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**RESEARCH ARTICLE**

# Cytogenetic characterization of six populations of *Narcissus tazetta* L. (Amaryllidaceae) from western Mediterranean

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## *Abstract*

*Narcissus tazetta* L. is one of the most widespread species of the genus *Narcissus* L., characterized by a high morphological and karyological variability. Six populations of the wild western Mediterranean *N. tazetta* were investigated for their chromosome numbers and cytogenetic analysis. The investigated populations exhibit a same diploid chromosome number ($2n$=20). The total length of the diploid chromosome set was 116.72 µm. Fluorochrome banding and FISH revealed variability in the number and intensity of CMA positive bands ($CMA^+$) and rDNA signals depending on population and presence or absence of B chromosomes. Two types of B chromosomes were detected, one of which carried 35S rDNA locus. Any correlation between presence of Bs and particular environmental conditions did not find in this study.



## INTRODUCTION

Due to the complexity of the *Narcissus* L. (Amaryllidaceae) taxonomy, diverse classifications have been proposed for the genus (Fernandes, 1968; Webb, 1980; Hanks, 2002; Mathew, 2002). But in the recent reviews, Graham and Barret (2004) and Zonneveld (2008) reported just 36 species, mainly distributed in the Mediterranean region, with a centre of diversity in the South Iberian Peninsula and Morocco.

The mechanisms of chromosomal rearrangements have caused the formation of two primary basic numbers of both subgenera *Narcissus* L. ($x$=7) and *Hermione* (Haw.) Spach ($x$=5) from the $x$=6 ancestor (Fernandes, 1951, 1967, 1975). A hybridisation between $x$=5 species and their ancestor ($x$=6), according to the same author, generates the secondary basic number $x$=11.

It seems that B chromosomes are widespread in the genus (observed in at least 12 species), frequently in horticultural varieties, and their number ranges from one to a maximal five (Fernandes, 1952; Wylie, 1952; Brandham and Kirton, 1987; Takhtajan, 1990; Zonneveld, 2008). B chromosomes are characterized by non-segregational and selfish behaviour, unique evolutionary path, unknown origin, and the presence of recombination barriers with A chromosomes. They are one of the major sources of genetic diversity, intraspecific variation in genome size and structural polymorphisms in natural populations (Camacho, et al. 2000; Jones and Houben, 2003; Muratovic et al., 2005; Jones et al., 2008). Some authors suggested genetic but not functional inertness of B chromosomes in plants, since it is noticed that when B chromosomes are present in large numbers they show adverse effects on exuberance, plant fertility and crossing-over frequency (Carchilan and Houben, 2007; Jones et al., 2008). But, sometimes plants with B chromosomes have a greater ability to survive than those without them (Jones, 1995),

probably thanks to the genes that they possess. However, the presence of active ribosomal genes on B chromosomes in many plants (Jones, 1995; Camacho et al., 2000; Carchilan and Houben, 2007; Muratović et al., 2006; Pustahija, 2011; Robert et al., 2011) does not support the opinion of their genetic inertness.
Molecular cytogenetics is an excellent tool for chromosome identification and studies of chromosome evolution. In eukaryotes, the rRNA genes are the most widely used chromosome markers. These genes are organized into two distinct families (i.e., 45S and 5S rDNA) that occur as tandem arrays at one or more specific chromosome regions. Due to their high copy number detection of the rRNA genes is highly reproducible and provides valuable information concerning chromosomal evolution. Copy number and chromosome distribution of rDNAs can change rapidly and rDNA transposition or dispersion in plant genomes is frequently observed (Schubert and Wobus, 1985; Raskina et al., 2004; Datson and Murray, 2006). These rearrangements generally correlate with species differentiation and speciation (Siljak-Yakovlev et al., 2014). To date only a small number of studies on rDNA and heterochromatin concern the genus *Narcissus* (De Dominicis et al., 2002; D'Amato, 2004; Dìaz Lifante et al., 2009; Pustahija, 2011; Wu et al., 2011).

Due to its ecological amplitude and morphological plasticity, *N. tazetta* (subgenus *Hermione*, section *Tazettae* Spach) is one of the most widespread species of the genus. It grows in most Mediterranean countries from sea level up to ca. 1500m, from the Atlantic part of the Iberian Peninsula across to the Middle East. It seems naturalised in Canary Islands, Persia, China and Japan (Fernandes, 1951).
In this work we studied the *N. tazetta* populations of the West Mediterranean Region, especially in Algeria. The present study is based on physical mapping of heterochromatin and rRNA genes in order to characterize the genome organization of this species.

We have undertaken the cytogenetic characterization of 6 *N. tazetta* populations by: 1) chromosome number determination by standard techniques; 2) heterochromatin distribution pattern on chromosomes by fluorochrome banding; 3) rDNA physical mapping by fluorescence *in situ* hybridisation.

## MATERIAL AND METHODS

### Plant material

Plants of wild *N. tazetta s.l.* were collected in Algeria (4 populations), Southern France (1 population) and Italy (1 population), between 2005 and 2013, and cultivated for cytological analysis (Table 1).

### Methods

### Feulgen staining and karyotype analysis

Root meristems obtained from potted plants, were pretreated in 0.05 % colchicine solution for 5h at room temperature and fixed in Carnoy I [3:1 (v/v) absolute ethanol: acetic acid] at 4°C for 24h.
For chromosome count, meristems were stained in Schiff's reagent after hydrolysis in 1N HCl for 8-10 min at 60°C, and then squashed in a drop of acetic orcein. Cover slips were removed following the method of Conger and Fairchild (1953) and slides were air-dried for 24h and then mounted in Depex. Cells with good spreading of chromosomes were photographed using a Zeiss Axioscope microscope coupled with a Sony CCD camera.
For karyotype analysis, chromosome arm lengths were measured in five well-spread metaphase plates obtained from five different individuals per population. Chromosome measurements were made using MicroMeasure version 3.3 (available via the Internet at http://www.colostate.edu/Depts/Biology/MicroMeasure).
The measurements did not take into account satellites. Centromere position and chromosome type were determined following the nomenclature of Levan et al. (1964). Arm ratio as: r=long arm/short arm and centromeric index as: Ci=100 x short arm/total length of the chromosome were estimated in this purpose. The asymmetry index regarding chromosome type was evaluated according to Arano and Saito (1980) as: Asl=Σ long arm/Σ total chromosome length x 100. The mean of centromeric asymmetry as: $M_{ca}$=Ax100 according to Peruzzi and Eroglu (2013) [A proposed by Watanabe et al. (1999) as Mean (long arm-short arm)/(long arm+short arm)] and the coefficient of deviation of chromosome lengths according to Paszko (2006) as $CV_{cl}$=$A_2$x100 [$A_2$ proposed by Romero Zarco (1986) as the ratio between standard deviation and the mean total length] were calculated to evaluate respectively intrachromosomal and interchromosomal asymmetry. The ratio between the longest and the shortest chromosome pairs (R) was also calculated.

### Fluorochrome banding and Fluorescent *In Situ* Hybridization (FISH)

Chromosome preparations: pretreated and fixed root tips were washed in citrate buffer (pH 4.6) for 15 min and then hydrolysed in an enzymatic mixture [4% RS cellulase (Yakult Honsha Co., Japan), 1% pectolyase Y-23 (Seishin, Co., Japan), 4% hemicellulase (Sigma, France)] at 37°C for 20-30 min, depending on the thickness of the root. Meristems were then washed in distilled water for 5-10 min before spreading in a drop of 45% acetic acid.

Fluorochrome banding: GC-rich DNA regions were detected using chromomycin $A_3$ staining (CMA, Sigma) following the technique of Schweizer (1976) with slight modifications described by Siljak-Yakovlev et al. (2002): preparations were stained in McIlvaine buffer pH 7 solution containing 0.2 mg/ml CMA and 5 mM $MgSO_4$ for 1h in the dark. Slides were then mounted in a drop of Citifluor AF2 (Agar Scientific Ltd, UK).

Chromosome preparations stained with CMA were discoloured in Carnoy I, dehydrated in cold graded ethanol series (70%, 90%, 100%) for 3 min in each one, and air-dried at room temperature overnight and then used for FISH experiment.

Fluorescent *in situ* hybridization (FISH):in order to determine the location and number of 35S and 5S rDNA gene loci, a FISH experiment using two DNA probes was performed according to Heslop-Harrison et al. (1991) with slight modifications: the pepsin treatment (0.1 mg/ml in HCl 0.01 N) was carried out for 10-15 min at 37° C;chromosomal DNA and probes were denatured for 10 min at 72°C.The probe for 35S was a clone of a 4 Kb *Eco*RI fragment including 18S-5.8S-26S rDNA sequences from *Arabidopsis thaliana* labelled with direct Cy3 fluorochrome (Amersham, Courtaboeuf, France) by nick translation. The 5S probe was the pTa794 clone (Gerlach and Dyer, 1980) including a 410 bp *Bam*HI fragment from wheat, labelled with digoxigenin-11-dUTP (Roche Diagnostics, Meylan, France) by a polymerase chain reaction. Counterstaining and mounting of slides were done after FISH in Vectashield medium containing DAPI (4′,6-diamidino-2-phenylindole, Vector Laboratories, UK).

Observation and image acquisition:chromosome preparations were observed using an epifluorescence Zeiss Axiophot microscope with different combination of excitation and emissions ZEISS filter sets (01, 07, 15 and triple 25). The acquisition and treatment of images were performed using a highly sensitive CCD camera (RETIGA 2000R, Princeton Instruments, Evry, France) and an image analyzer (MetaVue, Evry, France).

## RESULTS

### Karyotype analysis

The chromosome number was 2*n*=20 for all studied populations (Fig.1).

The analysis of karyotypes showed the existence of two chromosomal types (Table 2, Figs. 2A-F and J): submetacentric (3, 6, 8, 9 and 10 chromosome pairs) and subtelocentric (1, 2, 4, 5 and 7 chromosome pairs). Since the pair 7 bears a satellite, the corresponding chromosomal formula is 2*n*=2*x*=20=10sm+8st+2st-sat. A submetacentric B chromosome was observed in three Algerian populations (1, 2 and 3). Fifty metaphase plates on one individual bearing the B chromosome were observed for each population and the frequency of Bs reached 40.47%, 60% and 48% respectively. On five studied individuals for populations 1 and 3, the number of individuals with B chromosome was respectively 3 and 2. Two out six individuals from population 2 possess B chromosome.

The total chromosome length (TL) varied from 3.09 to 8.67 µm, The B chromosome length ranged from 1.7 to 2.06 µm. Ratio values between the biggest and the smallest chromosomal pair (R) and the total length of the diploid chromosome set are 2.8 and 116.72 µm. The asymmetry indexe (Asl) is 75.35%. This value show that the karyotype is asymmetric, which is predicted by the morphological types of chromosomes. The mean centromeric asymmetry (Mca) and the coefficient of chromosome length's variation (CVcl) are 50.68% and 37.5%.

### Heterochromatin revealed by CMA banding and DAPI after FISH

The chromomycin $A_3$ staining revealed interpopulation variability of number and intensity of bands and even, in two cases, between two homologous chromosomes (Figs.2A, C, E and J).

The common karyotype presented total of six GC-rich bands: two bands in the subterminal region of the long arms of chromosome pair 1, and four bands in the telomeric region of the short arms of the pair 8 as well as short arms and satellites of the chromosome pair 7 (Fig. 2J).

Some populations showed exceptions. Namely, populations 1 and 3 presented an additional band on their B chromosome: in the telomeric region of the short arm in population 1 (Fig. 2J Bs1) and in centromeric region in population 3 (Figs. 2A, J Bs2). In population 5, the chromosome pair 8 presented the GC-rich band on only one homologous chromosome (Fig. 2C). In the population 2, eight GC-rich bands were observed, where two additional bands are detected: one on the telomeric region of one homologous chromosome of pair 1 and the other on the short arm of B chromosome (Figs. 2E arrow, J).

DAPI after FISH revealed the presence of two bands of constitutive heterochromatin in all analysed populations, positioned in the subterminal region of the long arm of chromosome pair 1. Population 2 had one additional $DAPI^+$ band in the telomeric region of the long arm on one homologous chromosome of pair 1 (Figs. 2F, J). The B chromosome of population 3 possesses one $DAPI^+$ band in the centromeric region of the long arm. The obtained results showed that all $DAPI^+$ bands (constitutive heterochromatin) were also $CMA^+$ but with lower intensity.

### Physical mapping of 35S and 5S rRNA genes

All populations are characterized by two 35S loci collocated with GC-rich bands on chromosome pairs 7 and 8 (Figs. 2B, D, J), except populations 1 and 2 which possess additional 35S locus collocated with telomeric CMA

band on B chromosome (Figs. 2F, J). The B chromosome in population 3 does not carry the ribosomal genes. Fluorescence intensity of signals varied among populations (Figs. 2B, D, F) and sometimes between homologous sites. In population 5 only three 35S signals were detected: on the short arms of chromosome pair 7 (bearing the satellite) and in the telomeric region of the short arm of one homologous of pair 8 (Fig. 2D).

One 5S locus was observed in the subterminal region on the long arms of the chromosome pair 1. In population 2 a second 5S locus in terminal region on the long arm of one homologous of the same chromosome pair was noticed (Figs. 2F arrow, J).

Analysing obtained data, it is noticeable that in all investigated populations both 35S and 5S loci corresponded to the GC-rich DNA regions ($CMA^+$ bands) and only 5S locus exhibited also the $DAPI^+$ bands.

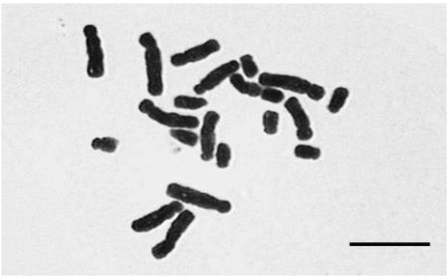
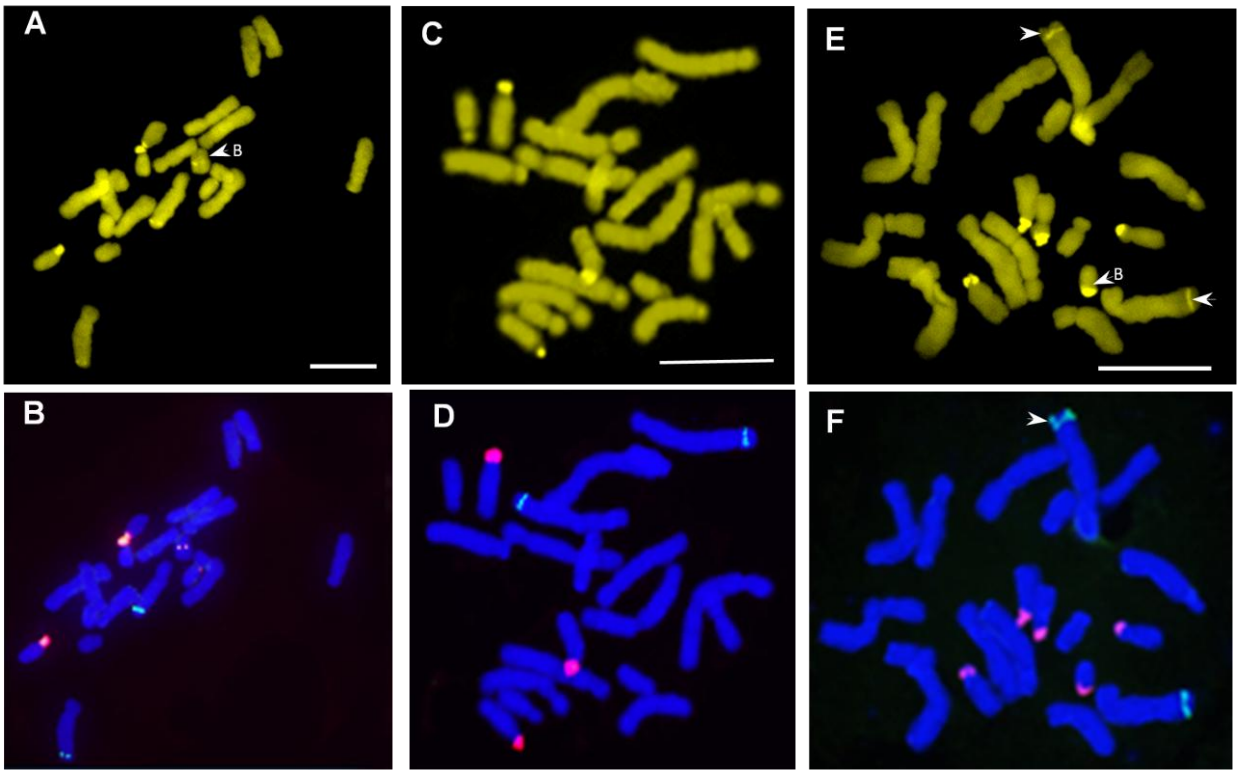


**Fig.1.** Mitotic metaphase of *N. tazetta*. Scale bar=10 µm.



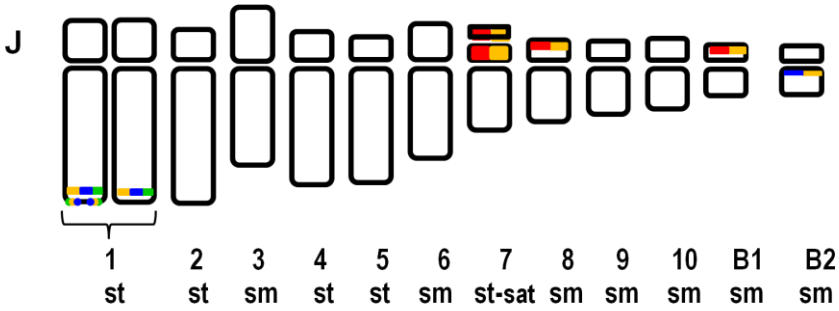



**Fig. 2.** Fluorochrome banding with chromomycine (yellow), DAPI after FISH (blue), physical mapping of 5S (green) and 35S (red) RNA genes and idiograms of *N. tazetta*. Population 3 - Amamcha (**A** and **B,** B chromosome arrowed), population 5 - Liguria (**C** and **D**), population 2 - Mila (**E** and **F,** one 5S locus is duplicated – arrowhead) and idiogram (**J**). Scale bar=10 µm.

**Table 1.** Geographic origin and altitude for each studied population

| Population number | Population accession | 2n | Localities | Altitude (m) |
|---|---|---|---|---|
| 1 | Alg-DjO1 | 20+B | Algeria, Constantine, Djebel Ouahch, Tafrent | 840 |
| 2 | Alg-Mila | 20+B | Algeria, Mila | 258 |
| 3 | Alg-Am | 20+B | Algeria, Setif, Amamcha | 1199 |
| 4 | Alg-DjA | 20 | Algeria, Guelma, Djebel Ancel | 828 |
| 5 | It-Liguria | 20 | Italy, Liguria, near Genova | 500 |
| 6 | Ga-Crau | 20 | France, Bouches du Rhône, Crau | 3 |

Alg = Algeria, Ga = France, It = Italy.

**Table 2.** Morphometric data concerning *N. tazetta* karyotype

| Chr. pair | Long arm (µm) | Short arm (µm) | Total length TL(µm) | Centromeric index (Ci) | Arm ratio (r) | Chr. type (t) |
|---|---|---|---|---|---|---|
| 1 | 6.65 (0.16) * | 2.02 (0.05) | 8.67 | 23.30 | 3.29 | st |
| 2 | 6.70 (0.25) | 1.54 (0.06) | 8.24 | 18.69 | 4.35 | st |
| 3 | 4.76 (0.09) | 2.71 (0.08) | 7.47 | 36.28 | 1.76 | sm |
| 4 | 5.80 (0.09) | 1.43 (0.03) | 7.23 | 19.78 | 4.05 | st |
| 5 | 5.66 (0.25) | 1.14 (0.09) | 6.80 | 16.76 | 4.96 | st |
| 6 | 4.46 (0.17) | 1.79 (0.08) | 6.25 | 28.64 | 2.49 | sm |
| 7 | 3.04 (0.09) | 0.75 (0.01) | 3.79 | 19.79 | 4.05 | st-sat |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | 2.64 (0.06) | 1.00 (0.05) | 3.64 | 27.47 | 2.64 | sm |
| 9 | 2.26 (0.07) | 0.92 (0.01) | 3.18 | 28.93 | 2.46 | sm |
| 10 | 2.00 (0.09) | 1.09 (0.02) | 3.09 | 35.27 | 1.83 | sm |

Total length of diploid chromosome set (TLC)=116.72µm; ratio between the longest and the shortest chromosome pair (R)=2.8; asymmetric index (AsI)=75.35%; coefficient of variation of chromosome length (CVcl)=37.5%; mean centromeric asymmetry (Mca)=50.68%; Chr=chromosome; morphological type of chromosome (t) according to Levan et al. (1964): submetacentric (sm), subtelocentric (st), sat=satellite; (*****)=standard deviation

## DISCUSSION

**Chromosome number and karyotype features**

The studied populations of *N. tazetta* present a chromosome number of 2*n*=20. This number has already been reported by numerous authors (Maugini, 1953; Hong, 1982; Brandham and Kirton, 1987; De Dominicis et al., 2002; Aquaro et al., 2008; Wu et al., 2011). Several chromosomal formulas have been established for 2*n*=20: 2sm+16st+2st-sat (Maugini, 1953); 4sm+10st+4t+2t-sat (Hong 1982); 4sm/m+14st+2st-sat (De Dominicis et al., 2002) and 8sm+8st+2t+2st-sat (Aquaro et al., 2008). The last one is similar to the observed formula in this study: 2*n*=20=10sm+8st+2st-sat.

**Heterochromatin and ribosomal genes distribution**

Three chromosome pairs can be easily recognized thanks to the chromomycin and DAPI after FISH signals distribution, and 5S and 35S rDNA physical mapping. The GC-rich bands localisation slightly differed from the those reported by De Dominicis et al. (2002) in Italian *N. tazetta* populations. In fact, the authors reported the same signals but on the long arms of the chromosome pair 1 and on the short arms of the pair 7, with the additional signal in terminal region on the short arms of chromosome pair 1 but without signal on pair 8.

Our FISH results concerning were slightly different from the results of Wu et al. (2011) who found a 5S locus on the long arm of the second chromosome pair.

In our study, 5S and 35S rDNA loci were always colocated with the GC-rich DNA regions. This type of colocalization seems generalized and has already been reported for both families of rRNA genes (Siljak-Yakovlev, 2002; Hamon et al., 2009; Muratovic et al., 2010; Bareka et al., 2012; Baziz et al., 2014). The 5S sites are DAPI positive in all analysed populations, and also they were visible in interphase nuclei, where they formed strongly fluorescent chromocenters (Martel et al., 1996; Ansari et al., 1999; Pustahija, 2011). In contrast, the 35S sites were DAPI negative corresponding to DNA AT-poor regions.

The chromosome pairs 7 and 8 exibited a polymorphism of the GC bands (Figs. 2A, C, E) and the one of 35S rDNA signals intensity. The 35S signals located in the telomeric region of pair 8 also vary in size and intensity among populations (Figs. 2B, D, F) and between the homologous sites within the same population (Fig. 2B). This heterogeneity is probably due to a difference in the number of gene copy repetitions, as has been reported in several plant species (Leitch and Heslop-Harrison, 1992; Murata et al., 1997; Ansari et al., 1999; Shan et al., 2003; Bogunic et al., 2011; Garnatje et al., 2012).

Certain discrepancies in the distribution of ribosomal genes in some populations, in relation to the common karyotype, were observed. Similar cases have already been documented in several species (Vaio et al., 2005; Hasterok et al., 2006; Fukushima et al., 2011; Jang et al., 2013). We presume that, in our study, the observed odd number of 35S rDNA signals in population 5 (Fig. 2D) and 5S loci in population 2 (Fig. 2F) is probably result of chromosomal rearrangements maintained by vegetative reproduction of the species.

**B chromosomes**

The existence of B chromosomes in *Narcissus* have been reported by several authors. Their number was variable: up to five Bs have been signalled in *N. bulbocodium* (Wylie, 1952; Fernandes, 1952, 1963), up to three in *N. tazetta* (Brandham and Kirton, 1987) and *N. triandrus* L.(Brandham and Kirton, 1987), two in *N. poeticus* L. (Pustahija, 2011) and one in *N. cyclamineus* DC. And *N. Minimus* Kunth (Wylie, 1952; Brandham and Kirton, 1987). All daffodils B chromosomes are about two-thirds the size of the smallest chromosomes, and they could be heterochromatic or not, with or without active ribosomal genes (Zonneveld, 2008; Pustahija, 2011).

In this study,two types of B chromosomes were detected: the first one (Bs1) carried $CMA^+$ signals colocated with 35S rDNA locus in the populations 1 and 2; and the second one (Bs2) without rDNA signals (population 3). Some Bs bearing rRNA genes have been reported in other plant species such as *Brachycome dichromosomatica* (Donald et

al., 1995), *Crepis capilaris* (L.) Wallr. (Maluszynska and Schweizer, 1989; Maluszynska, 1990; Jamilena et al., 1994), *Hepatica nobilis* Mill. (Matsuda et al., 1993), *Lilium martagon* L. (Muratovic et al., 2006), *Pennisetum glaucum* (L.) R.Br. (Robert et al., 2011). In *N.poeticus* two chromosomes B bearing active 35S loci have been observed in the populations growing under stressful environmental conditions (Pustahija, 2011). No correlation was obseved between presence of Bs and particular environmental conditions in our study.

## CONCLUSION

In this paper, a cytogenetic study was performed on six wild *N. tazetta* populations including four Algerian ones, investigated for the first time in this area of research. The present study shows interpopulation variability of number and intensity of GC-rich DNA regions and in physical mapping of 5S and 35S rRNA genes. We will expand our sampling of *N. tazetta* throughout the Mediterranean to detect a possible interpopulation variability in karyotypes and physical mapping of 5S and 35S rRNA genes.

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