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Comparative karyotype analysis of two Iranian cyprinids, *Alburnoides bipunctatus* and *Alburnus filippii* (Cypriniformes, Cyprinidae)

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This study provides new data on chromosomal characteristics of two cyprinid fish species found in Iran, *Alburnoides bipunctatus* and *Alburnus filippii*. The chromosomes of these species were compared using Giemsa staining and silver-staining (Ag) techniques to describe the karyotypes. Both species possess $2n = 50$ chromosomes with no detected sex heteromorphism. The karyotypes were distinct, composed of 7 pairs of metacentric, 10 pairs of submetacentric, and 8 pairs of subtelo-acrocentric chromosomes in *A. bipunctatus* and 6 pairs of metacentric, 9 pairs of submetacentric, 4 pairs of subtelocentric, and 6 pairs of acrocentric chromosomes in *A. filippi*. Both species possessed a single pair of Ag-stained nucleolar organizer region (NOR)-bearing chromosomes, but the species differed in its location and chromosomal type. In *A. bipunctatus,* Ag-NORs were located on the telomeres of two submetacentric chromosomes, and in *A. filippii*, on the telomeres of the short arm of a subtelocentric chromosome pair. The NOR patterns were nearly identical with those found in other representatives of Eurasian cyprinids and support the phylogenetic relationships proposed for species in the Leuciscinae. The results of cytogenetic studies of related species, mainly in the Leuciscinae, are compared and discussed.

Key words: Cypriniiformes; *Alburnoides bipunctatus*; *Alburnus filippii*, Fish cytogenetics, NOR, Cytotaxonomy

INTRODUCTION

The family Cyprinidae (Cypriniformes), with about 2,010 species in 210 genera, is the most speciose family among freshwater fishes (Coad, 2006). The genera *Alburnus* and *Alburnoides* are leuciscine cyprinids found in Europe and northern parts of southwest Asia and encompass 22 species, eight of which occur in Iran (Froese and Pauly 2008).

Alburnus eichwaldii (De Filippi, 1863), described from the "Kur presso Tiflis" (Kura River near Tbilisi, Georgia), is usually regarded as a Caspian Sea basin subspecies of *Alburnoides bipunctatus* (Bloch, 1782). Bănărescu (1991), stated that *A. eichwaldii* could not be distinguished from *Alburnoides bipunctatus fasciatus* (Nordmann, 1840) from the Black Sea basin. Holčík and Jedlička (1994) and Reshetnikov *et al.* (1997) consider that the subspecies status is disputable. Another nominal

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subspecies, *Alburnoides bipunctatus armeniensis* (Dadikyan, 1972), occurs in the Aras River drainage of Armenia, inhabiting the Arpa, Vorotan, Vedi, Marmarik, and Kasakh rivers and their tributaries.
Greek subspecies of *A. bipunctatus* appear to be paraphyletic based on *cytochrome b* sequence data. *Alburnoides bipunctatus strymonicus* is considered basal to a clade including *A. b. orhidanus* and the French *A. b. bipunctatus*, suggesting an eastern origin of European *Alburnoides* (Zardoya *et al.,* 1999). However, *A. bipunctatus* in Iran must be regarded as a species complex. According to Coad (2006), populations in the Caspian Sea basin and possibly the Orumiyeh, Namak Lake, and Tedzhen basins might represent members of cryptic species, while populations outside these basins, mainly from the Kor River basin of southern Iran, might be considered members of other genera or species.
Alburnoides bipunctatus species complex, commonly known as Spirilin, is widely distributed in Iran, commonly in small streams and less frequently in the main areas of large river basins of the Caspian Sea, Lake Orumiyeh, Tedzhen River, Kavir, Namak Lake, Zayandeh and Shur rivers (Esfahan), and Kor River (Abbasi *et al*., 1999; Kiabi *et al*., 1999; Abdoli, 2000). The Kura Bleak (*A. filippii*, Kessler, 1877) is a small fish distributed from the upper to the lower reaches of the Aras (Qareh Su) and Safid (Qezel Owzan) rivers and in the Anzali Wetland (Abbasi *et al.* 1999; Kiabi *et al.* 1999).
As taxonomic and systematic characteristics, chromosomal nucleolar organizer regions (NORs) have been described for about 70 fish species. The types of interspecific NOR heteromorphisms among the North American cyprinids differ qualitatively from their intraspecific NOR heteromorphisms, serving as taxonomically and systematically informative characteristics (Buth *et al.,* 1991).
Current data for *A. bipunctatus* are based on conventionally stained chromosomes, and no chromosome banding methods have yet been applied. The silver stained-NOR is a useful chromosome marker; NOR polymorphisms, including number, location and size, are often species-specific. The numbers of NORs, the morphology of NOR-bearing chromosomes, and the position of the NORs on the chromosomes have frequently been observed to differ even in closely related species with very similar karyotypes (Takai and Ojima, 1986, Sola *et al.,* 1992). The location of NORs is a useful karyotypic marker in fish cytotaxonomy and has been recently used for studying phylogenetic relationships among species (Boron, 2001; Ra´bova´, Ra´b and Ozouf-Costaz, 2001; Ra´bova´ *et al.,* 2003). Although NORs have been insufficiently analyzed in Eurasian cyprinids, a single NOR-bearing chromosome pair (single NORs) is present in most analyzed species of Leuciscinae (reviewed in Ra´b and Collares-Pereira, 1995) and is considered a plesiomorphic characteristic.
Although chromosome numbers of most *Alburnus* spp. have been described, no such data were found for *A. filippii*. Chromosome analysis of *Alburnus* species has shown no major differences in the diploid numbers; 2n = 50 seems to be fixed in the genus, with the exception of *Alburnus arborrela*, which has 2n = 52 (Vasil'ev, 1980; Simovic *et al.,* 1994).
Although knowledge regarding *Alburnus* and *Alburnoides* taxonomy has expanded, their systematics is poorly understood, and the taxonomy of some species requires further study. This study attempts to elucidate some of these questions through chromosome analysis, focusing on *A. bipunctatus*, and *A. filippii*. Our aim was to compare the karyology of *A. bipunctatus* and *A. filippii* from the Caspian Sea Basin and to present the chromosomal complement, number, and location of the nucleolar organizer regions (NORs) of these species. Data will be compared with those from European populations, followed by a taxonomic discussion of cytogenetic studies of related species.

MATERIAL AND METHODS

Six females and four males *Alburnus filippii* were collected in 2006 in the Anzali Wetland (37º 28' N, 49º 27' E), in Guilan, Iran. Samples of *Alburnoides bipunctatus* (five females and nine males) were captured in 2006 in the Anzali Wetland (38º 28' N, 49º 26' E) near the Siahdarvishan River in northern Iran. The fish were transported live to the laboratory and held for 72 h prior to processing.

Chromosomes were obtained directly from gill epithelium and kidney head cells (Collares-Pereira *et al.,* 1998). The colchicine dosage was 2μg/ml per g of total mass with an exposure time of 3–4 h. Hypotonicity was produced with 0.056% KCl for 40 min for kidney cells, and 55 min for gill epithelial cells. The cell suspension was fixed in a fresh solution of 3:1 methanol /glacial acetic acid and with three changes of fixative at 15 min intervals. Slides were air-dried and chromosomes stained 15 min with 4 – 5% Giemsa solution at pH 6.8, or alternatively, sequential Ag-NOR staining was applied according to Howell and Black (1980) with slight modifications to reveal the presence of active rDNA clusters. Well-spread mitotic metaphases were photographed, and enlarged prints were used to prepare 3– 4 karyotypes per specimen. Chromosome classification followed Levan et al. (1964). The distributions of Ag-NOR and nucleoli within each individual were scored for all karyotypes examined. At least 34 metaphase plates were used to analyze Ag-NOR and 70 nuclei were examined to determine the nucleoli distribution within each individual.

RESULTS

All specimens of *A. bipunctatus* and *A. filippii* were characterized by the same $2n = 50$ chromosomes. The karyotypes of both species were characterized by the presence of one pair of acrocentric chromosomes, the largest in the set, but no heteromorphic sex chromosomes. Despite the same diploid number, similar karyotypic macrostructure, and the absence of heteromorphic sex chromosomes, some differences were found. No variation in the number of NORs was observed, although an evident NOR size polymorphism between homologs of the NOR-bearing chromosome pairs was detected in both species.

Alburnoides bipunctatus

Chromosome counts from all specimens of *A. bipunctatus* revealed an invariable diploid chromosome number of $2n=50$ and a fundamental number (number of chromosome arms) of 84. Its karyotype comprised 7 pairs of metacentric (M), 10 pairs of submetacentric (SM), and 8 pairs of subtelo-acrocentric (ST– A) chromosomes (Fig. 1). The NORs, as revealed by Ag-staining, covered nearly the entire short arms of a mid -sized submetacentric (SM) chromosome pair, a pattern common in a number of other cyprinid fishes (Fig. 1).

Alburnus filippii

The number of diploid chromosomes in *A. filippii* was 2n = 50, of which 6 pairs were metacentric (M), 9 pairs were submetacentric (SM), 4 pairs were subtelocentric (ST) and 6 pairs were acrocentric (A). The chromosomes were arranged in decreasing size, and in this metaphase spread, the fundamental number was 88. A NOR was observed at the end of the short arms of one of the pairs of medium-sized ST chromosomes. The karyotype in Figure 1 has been ordered according to the length of chromosomes, indicating the morphological classification based on the centromere position. After silver staining, two Ag-NORs were found in the karyotype of *A. filippii* (Fig. 2) located on the short arm of a single subtelocentric chromosome pair.

DISCUSSION

Most karyotypes investigated for species of *Alburnoides* and *Alburnus* possess $2n = 50$ chromosomes (Table 1). Only Vasil'ev (1980) and Klinkhardt *et al.* (1995) reported a diploid number of $2n = 52$ chromosomes for *A. arborrela.* A pair of large acrocentric chromosomes has been proposed as a marker for the genus *Alburnus* (Gold and Avise, 1977) as well as for some other cyprinid genera, such as *Leuciscus*, *Phoxinus*, and *Chondrostoma*, belonging to the Leuciscinae (Cataudella *et al.,* 1977). The karyotypes of Leuciscinae are composed mainly of bi-armed elements, suggesting a high evolutionary position among other Cyprinidae (Ráb *et al.,* 2008).

TABLE 1. Summary of the chromosomal features of species of *Alburnoides* and *Alburnus* diploid numbers (2n), fundamental numbers (NF), and metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) chromosomes

	Karyotype							
Species	2n	NF	m	sm	st	a	NORs	References
Alburnoides								
A. bipunctatus	50	84	14	20	16	-	sm	Present study
A. bipunctatus	50	84	16	22	12	-	-	Kilic-Demirok and Ünlü, 2004
A. taeniatus	50	78	16	12	20	-	-	Sofradzija *et al.*, 1979
Alburnus								
A. akili	50	18	82	32	-	-	-	Arkhipchuk, 1999
A. alburnus	50	18	82	32	-	-	-	Arkhipchuk, 1999
A. alburnus	50	86	14	14	8	14	sm	Schmid *et al.*, 2006
A. arborella	52	87	-	-	-	-	-	Vasil'ev, 1980
A. atropatenae	50							Klinkhardt *et al.*, 1995
A. caeruleus	50							Klinkhardt *et al.*, 1995
A. doriae	50	86	18	14	14	14	-	Arkhipchuk, 1999
A. filippii	50	88	12	18	8	12	st	Present study
A. hebes (1)	50							Klinkhardt *et al.*, 1995
A. heckeli	50	78	12	12	16	10	-	Simovic *et al.*, 1994
A. hohenackeri	50							Klinkhardt *et al.*, 1995
A. kosswigi (2)	50							Klinkhardt *et al.*, 1995
A. microlepis(1)	50							Klinkhardt *et al.*, 1995
A. nasreddini	50							Vasil'ev, 1980
A. orontis	50							Vasil'ev, 1980
A. qalilus	50							Vasil'ev, 1980
A. scoranzoides	50							Vasil'ev, 1980
A. tarichi	50							Vasil'ev, 1980

(1) = *Alburnus sellal*, (2) = *Alburnus escherichii*

FIGURE 1.- Giemsa-stained karyotypes of *Alburnoides bipunctatus* from the Caspian Sea basin. The NOR-bearing chromosome pair, after silver staining, is shown in the box. Bar = 5 μm.

The karyotypes of only two Iranian *Alburnoides* species have been described, in which the chromosome numbers are commonly equal to 50 per somatic cell (Table 1). There are also six *Alburnus* species in Iran of which karyotypes have been described for three, including, *A. alburnus, A. akili* and *A. heckeli* in which the chromosome numbers were also equal to 50 per somatic cell (Table 1). Although they have the same diploid number of chromosomes, the two leuciscinae species under discussion differ in their karyotype formulae and the location of the NOR-bearing chromosomes. Whereas *A. bipunctaus* had an NF = 84 and a simple NOR system, *A. filippii* had an NF = 88 and a simple NOR system. Cytogenetic studies in various *Alburnoides* and *Alburnus* species have shown a constant diploid number of 2*n* = 50 chromosomes (Table 1). In spite of this trend towards conservation, differences in karyotype constitution and NOR location have indicated that they constitute a species complex, similar to that reported in other species of the same genus.

The cytogenetic data described above and in the literature (Table 1) show that the karyotype formulae vary among *A. bipunctaus* and *A. filippii* species, with NF ranging from 78 to 88, although the group's diploid number is conserved. These differences might be due to the occurrence of non-Robertsonian chromosome rearrangements such as pericentric inversions during the diversification of the "*A. bipunctaus-A. filippii* complex". Most of the cyprinid karyotypes are characterized by a relatively large number of bi-armed (meta- and submetacentric) compared to uni-armed (subtelo- and acrocentric) chromosomes, which is expected if one considers that NF is commonly above 80 in species with diploid numbers of 48 and 50 (Klinkhardt *et al.,* 1995).

Both of the species investigated in this study had one pair of NOR-bearing chromosomes, a pattern common in other Leuciscinae. The NORs of *A. bipunctatus* and *A. filippii* were observed at the end of the short arms of one pair of medium-sized SM and of one pair of medium-sized ST chromosomes, respectively. Similar results have been obtained in a number of Eurasian cyprinid fishes from the Leuciscinae (*Abramis*, *Aspius*, *Leucaspius*, *Leuciscus*, *Rutilus*, *Scardinius*, and *Vimba*; reviewed in Ráb and Collares-Pereira, 1995; Ra´bova´ *et al.,* 2003).

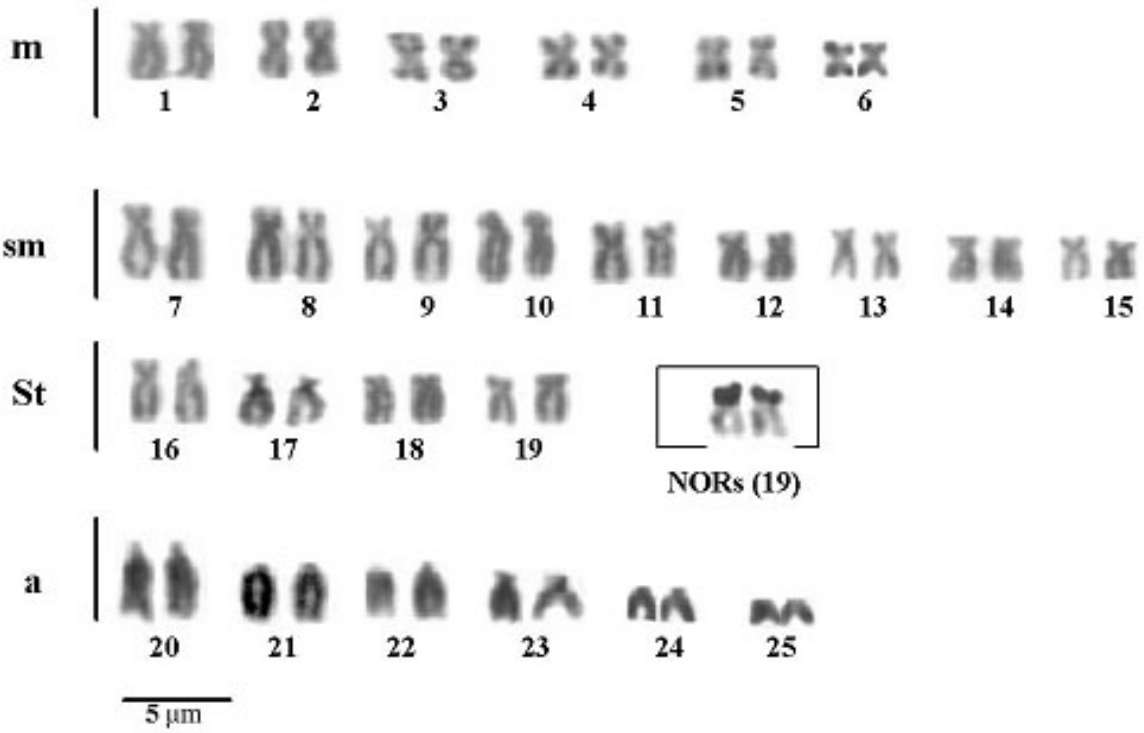

FIGURE 2.- Giemsa-stained karyotype of *Alburnus filippii*. The NOR-bearing chromosome pair, after silver staining, is shown in the box. = 5 µm.

It has been suggested that chromosomal NORs can play a role in inferring phylogenetic relationships, mainly based on a primitive pair of NOR-bearing chromosomes in most vertebrates (Ameniya and Gold, 1990). Our results reinforce this, with the two cyprinids having a pair of NORs. Indeed, the majority of the approximately 100 species of Cyprinidae investigated thus far (Klinkhardt *et al.,* 1995) show only one pair of NOR-bearing chromosomes, located in the terminal position of the short arms of ST or SM chromosomes. Buth *et al.* (1991), in their study of 69 North American cyprinid species, reported that most species had one or two pairs of NORs, while only three species had three pairs of NOR-bearing chromosomes.

The NOR can be found on the short arm, long arm, or in the regions close to the centromere. NOR number may range from one pair to eight. Although NORs are seen generally at the end of the short arms of ST and SM chromosomes, they may also sometimes be seen at the end of the long arms of SM and ST chromosomes, on the arms of M and A chromosomes, between telomeres and centromeres, or adjacent to the centromere (Galetti *et al*., 1984; Rab *et al.,* 1995). Although variants of NORs have been identified within and among some genera (e.g., *Alburnus albidus*, Bianco *et al.,* 2004; *Alburnus alburnus*, Schmid *et al.,* 2006; *Aspius aspius*, Ráb, Roth and Aref'ev, 1990; *Chondrostoma lusitanicum*, Rodriguez and Collares-Pereira, 1999; *Phoxinus phoxinus* and *Eupallasella perenurus*, Boron, 2001; *Vimba vimba* and *V. elongate*, Ra´bova´ *et al.,* 2003), the most common phenotype appears to be a single, smaller NOR-bearing SM chromosome pair with the NORs situated on the short arms (Ráb *et al.,* 1996; Collares-Pereira *et al.,* 1998). An exception is *Aspius aspius*, where the NORs are positioned on the long arm telomeric regions (Ra´b *et al.,* 1990). This characteristic 'leuciscine' NOR phenotype is also present in *Alburnoides* and *Alburnus*. Indeed, the two species of *Alburnoides* and

Alburnus possess similar karyotypes, chromosomal markers, and NOR phenotypes typical for other Eurasian leuciscine cyprinids.

NOR phenotypes have proven useful in cyprinid cytotaxonomy. For example, Buth et al. (1991) distinguished 15 or more NOR phenotypes in more than 100 North American cyprinids. Similarly, Takai and Ojima (1992) reviewed interspecific NOR variation in over 30 Japanese cyprinids. Among the European cyprinids, NOR variability has been insufficiently analyzed; in most cases, a single pair of NOR-bearing chromosomes was detected. However, Eurasian leuciscine cyprinids are not well known for this cytotaxonomic marker (see Ráb and Collares-Pereira, 1995).

The number of the NORs, the morphology of NOR-bearing chromosomes, and the position of the NORs on the chromosomes has been observed to vary, even in closely related species with similar karyotypes (Takai and Ojima, 1986). An apparently homologous NOR-bearing chromosome pair is present in the karyotypes of the two species under study. The differences in the location of NORs on chromosomes of *A. bipunctaus* and *A. filippii* seem to be species-specific in comparison with those of the European Cyprinidae. These NOR chromosome phenotypes might represent a derived condition among Cyprinidae.

Due to lack of available cytological information for *Alburnoides* and *Alburnus*, these chromosomal analyses provide valuable data to delineate intrageneric systematics. Knowledge of chromosome morphology becomes more important in cases of closely related taxa with the same chromosome number. In conclusion, further comparative cytotaxonomic and cytogenetic studies of the *A. bipunctaus-A. filippii* species complex are needed to determine whether chromosomes play an important role in diversification of these species in Iran. In addition, using classes of repeated sequences could also provide more information concerning their genomic structure.

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