stunžėnas | Received 30 December 2021 | Accepted 5 March 2022 | Published 5 April 2022

http://zoobank.org/D94E9294-570F-4136-BCB3-842AC4757759

Citation: Kawakami R, Taguchi T, Vacarizas J, Ito M, Mezaki T, Tominaga A, Kubota S (2022) Karyotypic analysis and isolation of four DNA markers of the scleractinian coral *Favites pentagona* (Esper, 1795) (Scleractinia, Anthozoa, Cnidaria). Comparative Cytogenetics 16(1): 77–92. https://doi.org/10.3897/compcytogen.v16.i1.79953

Abstract

We performed conventional and molecular cytogenetic studies on the *Favites pentagona* Esper, 1795, a scleractinian coral mostly found along the west coast of Japan. Karyotype analysis of *F. pentagona* by G-banding revealed a karyogram containing a homogenously staining region (HSR) on chromosome 10 in more than 50% of the examined metaphase spreads. This HSR consisted of sequences from 18S ribosomal RNA (rRNA) genes, as demonstrated by fluorescence in situ hybridization (FISH) and DNA sequencing. We highlighted the development of four chromosomal FISH markers from repetitive genes such as U2 small nuclear RNA linked to 5S rRNA sequence (U2 snRNA-5S), 18S rRNA, histone H3, and uncharacterized gene FP-9X. The chromosomal locations of the U2 snRNA-5S and 18S RNA were on the terminal end of long arm of chromosomes 2 and 10, respectively, while the histone H3 and the uncharacterized gene were located near the centromeres of chromosomes 1 and 9, respectively. These FISH markers will

improve the karyotyping of *F. pentagona* from mitotic preparations which helps in widening our understanding of coral genetic structure and chromosome organization. In addition, these improvements in karyotyping will provide the basis in constructing of chromosome-level genome assembly for *F. pentagona*.

Keywords
chromosome, FISH, histone, HSR, karyotype, rRNA, scleractinian coral

Introduction

Cytogenetic information from karyotypic analysis gives us a deeper understanding the way genetic material is packaged inside a nucleus of a cell and how certain genetic diseases are associated with chromosome defects and aberrations (Testa 1990; Super 1991). Cytogenetic information is also valuable in understanding the genome structure and organization of an individual which may vary greatly within and across species (Sullivan 2020). These variations in chromosome characteristics will give insight into their evolutionary process as they reflect genome shuffling, translocation, and chromosomal duplication/deletion (Guo et al. 2018). This information might be valuable for stony corals, in which taxonomic classification poses a great challenge as the integration of its morphological and molecular characteristics often reveal conflicting results (Fukami et al. 2004). This difficulty might be caused, in part, by hybridization of closely related species and by morphological plasticity. Confusion about the stony coral taxonomy necessitates the search for new coral characters such as cytogenetic information to understand coral systematics and evolution. However, the cytogenetic information of stony corals, such as karyotypes and gene maps, is limited. To date, molecular cytogenetic information on stony corals has only been reported for five species from three different families (2 species from Acroporidae, 2 species from Merulinidae, and 1 species from Lobophyliidae). These are *Acropora solitaryensis* Veron et Wallace,, 1984 and *Acropora pruinosa* Brook, 1893 (Acroporidae), *Coelastrea aspera* Verrill, 1866 and *Platygyra contorta* Veron, 1990 (Merulinidae), and *Echinophyllia aspera* Ellis et Solander, 1786 (Lobophylliidae) (Taguchi et al. 2013, 2014, 2016, 2017, 2020; Vacarizas et al. 2021). In those studies, new cytogenetic evidence was presented, including information regarding chromosome numbers, rRNA gene loci, the presence of a homogenously staining regions (HSR), and some repeated sequences shared with human satellite DNA.

In this study, we reported the detailed molecular cytogenetic analysis of stony coral *Favites pentagona* Esper, 1758, from family Merulinidae. *F. pentagona* is commonly observed along the west coast of Japan (Veron, 2000). Colonies of *F. pentagona* range from massive, encrusting to columnar forms. The valleys with colonies are usually long and relatively straight at colony margins, becoming increasingly short, sinuous, and contorted towards the colony center; septa have thin walls and are highly irregular (Veron, 2000).

Cytogenetic analysis of *F. pentagona* was conducted using conventional and molecular cytogenetic techniques, such as fluorescence in situ hybridization (FISH). We

identified a homogenously staining region (HSR) on *F. pentagona* chromosome 10 using G-banding and 4',6-diamidino-2-phenylindole (DAPI) staining, followed by karyotyping. Furthermore, the chromosomal locations of four tandemly repetitive genes were identified for *F. pentagona*. The development of four FISH markers was described, and the FISH signals of each gene were characterized showing the effectivity of these markers to identify specific chromosomes from mitotic cells.

Materials and methods

Coral collection

F. pentagona gametes were collected from spawning colonies at Nishidomari (32°46'N, 132°43'E), Kochi Prefecture, Japan (Fig. 1). The release of gamete bundles was observed between 8:00 pm and 9:30 pm on July 24, 2019. Coral bundles were collected using plastic cups placed over the colonies during spawning. After collection, eggs and sperm from the spawned bundles were separated. The separated gametes were then transferred to a new container to allow fertilization. Successful cell divisions were observed under the light microscope. Embryos were then rinsed in 0.2 μm filtered seawater (ADVANTEC cartridge filter; Advantec Toyo Corp., Tokyo, Japan) to remove external contaminants.

Chromosome preparations and G-banding

Coral chromosome preparations were conducted according to the method described by Taguchi et al. (2013). About 10–14 hours after artificial fertilization, embryos were treated in filtered seawater supplemented with 0.01% (v/v) colchicine (Sigma, St. Louis, MO, USA)

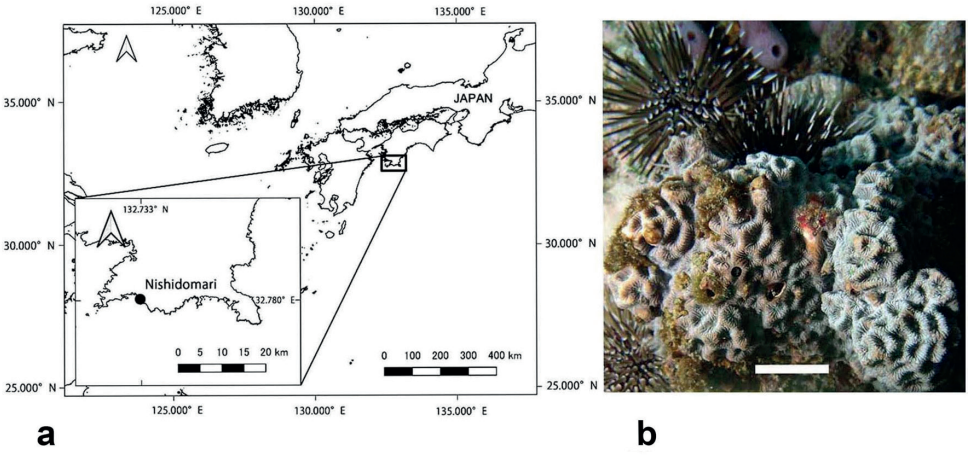

Figure 1. Map of the sampling site of *Favites pentagona* in Shikoku Island. Nishidomari, Otsuki-cho, Hata county, Kochi 788-0333, Japan (32°,46'44"N, 132°43'57"E) (**a**). Inset: Enlarged image of the collection area. Appearance of *Favites pentagona* in the sea (**b**). Scale bar: 2 cm.

for 1 h, followed by treatment with hypotonic solution (0.5 × sea water; diluted in distilled water) to spread the chromosomes. Embryos were fixed using a freshly prepared fixative containing absolute methanol and glacial acetic acid (3:1). Fixed embryos were soaked in diethyl ether overnight to remove intracellular lipids and incubated again in the fixative. Approximately 20 to 50 embryos were isolated using a fine needle to tear the embryos apart into their constituent cells. Suspensions containing embryo cells were transferred into a 1.5 ml tube filled with the fixative. The tube was centrifuged at 2000 *g* for 2 min and the pellet was re-suspended in 0.5 ml of fresh fixative. A drop containing separated cells was placed on a clean slide and then air- or flame-dried to spread the chromosomes. For G-banding, slides were treated with 0.025% trypsin solution at room temperature (approximately 25 °C) for 1 min (Seabright, 1973) and then stained with 5% Giemsa.

DNA extraction

Genomic DNA was extracted from *F. pentagona* sperm (approximately 0.1 ml) using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

PCR, DNA cloning and FISH probe preparation

Target genes (18S rRNA, U2 snRNA-5S, histone H3, and uncharacterized gene FP-9X) were amplified by PCR using the Emerald PCR master mix (Takara, Japan). The primer sets used are shown in Table 1. PCR was performed in a thermal cycler (WK-0518, Wako, Osaka, Japan) under the following conditions: initial denaturation for 2 min at 98 °C, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. PCR products were ligated into the pGEM-T Easy Vector (Promega, Madison, USA) and 30 ng of the ligation products were used to transform competent cells (JM109, pGMT-T Easy-Vector Systems, Promega). Transformed cells were plated onto Luria broth (LB) plates containing 100 μg/ml ampicillin, 40 μg/ml 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-Gal) and 0.05 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). Isolated colonies were screened by FISH. Probes using FISH screening were prepared by random prime labeling with digoxigenin-12-dUTP (DIG-dUTP) or cyanine-3-dUTP (Cy3-dUTP) in accordance with the kit

Table 1. PCR primer sets used in this study.

Primer set	Genes	Sequence (5'-3')	Reference	Species
1	18S rRNA	F-GGTTGATCCTGCCAGTAGTCATATGCTTG	Rowan and Powers (1992)	Zooxanthella
		R-GATCCTTCCGCAGGTTCACCTACGGAAACC		
2	histone H3	F-ATGGCTCGTACCAAGCAGACVGC	Huang et al., (2011)	Stony coral
		R-ATATCCTTRGGCATRATRGTGAC		
3	U2 snRNA-5S	F-CTTCCGTGATCGGACGAGAA	Stover and Steele (2001)	Hydra
		R-TATAATATTGGAACAGAATT		
4	Uncharacterized gene FP-9X	F-CTTCCGTGATCGGACGAGAA	Stover and Steele (2001)	Hydra
		R-CCAATTTTGTAGACATCTTGAAG	Kawaida et al., (2010)	Hydra

protocol (Invitrogen, Tokyo, Japan). Then, FISH-positive clones were later transferred into 15 ml test tubes containing 1.5 ml of LB/ampicillin medium and grown at 37 °C overnight. Plasmids from the resulting clones were extracted according to the manufacturer's protocol using a Mini Plus Plasmid DNA Extraction System (Viogene, NACALAI TESQUE, INC., Kyoto, Japan).

DNA sequencing and homology search

DNA inserts from plasmids were sequenced with the M13 forward and reverse primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (GE Healthcare, Japan) and ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific, Tokyo, Japan). DNA sequences were aligned, and homology searches were performed using Gapped BLAST and PSI-BLAST: a new generation of protein database search programs to search the GenBank database (http://www.ddbj.nig.ac.jp).

FISH analysis

FISH analysis was performed as previously reported (Taguchi et al. 1993), with slight modifications. Metaphase preparations were denatured in 70% formamide/2x Saline-sodium citrate (SSC) at 70 °C for 2 min; 0.8 µl of the prepared probe was mixed with 10 µl of hybridization solution (H7782, Sigma, Japan) and denatured at 82 °C for 10 min. Hybridization was performed at 37 °C in a CO_2 incubator for 12–15 h. After hybridization, samples were washed twice in 2x SSC and 1x Phosphate buffered detergent (PBD; 0.05% Tween20/4xSSC). Chromosomes were counterstained with DAPI.

Image acquisition and processing

FISH slides were examined under a fluorescence microscope (Olympus BX-50, Tokyo, Japan) equipped with a cooled charge-coupled device. Images of suitable metaphase spreads were acquired using an Olympus DP70 workstation and the FISH analysis software. The mirror units used for each fluorescence light (FITC, Cy-3, and DAPI) were U-NIBA, U-MWU, and U-MWIB (Olympus), respectively.

Results

Diploid karyotypes in *F. pentagona*

Chromosomes in metaphase cells were karyotyped by conventional trypsin G-banding, and an HSR in terminal end of one of the chromosomes was observed in approximately 50% of the observed metaphase spreads (Fig. 2). HSRs were shown in G-banded karyograms as long and lightly stained region of the chromosomes. The HSRs were also revealed using the inverted images of DAPI fluorescent staining showing the similar characteristics (Fig. 2, inset). Chromosomes were arranged in decreasing order

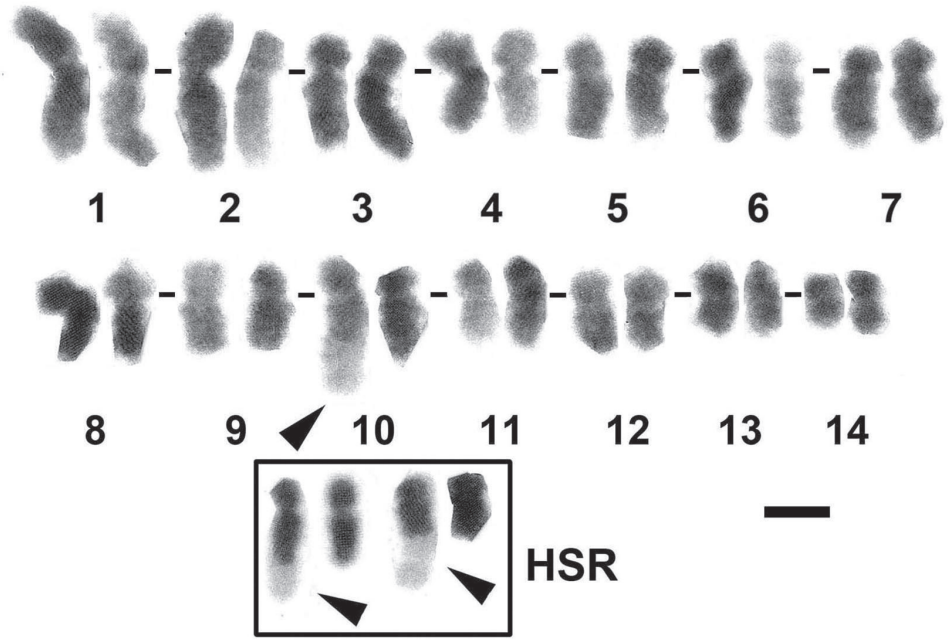

Figure 2. G-banded karyogram of *Favites pentagona* (2n = 28). The arrow indicates an HSR. Inset: two chromosome pairs with HSRs from two other metaphase spreads stained with DAPI (inverted images). One of the homologues has an HSR in each pair (arrowheads). Scale bar: 2 μm.

Table 2. Relative lengths and centromere indices of the 14 chromosome pairs, shown as means and standard deviations obtained from the eight metaphase spreads.

Chromosome number	Short arm (μm)	Long arm (μm)	Total length (μm)	Arm ratio	Overall length ratio	Chromosome type*
1	2.21±0.75	3.94±0.95	6.15±1.5	1.88±0.57	0.94±0.08	sm
2	1.52±0.48	3.3±0.82	4.82±1.12	2.3±0.71	0.74±0.06	sm
3	1.55±0.52	2.91±0.83	4.46±1.08	1.99±0.62	0.68±0.04	sm
4	1.48±0.53	2.74±0.62	4.22±0.98	1.98±0.61	0.65±0.04	sm
5	1.41±0.35	2.63±0.74	4.04±0.95	1.92±0.48	0.62±0.03	sm
6	1.49±0.26	2.36±0.74	3.85±0.9	1.6±0.38	0.59±0.03	m
7	1.39±0.26	2.34±0.62	3.74±0.8	1.7±0.36	0.57±0.04	sm
8	1.47±0.38	2.15±0.43	3.62±0.79	1.49±0.17	0.56±0.03	m
9	1.32±0.28	2.14±0.51	3.46±0.73	1.64±0.32	0.53±0.04	m
10	1.26±0.23	2.05±0.49	3.31±0.67	1.63±0.26	0.51±0.04	m
11	1.31±0.3	1.9±0.41	3.21±0.63	1.49±0.33	0.5±0.04	m
12	1.2±0.34	1.85±0.4	3.05±0.66	1.6±0.33	0.47±0.04	m
13	1.12±0.23	1.53±0.35	2.65±0.49	1.41±0.38	0.41±0.04	m
14	0.94±0.23	1.25±0.27	2.2±0.45	1.37±0.31	0.34±0.05	m

*Types were categorized according to the reference of Levan et al. (1964).
m: metacentric, sm: submetacentric. The part of an HSR on chromosome 10 was excluded.

of chromosome length from 1 to 14, which revealed that the chromosome with HSR is chromosome 10 (Fig. 2). The length and arm ratio of chromosomes were measured using eight metaphase spreads, as summarized in Table 2. The modal number of chromosomes per metaphase spread, which determined from 100 examined *F. pentagona*

cells, was 28 (2n = 28). The percentage of metaphase spreads with an HSR from all cells was greater than 50% (43/80). Based on the arm ratio (Levan et al. 1964), this karyogram consisted of six submetacentric (1, 2, 3, 4, 5 and 7) and eight metacentric (6, 8, 9, 10, 11, 12, 13, and 14) chromosomes (Table 2).

Physical mapping of four FISH markers

FISH signals for the 18S rRNA gene locus were identified at the terminal ends of the long arm of chromosome 10 (Fig. 3a, c). The HSR, previously observed on chromosome 10 by G-banding, is hybridized by 18S rRNA gene probe, exhibiting a broad and intense hybridization signal (Fig. 3a and c, green signals indicated by arrows). The HSR was also recognized as a pale part of chromosome 10 in DAPI staining metaphase (Fig. 3b, arrow). FISH probe from the uncharacterized gene FP-9X was mapped on the centromeres of chromosome 9 (Fig. 3a, c, red signals indicated by arrowheads,). The histone H3 gene, on the other hand, was mapped near the centromere of the long arm of chromosome 1 (Fig. 4a, c), whereas the U2 snRNA-5S gene locus was mapped on the terminal ends of the long arm of chromosome 2 (Fig. 5a, c).

Cloning and Sequence analysis of FISH probes

Cloning and sequencing were performed for the amplicons from which FISH probes were prepared. Positive clones were designated as FP-18S for 18S rRNA gene, FP-H3 for histone H3 gene, FP-U2-5S for U2 snRNA-5S, and FP-X9 for uncharacterized gene FP-X9. Sequence analysis of FP-18S clone (1,732 bp) with the GenBank database revealed a difference of a single nucleotide from the partial sequence of *F. pentagona* 18S rRNA gene (Accession No. LC644154). The FP-H3 clone (329 bp) completely matched with the partial sequence of the *F. pentagona* histone H3 gene (Accession No. LC644156). The FP-U2-5S (824 bp) sequence contained U2 spliceosomal small nuclear RNA (snRNA) gene sequence and region of 5S rRNA gene (Accession No. LC644155) (Figs 6A, 7). Lastly, the FP-X9 (357 bp) (Accession No. LC644157) showed homology to the 5'-untranslated region (UTR) of the uncharacterized mRNA of *Orbicella faveolata* (Accession No. XM020759959) (Fig. 6B).

Discussion

To solve the difficulties in taxonomically classifying stony corals (Fukami et al. 2004) and promote research on coral genetics, we have accumulated molecular cytogenetic data on several coral species. At this point, we have published six molecular cytogenetic reports with both conventional and molecular cytogenetic analyses of scleractinian corals, such as *Acropora solitaryensis*, *Acropora pruinosa*, *Echinophyllia aspera*, *Coelastrea aspera*, and *Platygyra contorta* (Taguchi et al. 2013, 2014, 2016, 2017, 2020; Vacarizas 2021), which are commonly found on the western coast of Japan (Wallace, 1999). The present study focuses on *F. pentagona*, belonging to the genus *Favites* and the family Merulinidae.

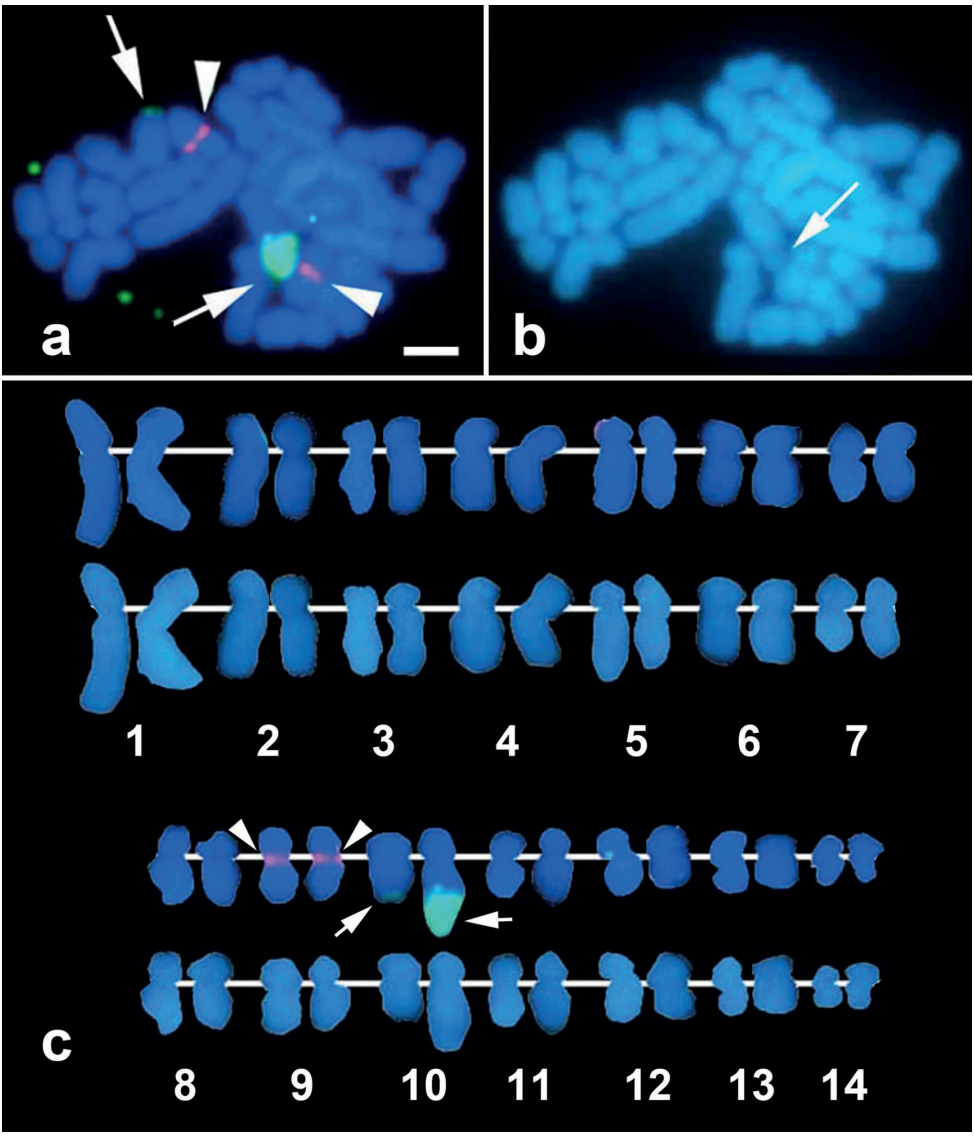

Figure 3. FISH image showing hybridization signals of uncharacterized gene FP-9X probe (red; arrowheads) and 18S rRNA gene probe (green; arrows) (**a**). Chromosomes were karyotyped according to size and centromere positions showing uncharacterized gene FP-X9 and 18S rRNA gene probe hybridization on chromosome 9 and chromosome 10, respectively (**c**, above alignment). DAPI-only channel revealing the HSR region pointed by the arrow (**b**) and its karyogram (**c**, below alignment). Scale bar: 2 μm.

We carried out conventional G-bandings to establish the karyotype of *F. pentagona* embryos. In general, obtaining high-quality G-banding in invertebrate chromosomes is difficult because of the relatively small chromosome size and the weak effect of trypsin on G-banding. Karyotyping of *F. pentagona* revealed the three chromosome groups that cannot be precisely identified because of their similar lengths. Furthermore, differences

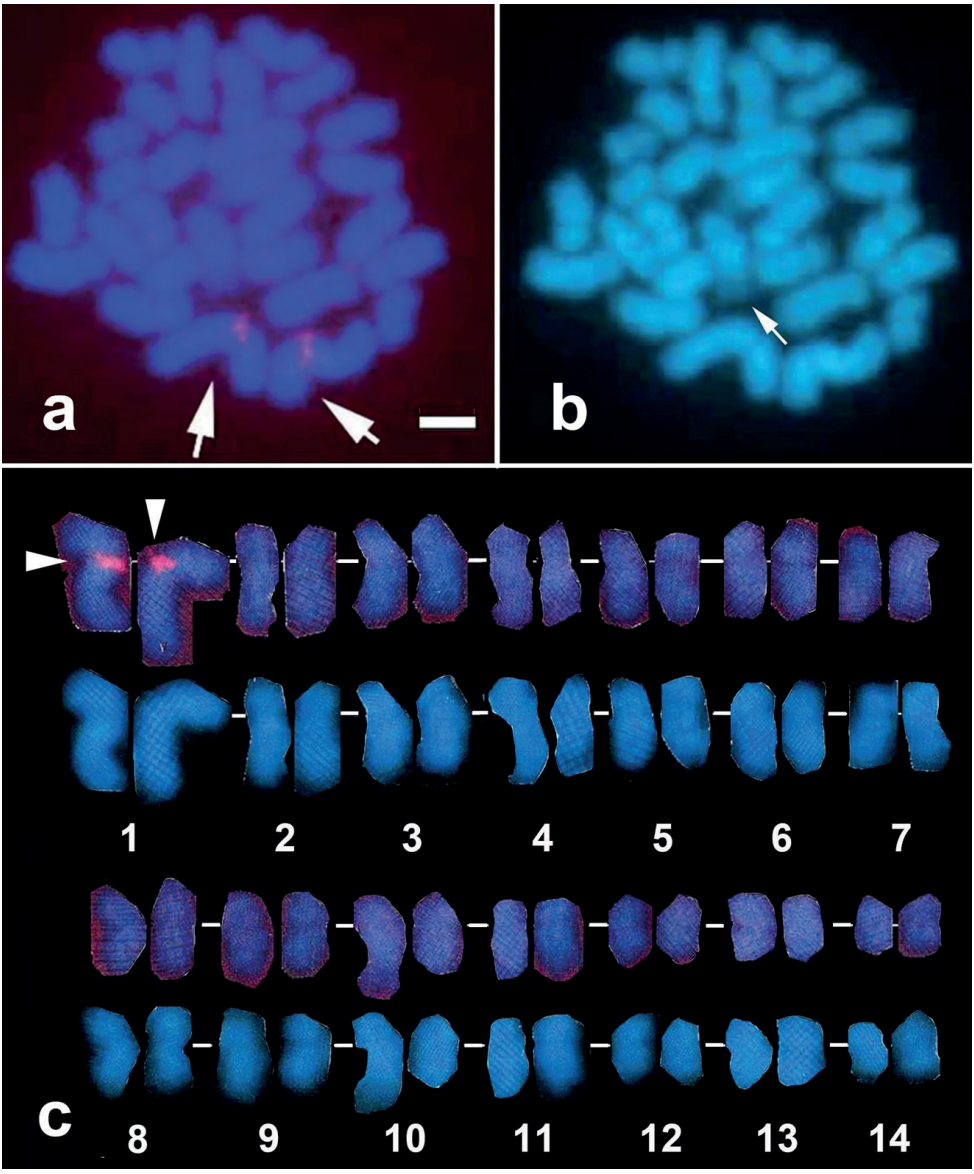

Figure 4. FISH image showing hybridization signal of histone H3 gene probe (red; arrowheads) (**a**). Chromosomes were karyotyped according to size and centromere positions showing the hybridization signal on chromosome 1 (**c**, above alignment). DAPI-only channel revealing the HSR region pointed by the arrow (**b**) and its karyogram (**c**, below alignment). Scale bar: 2 μm.

in chromosome condensation depending on the stage of the cell cycle at which cells were fixed sometimes made it difficult to measure the precise lengths of chromosomes. To develop a coral chromosome study, it is necessary to identify each chromosome precisely using a painting probe (Gokhman et al. 2019). Nonetheless, G-banded chromosomes revealed the presence of HSRs. HSRs are created by the amplification

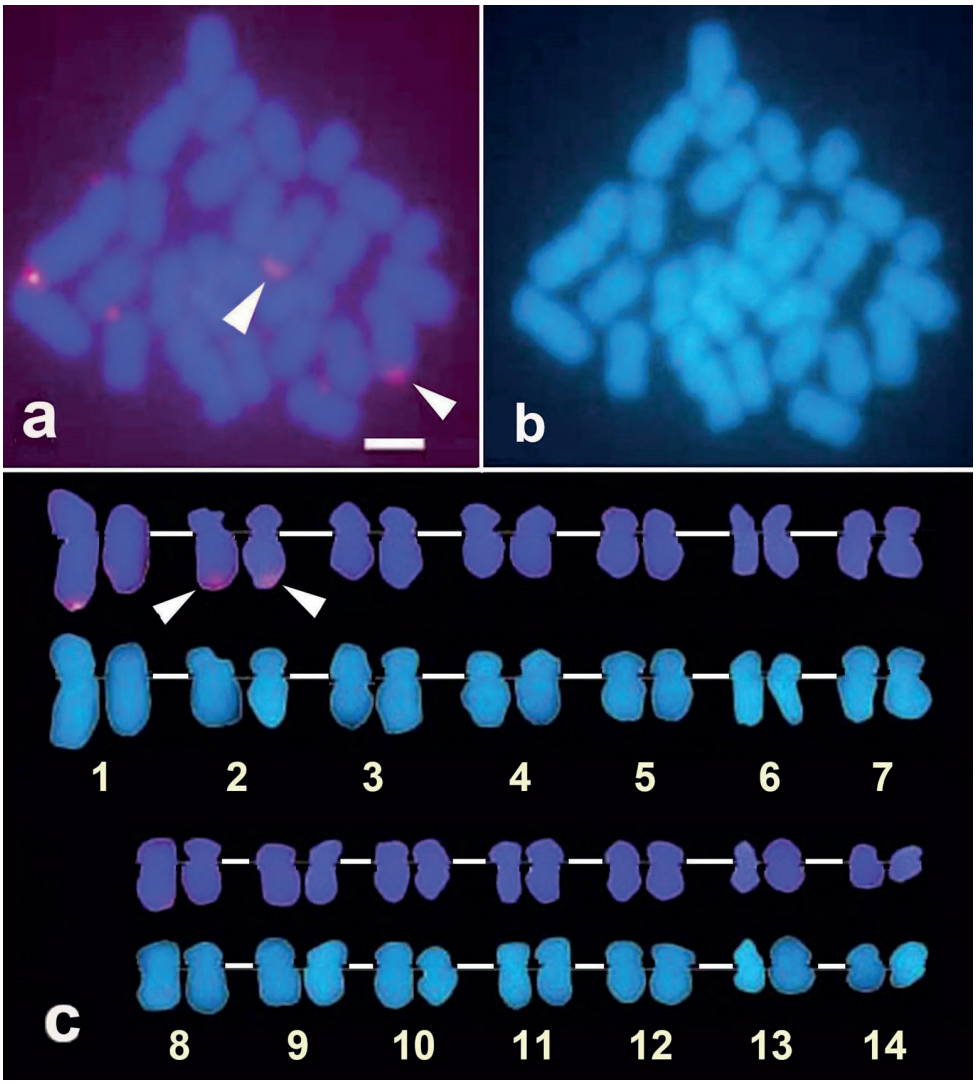

Figure 5. FISH image showing hybridization signal of U2 snRNA-5S gene probe (red; arrowheads) (**a**). Chromosomes were karyotyped according to size and centromere positions showing hybridization signal on chromosome 2 (**c**, above alignment). Few background signals were seen; one background signal was on long arm telomere of chromosome 1 (**a** and **c**). DAPI-only channel without HSR region (**b**) and its karyotype (**c**, below alignment). Scale bar: 2 μm.

and accumulation of certain DNA region within chromosomes. The amplification can be detected by conventional G-banding and FISH using locus-specific probes, which show intense hybridization signals on a single chromosome, as opposed to two copies with normal-homologous chromosomes (Biedler and Spencer 1976). In this study, we demonstrated that the HSR in the *F. pentagona* chromosomes is composed mainly of sequences from 18S rRNA gene. Generally, genetic amplification at the chromosomal level is manifested in the form of HSRs in tumor cells (Takaoka et

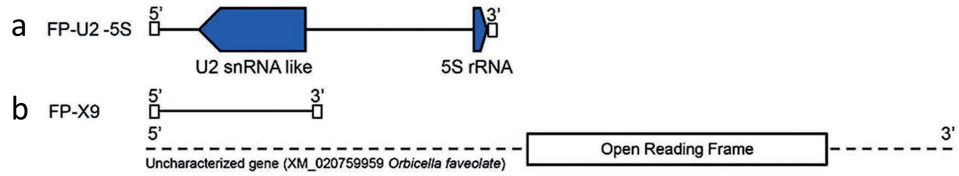

Figure 6. Schematic diagram of FISH probe sequences of FP-U2-5S which contains the U2 spliceosomal snRNA-like region and partial sequence of 5S rRNA gene sequence (**a**). FP-X9 is similar to the 5'-untranslated region of uncharacterized gene of a Merulinidae coral (*Orbicella faveolata* (Ellis et Solander, 1786)) (**b**). Rectangles in both ends of bars show the primer positions and relative lengths.

al. 2012). We arranged each chromosome in decreasing order of their lengths and located the long chromosome 10 with an HSR. In our previous studies (Taguchi et al. 2013, 2016, 2017), using G-banding and FISH analyses, we revealed the presence of HSRs in chromosomes 11, 12, and 13 in each of the following three corals, *C. aspera*, *E. aspera*, and *P. contorta*. Interestingly, based on our recent studies on five stony coral species, including *F. pentagona*, HSRs are commonly found in the coral chromosomes of non-*Acropora* species. This indicates that the presence of an HSR is a cytogenetic characteristic of certain taxa of stony corals (Taguchi et al. 2013, 2014, 2016, 2017, 2020; Vacarizas et al. 2021).

The FISH marker for *histone H3* gene was observed at the centromeric region of chromosome 1. The core histone genes are highly conserved and repetitive, and their loci can thus be detected using FISH probes containing the sequence of a single array composed of tandem repeats (Huang et al. 2011). Previous molecular cytogenetic study on stony coral *Acropora pruinosa* has shown that the core histone gene locus which contains the H2a and H2b sequences were on the centromeric region of chromosome 8 (Vacarizas et al. 2021). These results imply that the chromosomal location of a highly conserved core histone varies across different family (Acroporidae and Merulinidae) of order Scleractinia (stony corals).

Information on 5S rDNA gene among stony corals is very limited (Taguchi et al. 2017; Taguchi et al. 2020; Vacarizas et al. 2021). We therefore developed the 5S rRNA gene primers reported not only in scleractinian corals, but also from other distant taxa such as hydra, starfish, and jellyfish (Hori et al. 1982; Walker and Doolittle 1983; Hendriks et al. 1987; Stover and Steele 2001) in which cytogenetic studies have identify specific FISH markers for 5S rRNA gene. The 5S rRNA gene primer from *Hydra* utilized by Stover and Steele (2001) were used to amplify suitable FISH probe sequences for this coral *F. pentagona*. Surprisingly, the sequence contain not only sequence of 5S rRNA gene but also the complete region of the U2-snRNA. The 5S rRNA gene and the U2-snRNA gene sequence were homologous to those of *C. aspera* (LC120341) and *A. pruinosa* (LC 557013), respectively (Fig. 6). In this study, we showed that chromosomal location of this U2 snRNA-5S of *F. pentagona* was near the telomere region of chromosome 2. This is different from the chromosomal location of 5S-snRNA in *C. aspera* (Taguchi et al. 2017) and *A. pruinosa* (Vacarizas et al. 2021) chromosomes. Based on the alignments (red rectangle in Fig. 7), several insert nucleotide sequences were found only in the *U2 snRNA* gene of *F. pentagona*. This suggests

U2 snRNA
5S rDNA

that *U2 snRNA* and *5S rRNA* genes seemed to be fused, as cloning-sequence analysis and the sequence of the cloned insert is a part of the pseudogene.

Each of the FISH markers derived from the four different amplicons (FP-18S, FP-H3, FP-U2-5S, and FP-X9) were observed at a single site (locus), even in uncontracted prometaphase spreads with elongated chromosomes. As the specificity of the markers is very high due to the highly repetitive and conserved nature of these genes, these markers will be useful for karyotyping and identifying specific chromosomes containing similarity of sequences within closely related species.

Conclusion

The series of cytogenetic studies on stony corals including the development of specific FISH markers may contribute to understand changes in chromosomal structures across taxa thereby gain insight into its evolutionary processes (Guo et al. 2018). Thus, these results might shed light on coral diversity and improve the classification of corals. However, it is important to develop more suitable FISH markers which can identify specific gene loci. In this study, we isolated FISH markers that provided distinct and bright hybridization signals on chromosomes that suitable for routine observation.

We highlighted the development of four chromosomal FISH markers for U2 snRNA-5S, 18S rRNA, histone H3, and uncharacterized FP-9X genes, which were found to be located on chromosomes 2, 10, 1, and 9, respectively. The loci of the gene histone H3 were mapped in the chromosomes of stony corals for the first time. The isolation of four FISH markers for *F. pentagona* will also promote gene mapping and understand genome structure and organization for this species. This cytogenetic information on stony coral along with morphological and molecular characteristics may contribute to understand its evolutionary processes and resolve taxonomic problems in stony coral taxonomy.

Authors' contribution

TT: designed and performed experiments, organized the figures, and drafted manuscript. RK, MI, and JV: performed experiments, organized the figures and the tables. TM: did sampling and identification of the specimens. RK and JV: corrected the manuscript. AT, SK, and TT: supervised the research and corrected the manuscript. All authors read, discussed, and approved the final version of the manuscript.

Figure 7. Comparison of FP-U2-5S sequence to *Coelastrea aspera* (Acession No. LC120342) and *Acropora pruinosa* (Acession No. LC557013) sequences. 5S rDNA sequences are highly conserved (793–823). There are 6 inserts in FP-U2-5S sequence (red rectangles: 102–107, 125–134, 165–208, 213–218, 225–238 and 246–250).

Acknowledgements

This work was supported by grants from the Japan Society for the Promotion of Science (15K14789, 17H03861, and 21K05734: all to T.T.).

References

Biedler JL, Spengler BA (1976) Metaphase chromosome anomaly: association with drug resistance and cell-specific products. Science 16 (191): 185–187. https://doi.org/10.1126/science.942798

Fukami H, Budd AF, Paulay G, Solé-Cava A, Chen CA, Iwao K, Knowlton N (2004) Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. Nature 427: 832–835. https://doi.org/10.1038/nature02339

Gokhman VE, Cioffi MB, König C, Pollmann M, Gantert C, Krogmann L,Steidle JLM, Kosyakova N, Liehr T, Al-Rikabi A (2019) Microdissection and whole chromosome painting confirm karyotype transformation in cryptic species of the *Lariophagus distinguendus* (Förster, 1841) complex (Hymenoptera: Pteromalidae). PLoS ONE 14(11): e0225257. https://doi.org/10.1371/journal.pone.0225257

Guo L, Accorsi A, He S, Guerrero-Hernández C, Sivagnanam S, McKinney S, Gibson M, Alvarado AS (2018) An adaptable chromosome preparation methodology for use in invertebrate research organisms. BMC Biology 16: e25. https://doi.org/10.1186/s12915-018-0497-4

Hendriks L, De Baere R, Vandenberghe A, De Wachter R (1987) The nucleotide sequences of 5S ribosomal RNA of *Actinia equine* and *Sepia officinalis*. Nucleic Acids Research 15: e2773. https://doi.org/10.1093/nar/15.6.2773

Hori H, Ohama T, Kumazaki T, Osawa S (1982) Nucleotide sequences of 5S rRNAs from four jellyfishes. Nucleic Acids Research 10: 7405–7408. https://doi.org/10.1093/nar/10.22.7405

Huang D, Licuanan WY, Baird AH, Fukami H (2011) Cleaning up the 'Bigmessidae': Molecular phylogeny of scleractinian corals from Faviidae, Merulinidae, Pectiniidae and Trachyphylliidae. BMC Evolutionary Biology 11: e37. https://doi.org/10.1186/1471-2148-11-37

Kawaida H, Shimizu H, Fujisawa T, Tachida H, Kobayakawa Y (2010) Molecular phylogenetic study in genus *Hydra*. Gene 468: 30–40. https://doi.org/10.1016/j.gene.2010.08.002

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Rowan R, Powers DA (1992) Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). Proceedings of the National Academy of Sciences of the United States of America 89(8): 3639–3643. https://doi.org/10.1073/pnas.89.8.3639.

Seabright M (1973) Improvement of trypsin method for banding chromosomes. Lancet 2: 1249–1250. https://doi.org/10.1016/S0140-6736(73)90561-8

Stover NA, Steele RE (2001) Trans-spliced leader addition to mRNAs in a cnidarian. Proceedings of the National Academy of Sciences of the United States of America 98: 5693–5698. https://doi.org/10.1073/pnas.101049998

Sullivan BA (2020) A sampling of methods to study chromosome and genome structure and function. Chromosome Research 28: 1–5. https://doi.org/10.1007/s10577-020-09629-y

Super M (1991) Reviews in Medicine: Medical genetics. Postgraduate Medicine Journal 67: 613–631. https://doi.org/10.1136/pgmj.67.789.613

Taguchi T, Bellacosa A, Zhou JY, Gilbert DJ, Lazo PA, Copeland NG, Jenkins NA, Tsichlis PN, Testa JR (1993) Chromosomal localization of the Ox-44 (CD53) leukocyte antigen gene in man and rodents. Cytogenetics and Cell Genetics 64: 217–221. https://doi.org/10.1159/000133580

Taguchi T, Kubota S, Mezaki T, Sekida S, Okuda K, Nakachi S, Shinbo S, Iiguni Y, Tominaga A (2013) Detection of characteristic heterochromatin distribution, highly amplified rRNA genes and presence of the human satellite III DNA motif in the scleractinian coral *Echinophyllia aspera* Ellis and Solander 1788. Chromosome Science 16: 33–38.

Taguchi T, Mezaki T, Iwase F, Sekida S, Kubota S, Fukami H, Okuda K, Shinbo T, Oshima S, Iiguni Y, Testa JR, Tominaga A (2014) Molecular cytogenetic analysis of the scleractinian coral *Acropora solitaryensis* Veron & Wallace 1984. Zoological Science 31: 89–94. https://doi.org/10.2108/zsj.31.89

Taguchi T, Kubota S, Mezaki T, Tagami E, Sekida S, Nakachi S, Okuda K, Tominaga A (2016) Identification of homogeneously staining regions by G-banding and chromosome microdissection, and FISH marker selection using human Alu sequence primers in a scleractinian coral *Coelastrea aspera* Verrill, 1866 (Cnidaria). Comparative Cytogenetics 10: 61–75. https://doi.org/10.3897/CompCytogen.v10i1.5699

Taguchi T, Kubota S, Tagami E, Mezaki T, Sekida S, Okuda K, Tominaga A (2017) Molecular Cytogenetic Analysis and Isolation of a 5S rRNA-Related Marker in the Scleractinian Coral *Platygyra contorta* Veron 1990 (Hexacorallia, Anthozoa, Cnidaria). Cytologia 82(2): 205–212. https://doi.org/10.1508/cytologia.82.205

Taguchi T, Tagami T, Mezaki T, Vacarizas JM, Canon KL, Avila TN, Bataan DAU, Tominaga A, Kubota S (2020) Karyotypic mosaicism and molecular cytogenetic markers in the scleractinian coral *Acropora pruinosa* Brook, 1982 (Hexacorallia, Anthozoa, Cnidaria). Coral Reefs 39: 1415–1425. https://doi.org/10.1007/s00338-020-01975-x

Takaoka E, Sonobe H, Akimaru K, Sakamoto S, Shuin T, Daibata M, Taguchi T, Tominaga A (2012) Multiple sites of highly amplified DNA sequences detected by molecular cytogenetic analysis in HS-RMS-2, a new pleomorphic rhabdomyosarcoma cell line. American Journal of Cancer Research 2: 141–152.

Testa JR (1990) Chromosome translocations in human cancer. Cell Growth Differentiation 1: 97–101.

Vacarizas J, Taguchi T, Mezaki T, Okumura M, Kawakami R, Ito M, Kubota S (2021) Cytogenetic markers using single sequence probes reveal chromosomal locations of tandemly repetitive genes in scleractinian coral *Acropora pruinosa*. Scientific Reports 11: e11326. https://doi.org/10.1038/s41598-021-90580-1

Veron JEN (2000) Corals of the World. Vol 3. Townsville: Australian Institute of Marine Sciences, 1410 pp.
Walker WF, Doolittle WF (1983) Nucleic Acids Research 11: 5159–5164. https://doi.org/10.1093/nar/11.15.5159
Wallace CC (1999) Staghorn Corals of the World. CSIRO Publishing, Collinwood, 421 pp. https://doi.org/10.1071/9780643101388

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