

DNA–Feulgen cytophotometric determination of genome size for the freshwater-invading copepod *Eurytemora affinis*

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Abstract: Variation in nuclear DNA content within some eukaryotic species is well documented, but causes and consequences of such variation remain unclear. Here we report genome size of an estuarine and salt-marsh calanoid copepod, *Eurytemora affinis*, which has recently invaded inland freshwater habitats independently and repeatedly in North America, Europe, and Asia. Adults and embryos of *E. affinis* from the St. Lawrence River drainage were examined for somatic cell DNA content and the presence or absence of embryonic chromatin diminution, using Feulgen–DNA cytophotometry to determine a diploid or 2C genome size of 0.6–0.7 pg DNA/cell. The majority of somatic cell nuclei, however, have twice this DNA content (1.3 pg/nucleus) in all of the adults examined and possibly represent a population of cells arrested at the G2 stage of the cell cycle or associated with some degree of endopolyploidy. Both suggestions contradict assumptions that DNA replication does not occur in adult tissues during the determinate growth characteristic of copepods. Absence of germ cell nuclei with markedly elevated DNA values, commonly found for species of cyclopoid copepods that show chromatin diminution, indicates that *E. affinis* lacks this trait. The small genome size and presumed absence of chromatin diminution increase the potential utility of *E. affinis* as a model for genomic studies on mechanisms of adaptation during freshwater invasions.

Key words: copepod, genome size, DNA–Feulgen, calanoid, *Eurytemora*.

Résumé : L'existence d'une variation du contenu nucléaire en ADN chez certains eucaryotes est bien documentée, mais les causes et les conséquences d'une telle variation demeurent nébuleuses. Les auteurs rapportent ici la taille du génome d'un copépoïde calanoïde des estuaires et marais, *Eurytemora affinis*, qui a récemment envahi des habitats d'eau douce indépendamment et de manière répétée en Amérique du Nord, en Europe et en Asie. Des adultes et des embryons de l'*E. affinis* provenant du fleuve St-Laurent ont été examinés pour leur contenu en ADN génomique ainsi que pour la présence/absence d'une diminution de la chromatine chez les embryons au moyen de la cytophotométrie d'ADN coloré au Feulgen. La taille du génome diploïde ou 2C a été estimée entre 0,6 et 0,7 pg d'ADN par cellule. La majorité des noyaux des cellules somatiques possèdent cependant deux fois cette quantité d'ADN (1,3 pg par noyau) chez tous les adultes examinés et représentent possiblement une population de cellules arrêtées au stade G2 du cycle cellulaire ou montrant de l'endopolyploïdie. Ces deux hypothèses vont à l'encontre de la présomption voulant que la réplication de l'ADN n'ait pas lieu au sein des tissus adultes lors de la croissance déterminée chez les copépoïdes. L'absence de noyaux de cellules germinales montrant des contenus en ADN beaucoup plus élevés, une observation répandue chez les copépoïdes montrant une diminution de la chromatine, indique que l'*E. affinis* ne possède pas ce caractère. La petite taille du génome et l'apparente absence de diminution de la chromatine confèrent à l'*E. affinis* une utilité potentielle en tant que modèle pour des études génomiques sur les mécanismes d'adaptation lors de l'invasion de milieux d'eau douce.

Mots clés : copépoïde, taille du génome, ADN coloré au Feulgen, calanoïde, *Eurytemora*.

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Introduction

Copepods are an abundant and geographically widespread subclass within the subphylum Crustacea and include many different species that span marine, brackish, and freshwater habitats (reviewed by Heron 1976; Mauchine 1998). They constitute important members of planktonic food webs as the dominant grazers on phytoplankton, and are a major food source for many species of fishes (Mauchine 1998). There are three major orders of free-living copepods: Calanoida, Cyclopoida, and Harpactoida. Although there is a sizable body of literature on the ecology, systematics, morphometrics, and demography of some copepod species (cf. Mauchine 1998 and Dodson et al. 2003 for recent reviews), information on variation in genome sizes for particular species of copepods is relatively sparse (Beermann 1977; McLaren et al. 1988, 1989; Grishanin et al. 1996; Gregory et al. 2000; Wyngaard and Rasch 2000). The most complete and current listings are available from the Animal Genome Size Database (Gregory 2001).

The calanoid copepod *Eurytemora affinis* is a predominantly estuarine and salt marsh species with a broad geographic range in the northern hemisphere. Within the past century, populations have invaded freshwater lakes and reservoirs multiple times independently from genetically distinct sources in North America, Europe, and Asia (Lee 1999). Such transitions from saline to freshwater habitats characterize invasion pathways of many recent invertebrate invaders (Dermott et al. 1998; Lee and Bell 1999; Smith et al. 2002).

Freshwater-invading populations of *E. affinis* experience strong selection and heritable shifts in tolerance and performance in response to salinity (Lee et al. 2003). Given that selection is occurring, what genes are under selection? Also, are the same or different genes under selection during independent invasion events? Multiple independent invasions provide replicated tests for adaptation during invasions and allow testing of whether the same or distinct genetic mechanisms are involved in freshwater adaptation within and among genetically distinct clades. In this study, genome size of *E. affinis* was measured to determine its feasibility as a model system for genomic studies to elucidate mechanisms of freshwater adaptation, for assessing the role of nucleotypic effects in adaptation (Cavalier-Smith 1985; Escribano et al. 1992; Vinogradov 1997), and for examining the significance of hierarchical selection in evolutionary processes (Gregory 2004).

Many copepod species exhibit the phenomenon of chromatin diminution, a process that involves chromosome fragmentation, marked decreases in nuclear DNA content, and genomic reorganization during early cleavage stages of embryogenesis (Beermann 1977; Rasch and Wyngaard 1996, 2001a; Einsle 1996; Grishanin et al. 1996). An unusual and apparently rare type of chromatin diminution has been described for the calanoid copepod, *Pseudocalanus* (Robbins and McLaren 1982), now believed to be *P. acuspes* (Nanton 1993; I.A. McLaren, personal communication). Moreover, in addition to the report by McLaren et al. (1966) of polyteny as a source of cryptic speciation in copepods, the genome sizes determined for a great majority of saltwater calanoid species are remarkably large, ranging from 4 to 24 pg DNA per 2C somatic cell (McLaren et al. 1989; Escribano et al.

1992; Gregory et al. 2000). Freshwater cyclopoid copepods, on the other hand, possess relatively small genomes, ranging from 0.5 to 4.1 pg DNA per diploid somatic cell (Wyngaard and Rasch 2000). No data are available for genome sizes of estuarine species of copepods.

The occurrence of chromatin diminution and (or) a large genome size in *E. affinis* might well pose problems for its use in future genomic analyses. To address these issues, we have used static DNA–Feulgen cytophotometry to estimate its somatic cell (2C) genome size and to examine specimens for germline nuclei with markedly elevated levels of DNA, a diagnostic characteristic among cyclopoid copepods that exhibit chromatin diminution (Rasch and Wyngaard 1992, 2001a).

Materials and methods

Specimens of *Eurytemora affinis* were collected from the L'Isle Verte salt-marsh in the St. Lawrence River drainage in May 2001. This population belongs to the Atlantic clade of the *E. affinis* species complex (Lee 2000; Lee and Frost 2002).

Individual animals were fixed in 3:1 volume fraction of methanol – acetic acid, sexed, and squashed in a drop of 45% v/v acetic onto a glass slide coated with a thin film of gelatin. After freezing in liquid nitrogen to remove the siliconized coverglass and thawing in absolute ethanol, slides were air dried and held in darkness to accumulate an entire set for simultaneous staining with the Feulgen reaction for DNA, as described elsewhere (Rasch 1985, 2003). The inclusion of reference standards of chicken red blood cells (CRBC, 2.5 pg DNA per nucleus) and trout red blood cells (TRBC, 5.0–5.1 pg DNA per nucleus) allowed us to express relative integrated absorbance (RIA) values for the Feulgen–DNA dye complex in terms of picograms of DNA per nucleus for an unknown cell sample (Rasch 1985; Hardie et al. 2002). Slides were held in darkness until used for DNA measurements.

Specimens and their reference standards for cytophotometry were mounted in matching refractive index liquids (n_D 1.524–1.556) to minimize non-specific light loss owing to scatter from carapace fragments in areas of dispersed organs and tissues. All measurements of RIA were made using the subtractive method described by Bedi and Goldstein (1976). According to their procedures, the absorbance of a clear or “background” area (delimited by a mask size that is suitable for the nucleus to be measured) is subtracted from the net RIA determined for a single nucleus centered in the same size mask and at the same focal plane (Rasch 2003). The subtractive method generates a pair of microscopic glass “cuvettes” that yield estimates of concentration (C) and volume (V) of the Feulgen–DNA dye complex. These values are used to estimate the actual amount (M) of DNA (pg/nucleus), using appropriate reference standards and validated correction curves (Rasch 1974, 1985, 2003; Hardie et al. 2002).

Results and discussion

The 2C DNA content of somatic cells in *E. affinis* is approximately 0.6–0.7 pg or approximately 640 Mb DNA (Ta-

Table 1. Estimated 2C genome size for embryonic cells (E) and for somatic cells of adult females (F) of *Eurytemora affinis*.

Specimen	Cell type/nucleus	DNA–Feulgen per nucleus		Estimated DNA per nucleus (pg)		n
		Mean	±SE	Mean	±SE	
52999	CRBC	42.19	0.108	2.50	0.065	100
50883	TRBC	84.50	1.077	5.001	0.064	50
E No. 905A	Interphase	22.06	1.140	1.31	0.067	31
E No. 905B	Interphase	21.59	1.218	1.28	0.072	20
E No. 905A	Telophase	12.11	0.540	0.72	0.032	7
E No. 905B	Telophase	10.66	0.197	0.63	0.012	5
E No. 905B	Metaphase	22.51	0.438	1.33	0.026	9
E No. 909A	Interphase	22.83	0.568	1.35	0.033	24
E No. 909A	Telophase	10.84	0.323	0.64	0.019	8
F No. F905	Interphase	21.77	0.286	1.29	0.017	186
F No. F906	Interphase	22.20	0.144	1.32	0.027	54
F No. F907	Interphase	22.52	0.561	1.33	0.033	28
F No. F908	Interphase	22.17	0.541	1.31	0.032	24
F No. F908	Interphase	21.16	0.236	1.25	0.014	60
F No. F909	Interphase	22.33	0.487	1.32	0.029	135
F No. F909	Interphase	21.63	0.810	1.28	0.048	81

Note: Chicken red blood cell (CRBC) nuclei were used as a reference standard of 2.5 pg/nucleus. The DNA content estimated for trout red blood cells (TRBC) is within 3%–4% of the expected amount of 5.1–5.2 pg/nucleus (Rasch 1985). Values shown for *E. affinis* represent scans of 672 individual nuclei. See text for additional details.

Table 2. Estimated 2C genome size based on the DNA content of somatic cell nuclei from adult males (M) of *Eurytemora affinis*.

Specimen	Cell type/nucleus	DNA–Feulgen per nucleus		Estimated DNA per nucleus (pg)		n
		Mean	±SE	Mean	±SE	
52999	CRBC	42.19	0.108	2.50	0.065	100
50883	TRBC	84.50	1.077	5.00	0.064	50
M No. 910	Somatic	20.34	0.451	1.20	0.027	34
M No. 910	Somatic	21.44	0.972	1.27	0.057	51
M No. 910	Somatic	22.23	0.444	1.32	0.026	35
M No. 911	Somatic	11.16	0.161	0.66	0.010	9
M No. 911	Somatic	21.74	0.841	1.29	0.049	54
M No. 911	Somatic	20.45	0.230	1.21	0.014	75
M No. 911	Somatic	21.04	0.510	1.25	0.031	33

Note: Chicken red blood cell (CRBC) nuclei were used as a reference standard of 2.5 pg/nucleus. The DNA content estimated for trout red blood cells (TRBC) is within 3%–4% of the expected amount of 5.1–5.2 pg/nucleus (Rasch 1985). Values shown for *E. affinis* represent scans of 291 individual nuclei. See text for additional details.

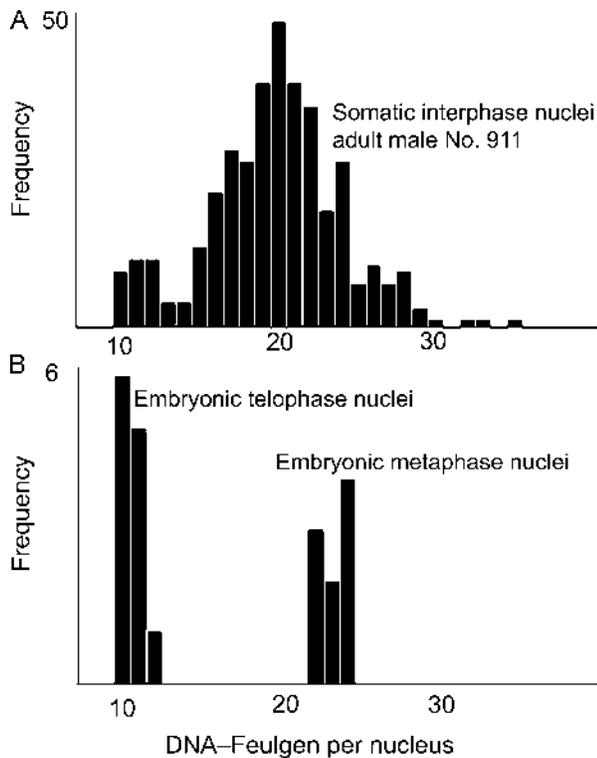
bles 1 and 2) and is about double the 2C value for *Drosophila melanogaster* (0.36 pg; Rasch et al. 1971).

Nuclei with a 2C DNA content occur primarily in embryos (E) and are defined as 2C by the DNA levels found for several late telophase and mid metaphase chromosome configurations measured in three embryos (E No. 905A, E No. 905B, and E No. 909A in Table 1) that were found on slides from two different mothers (F No. 905 and F No. 909 in Table 1). Nuclei with twice this amount of DNA, i.e., 1.3 pg or a 4C DNA level, constitute the predominant population in random samples of somatic cells from five adult females (F Nos. 905–909, Table 1) and two adult males (M Nos. 910 and 911, Table 2). Because nuclei of the 4C DNA class are the most commonly encountered population in Feulgen-stained squash preparations (cf. Fig. 1A), we initially thought that 1.3 pg DNA represented the diploid or 2C

genome size for this species (Rasch and Wyngaard 2001b). We now have evidence, to be presented below, that our previous estimate was derived from inadvertently sampling only adult somatic cell nuclei in the earlier study.

The presence of a discrete, minority population of very small nuclei first became evident from analysis of the pooled values from three different areas of the tissue squash used to measure nuclei from male No. 911 (Table 2). A small group ($n = 9$, or 5.3% of the total of 171 somatic nuclei) of tiny, pale nuclei with low DNA–Feulgen values were evident in histograms of somatic cells from this animal (Table 2; Fig. 1A). The mode for this low frequency class of nuclei coincides with that obtained for individual telophase nuclei seen as pairs of small, densely stained groups of chromosomes in several embryos that had been coincidentally squashed at the time of preparing their mothers for analysis

Fig. 1. (A) Histogram of DNA–Feulgen values from three sets of nuclear scans from independently selected areas in the tissue squash of adult male (No. 911) *Eurytemora affinis* ($n = 171$). Note the low-frequency group of interphase nuclei with half of the DNA content of the predominant cell population. (B) Histogram of DNA–Feulgen values for telophase and metaphase nuclei from embryos 905A, 905B, and 909A that were on slides with *Eurytemora affinis* adult females No. 905 and No. 909. The mode of the telophase nuclei coincides with that of the small group of interphase nuclei identified among the somatic cells of male No. 911.



(F Nos. 905 and 909 in Table 1). The distribution of DNA–Feulgen values for the embryonic telophase nuclei matched that for the small pale nuclei of male No. 911 (Figs. 1A and 1B). DNA content of the few metaphase configurations identified in the embryo squashes (Table 1) confirmed our assignment of 4C to the predominant somatic cell populations of adult forms (Table 1; Fig. 1B). DNA values of 1.2–1.3 pg/nucleus characterized all maternal somatic tissues examined (Table 1). Were it not for the fortuitous inclusion of the embryos from females carrying egg sacs at the time of their fixation, the sparse and somewhat cryptic 2C DNA class of somatic cell nuclei might have gone undetected. Failure to identify this class of small nuclei in DNA measurements for male No. 910 and females Nos. 905–909 points out the rarity of 2C somatic cells in adults of this species.

From the absence of nuclei with prominent amounts of heterochromatin (Beermann 1977) and markedly elevated levels of DNA in the ovaries of the adult females observed here (Rasch and Wyngaard 2001), we tentatively conclude that *E. affinis* lacks chromatin diminution during early embryogenesis. As we have reported earlier, the occurrence

of large, densely stained, heterochromatin-rich nuclei in germ cells before the initiation of meiosis is a diagnostic criterion for the subsequent occurrence of chromatin diminution in *C. strenuus*, *M. edax*, and *M. longisetus* (Rasch and Wyngaard 1996). No such nuclei were identified here in any of the females of *E. affinis*.

The functional significance of having only a minority population of 2C somatic cell nuclei in adult somatic tissues is unclear. McLaren and coworkers have previously noted the sporadic occurrence of 4C nuclei in somatic cells of several species of *Pseudocalanus* (McLaren et al. 1988, 1989; Escribano et al. 1992). The frequent occurrence of polyploid nuclei in somatic cells of *Cyclops strenuus* has also been reported by Grishanin et al. (1996). The generation of 4C nuclei in somatic tissues of *E. affinis* may be associated with its small genome size in comparison with that of its congeneric species, *E. composita* (1.58 pg DNA) and *E. herdmanni* (1.46 pg DNA) (Robbins and McLaren 1982). The predominating 4C DNA nuclear class found in the soma of *E. affinis* may represent cells arrested at the G₂ stage of the cell cycle. Alternatively, the prevalence of 4C nuclei may reflect some level of endopolyploidy as a mechanism of dosage compensation for the small size of the operative genome, as seems evident in the soma of males in several species of parasitoid wasps (Rasch et al. 1977). In either case, the unexpected preponderance of a 4C level of DNA in the somatic cells of *E. affinis* might be a mechanism to modulate genome size by making more DNA available for transcription (White and McLaren 2000). Comparisons of genomes, adult body sizes, and development rates of congeneric species of *Eurytemora* maintained in cultures at a common temperature would clarify the significance of these issues.

All three species of *Eurytemora* thus far studied have very small genomes when compared with those reported in the Animal Genome Size Database (Gregory 2001). Species of *Calanus*, *Hesperodiaptomus*, and *Pseudocalanus* may have as much as an order of magnitude more DNA than the 2C genome size reported here for *E. affinis* and its two congeners. It will be of considerable interest to determine genome size and genetic diversity of *E. velox*, another successful calanoid freshwater invader (Lee and Bell 1999), as well as other species within the genus *Eurytemora* (Heron 1976).

Although the small telophase nuclei containing 0.6–0.7 pg DNA per cell undoubtedly represent very early cleavage stages of embryogenesis, the absence of evidence for chromatin diminution and chromatin granules in the equatorial plane of the division figures observed here, makes it probable that we are observing levels of DNA per telophase nucleus that are the progenitors of the nuclei of the adult soma. Likewise, the small, pale nuclei observed in male No. 911 might represent the round to oval non-flagellate crustacean sperm described by earlier authors (Heberer 1924, plate II, Figs. 59–63; Rousset et al. 1981). This inference is unlikely, however, because the small nuclei occur in somatic tissues and have a DNA content that is equivalent to that of embryonic telophase nuclei.

In summary, both the small genome size (C value = 0.30 to 0.35 pg DNA) and presumed absence of chromatin diminution increase the potential utility of *E. affinis* for further genomic studies on mechanisms of adaptation during fresh-

water-invasion events, including quantitative trait loci mapping and microarray analysis.

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