Marine Biology (1997) 129: 33–39

M. A. Garrido-Ramos · M. C. Soriguer · R. de la Herrán
M. Jamilena · C. Ruiz Rejón · A. Domezain
J. A. Hernando · M. Ruiz Rejón

Morphometric and genetic analysis as proof of the existence of two sturgeon species in the Guadalquivir river

Received: 28 November 1996 / Accepted: 10 March 1997

Abstract Morphometric and genetic methods were used to identify two sturgeon species, *Acipenser naccarii* Bonaparte, 1836, and *A. sturio* Linnaeus, 1758, captured in some of the principal rivers of the Iberian Peninsula, including the Guadalquivir. After measuring 25 Iberian specimens from a fishery and several Spanish and Portuguese museums and applying stepwise discriminant analysis (SDA), four specimens preserved in different museums [two specimens from the Guadalquivir river (EBD-8173 and EBD-8174), one specimen from the Tagus river (MUC1) and one specimen from the Mondego river (MUC46B)], as well as five specimens captured in the Guadalquivir river in the 1940s but not preserved (CM1, CM2, CM3, CM4 and CM5), were identified as *A. naccarii*. After cloning and characterisation of a satellite-DNA family, *Hind*III, from *A. naccarii* genome, its absence from the genome of *A. sturio* was determined. Using this satellite-DNA as a genetic marker and by means of dot-blotting, we demonstrate that the DNA of the two specimens captured during the mid-1970s in the Guadalquivir river cross-hybridised with *Hind*III satellite-DNA sequences of *A. naccarii*. We conclude that *A. naccarii* is autochthonous to the Iberian Peninsula and is not, as was previously believed, endemic to the Adriatic Sea.

Communicated by A. Rodríguez, Puerto Real

M.A. Garrido-Ramos · R. de la Herrán · M. Jamilena · C. Ruiz Rejón · M. Ruiz Rejón (✉)
Departamento de Génetica, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain

M.C. Soriguer · J.A. Hernando
Departamento de Biología Animal, Biología Vegetal y Ecología, Facultad de Ciencias del Mar, Universidad de Cádiz, E-11510 Puerto Real, Cádiz, Spain

A. Domezain
Departamento de Invetigación y Desarrollo, Piscifactoría Sierra Nevada, E-18313 Riofrío, Granada, Spain

Introduction

The sturgeons (family Acipenseridae) are among the most ancient ray-finned fishes. This extremely endangered group of animals should be the object of biological conservation programmes (Birstein 1993) which, to be effective, require preliminary study of the specific status of sturgeons in the different regions of their distribution area. As part of a general study of the Iberian Peninsula, we conducted morphometric and genetic analyses to determine which sturgeon species have inhabited the Guadalquivir river (Southwest Spain) up to the present. It is generally accepted that the main sturgeon species in Western Europe, from Scandinavia to the Mediterranean, is the common sturgeon *Acipenser sturio* Linnaeus, 1758. This name was assigned to sturgeons throughout the Iberian Peninsula (Classen 1944; Elvira et al. 1991). However, some authors have reported the presence of the Adriatic sturgeon *A. naccarii* Bonaparte, 1836, a species currently considered to be endemic to the Adriatic region (Svetovidov 1989), in Portuguese rivers (Capello 1869; Gonçalves 1942). According to historical evidence, a large sturgeon population has inhabited the Guadalquivir river since Iberian times, and was exploited for caviar for centuries until 1956 (Javierre 1984). Today, sturgeons have almost disappeared from the river, with only three specimens captured since 1974 (Hernando 1975; Elvira and Almodóvar 1993). Therefore, for a definitive species identification, museum specimens have had to be analysed in addition to live specimens. In the present work we used morphometric and genetic methods to determine whether *A. naccarii* has been captured from some of the major rivers of the Iberian Peninsula, in particular the Guadalquivir river. Results have been compared to those obtained from *A. sturio* specimens of confirmed identification (two specimens from the Garonne river, France), and from several *A. naccarii* specimens of confirmed identification from a Spanish fish farm originating from fish stocks from Azienda Agrícola VIP (Brescia, Llanura Padana,

Italy) and widely recognised in the literature as belonging to this latter species (e.g. Fontana 1994; Arlati et al. 1995).

Materials and methods

Morphometric data for 25 specimens *Acipenser* spp. are shown in Table 1 together with the years in which they were captured. The legend to Table 1 gives the names of the museums or commercial fisheries, and the rivers of origin. Following Classen (1944) and the keys of Holcik et al. (1989), Sokolov (1989) and Svetovidov (1989), we measured: total length (TL), the distance from the tip of the snout to the barbels (A), the width of the snout at the base of the barbels (B), the distance between the base of barbels and the cartilaginous arch of the mouth (C), the distance between the tip of the snout and the cartilaginous arch of the mouth (F), and the length from the tip of snout to the frenulum (LFR). In addition, C-A and F:B values were calculated (the relative position of the barbels on the cartilaginous arch of the mouth and the ratio between the snout length and width, respectively). To investigate the possibility of any allometry between these indices and total length, we carried out a Student's *t*-test on the slope of the regression for TL vs C-A and TL vs F:B (Table 2) using the software Statgraphics^{TM} Plus (1995). Stepwise discriminant-analysis (SDA) using BMDP^{TM} software (Jennrich and Sampson 1990) was applied to the six morphometric variables (TL, A, B, C, F and LFR) measured and to both indices (C-A and F:B) for each specimen.

Genetic analysis was carried out on the specimens in Table 3. The DNA of live *Acipenser naccarii* specimens from Sierra Nevada

Table 1 *Acipenser* spp. Specimens examined in present study. Specimens from Guadalquivir river were: EBD-8173 and 8174 (Biological Station of Doñana, Seville); CM-1 to CM-5 [5 males captured from Guadalquivir river in 1940, full data for which are given in Table 1 of Classen's (1944) pioneer work]; SE-1 to SE-3 (Department of Animal Biology, University of Seville); Z1582 (Museum of Natural Sciences of Madrid); and AEC (Aguilar and Eslava Museum, Cordoba Province). PSN-1: live specimen of *A. naccarii* obtained from Sierra Nevada Fishery at Riofrío, Granada, Spain. Specimens from Portugal were: MUC1 and MUC46B [(Museum of University of Coimbra, captured from Tagus river (MUC1) and from Mondego river (MUC46B)]; AVG (Vasco da Gama Aquarium, Dafundo, Lisboa, captured from Miño River); MAN2 (Augusto Nobre Museum, University of Porto, from unknown river). Specimens from the Ebro river were: MNC-44145, MNCMD1, MNCMD2, MNC-33 and MNCMD3 (Natural Sciences Museum of Madrid) and B-82-5337, B-82-5340 and B-82-5342 (Zoological Museum of Barcelona) (*TL* total length; *A* distance from tip of snout to barbels; *B* width of snout at base of barbels; *C* distance between base of barbels and cartilaginous arch of mouth; *F* distance between tip of snout and cartilaginous arch of mouth; *LFR* length from snout to frenulum; *C-A* relative position of barbels on cartilaginous arch of mouth; F:B ratio between snout length and width)

Specimen No.	Preservation	Year collected	TL (cm)	A	B	C	F	LFR	C-A	F:B
EBD-8173	Ethanol-preserved	1974	175.50	5.80	11.60	7.40	13.20	24.50	1.60	1.14
EBD-8174	Stuffed	1975	152.00	5.20	11.40	12.60	12.60	–	2.20	1.10
CM-1	Live[a]	1940	163.00	7.00	8.50	9.50	16.50	23.50	2.50	1.94
CM-2	Live[a]	1940	145.00	6.00	8.50	8.00	14.00	22.00	2.00	1.65
CM-3	Live[a]	1940	152.00	6.00	9.30	7.20	13.20	22.70	1.20	1.42
CM-4	Live[a]	1940	154.00	7.00	8.50	8.50	15.50	24.00	1.50	1.82
CM-5	Live[a]	1940	145.00	6.00	7.00	7.50	13.50	21.00	1.50	1.93
MUC1	Stuffed	1890	203.00	4.20	9.25	6.80	10.60	25.40	2.60	1.15
MUC46B	Stuffed	1897	153.00	5.30	6.14	6.40	11.72	23.20	1.10	1.91
PSN-1	Live	1994	117.00	3.30	7.60	4.30	7.45	21.00	1.00	0.98
SE-1	Stuffed	End of 19th century	30.00	2.40	2.20	1.50	4.60	6.10	−0.90	2.09
SE-2	Stuffed	End of 19th century	31.50	2.15	2.00	1.30	4.30	6.15	−0.85	2.15
SE-3	Stuffed	End of 19th century	20.00	1.76	1.40	0.80	3.20	4.74	−0.96	2.29
Z1582	Ethanol-preserved	Between 1936 and 1944	23.50	2.15	1.94	1.44	3.55	5.50	−0.96	1.83
AEC	Stuffed	End of 19th century	125.00	6.50	5.60	6.50	13.00	24.50	0.00	2.32
MNC44145	Stuffed	?	83.00	4.50	2.70	4.05	8.55	16.00	−0.45	3.17
MNCMD1	Ethanol-preserved	?	34.50	2.75	2.60	2.24	4.99	7.60	−0.51	1.92
MNCMD2	Ethanol-preserved	?	33.30	2.80	2.24	2.20	5.00	7.70	−0.60	2.23
MNC-33	Ethanol-preserved	?	25.50	2.24	1.77	1.60	3.84	5.50	−0.64	2.17
MNCMD3	Ethanol-preserved	?	24.50	2.38	2.00	1.74	4.12	6.50	−0.64	2.06
B-82-5337	Stuffed	?	129.00	9.40	7.00	9.40	18.80	29.00	0.00	2.69
B-82-5340	Stuffed	1983?	48.00	3.70	2.20	3.70	7.40	10.30	0.00	3.36
B-82-5342	Stuffed	1983?	72.00	4.40	2.80	4.40	8.80	14.00	0.00	3.14
AVG	Stuffed	1961	175.00	3.40	2.60	3.40	6.80	12.60	0.00	2.61
MAN2	Stuffed	End of 19th century	42.00	3.20	2.05	3.20	6.40	9.20	0.00	3.12

[a] Measurements all made on live specimens by Classen (1944)

Table 2 *Acipenser* spp. Results of Student's *t*-test. Group codes are 1 = *naccarii*, 2 = *sturio* (*Abbreviations* as in legend to Table 1)

Variables, Group	*p* value	R^2	Intercept	Slope
TL vs C-A				
1	0.0434	41.78	−0.888	0.0167
2	0.2055	12.02	−0.630	0.0027
TL vs F:B				
1	0.8080	0.78	1.743	−0.0015
2	0.1949	12.56	2.250	0.0037

Fishery at Riofrío, Granada, Spain and frozen *A. sturio* specimens from CEMAGREF (Centre National du Machinisme Agricole, du Génie Rural, des Eaux et Forêt), Bordeaux, France were isolated from liver and muscle, respectively, following the procedure described by Sambrook et al. (1989). Cloning, sequencing, Southern-blot and dot-blot hybridisations were performed as previously described (Garrido-Ramos et al. 1994). The DNA from museum specimens were isolated as previously described Pääbo (1989).

Results and discussion

Morphometric analysis of *Acipenser* spp.

As shown in Table 1, Specimens EBD-8173, EBD-8174 and CM-1 to CM-5 from the Guadalquivir river, and Specimens MUC-1 and MUC46B from the Tagus and Mondego rivers (Fig. 1), formed a first group related to their C-A values (C-A > 0.0); these values resembled that obtained from one live specimen of *Acipenser naccarii* from the Sierra Nevada Fishery (Individual

Table 3 *Acipenser* spp. Genetic data from dot-blotting hybridisation indicating presence (+) or absence (−) of *Hind*III satellite-DNA family in genome of different sturgeon specimens. River of origin, year of collection and classification according to morphometric data are indicated

Specimen No.	Origin (river and year collected)	Preservation	Classification	Presence/absence of *Hind*III satellite
EBD-8173[a]	Doñana Biological Station (Guadalquivir river, 1974)	ethanol	*A. naccarii*	+
EBD-8174[a]	Doñana Biological Station (Guadalquivir river, 1975)	stuffed	*A. naccarii*	+
PSN-1	Sierra Nevada Fishery (1994)	live specimen	*A. naccarii*	+
PSN-2[b]	Sierra Nevada Fishery (1994)	live specimen	*A. naccarii*	+
Z1582	Natural Sciences Museum (Guadalquivir river, 1936–1944)	ethanol	*A. sturio*	−
CEM-1[b]	CEMAGREF. Bordeaux (Garonne river)	frozen	*A. sturio*	−
CEM-2[b]	CEMAGREF. Bordeaux (Garonne river)	frozen	*A. sturio*	−
UGRA1[b]	University of Granada (Guadalquivir river)	stuffed	*A. sturio*	−

[a] Specimens EBD-8173 and EBD-8174 previously classified as *A. sturio* by Hernando (1975)
[b] Morphometric data for these specimens are not complete; they were therefore not included in Table 1

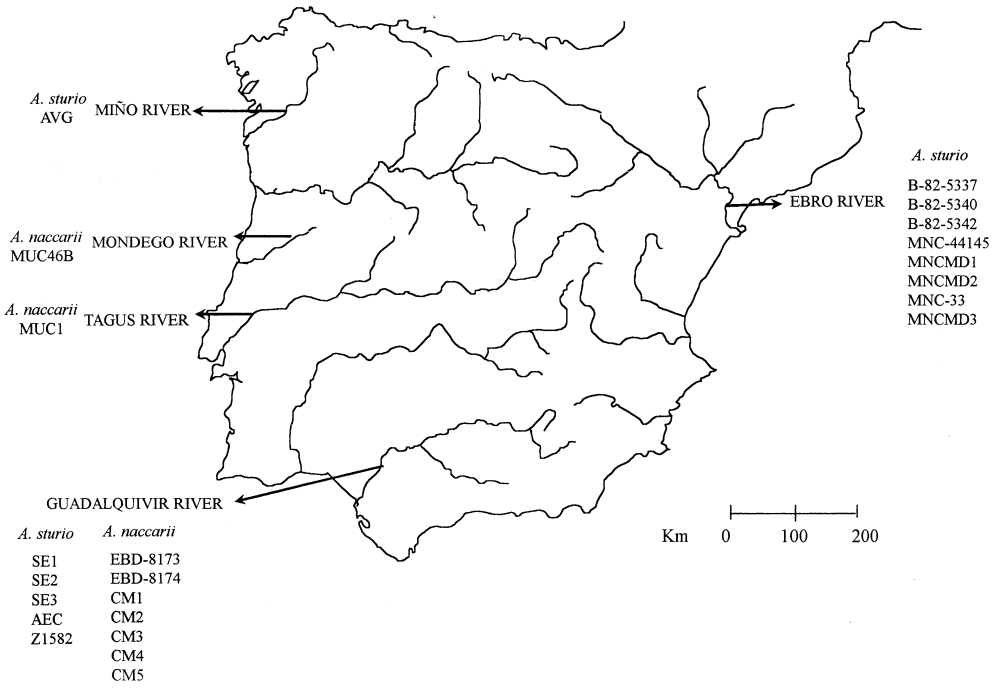

Fig. 1 Map of Iberian Peninsula, showing rivers from which analysed specimens of *Acipenser* spp. originated (Specimen descriptions are given in Table 1)

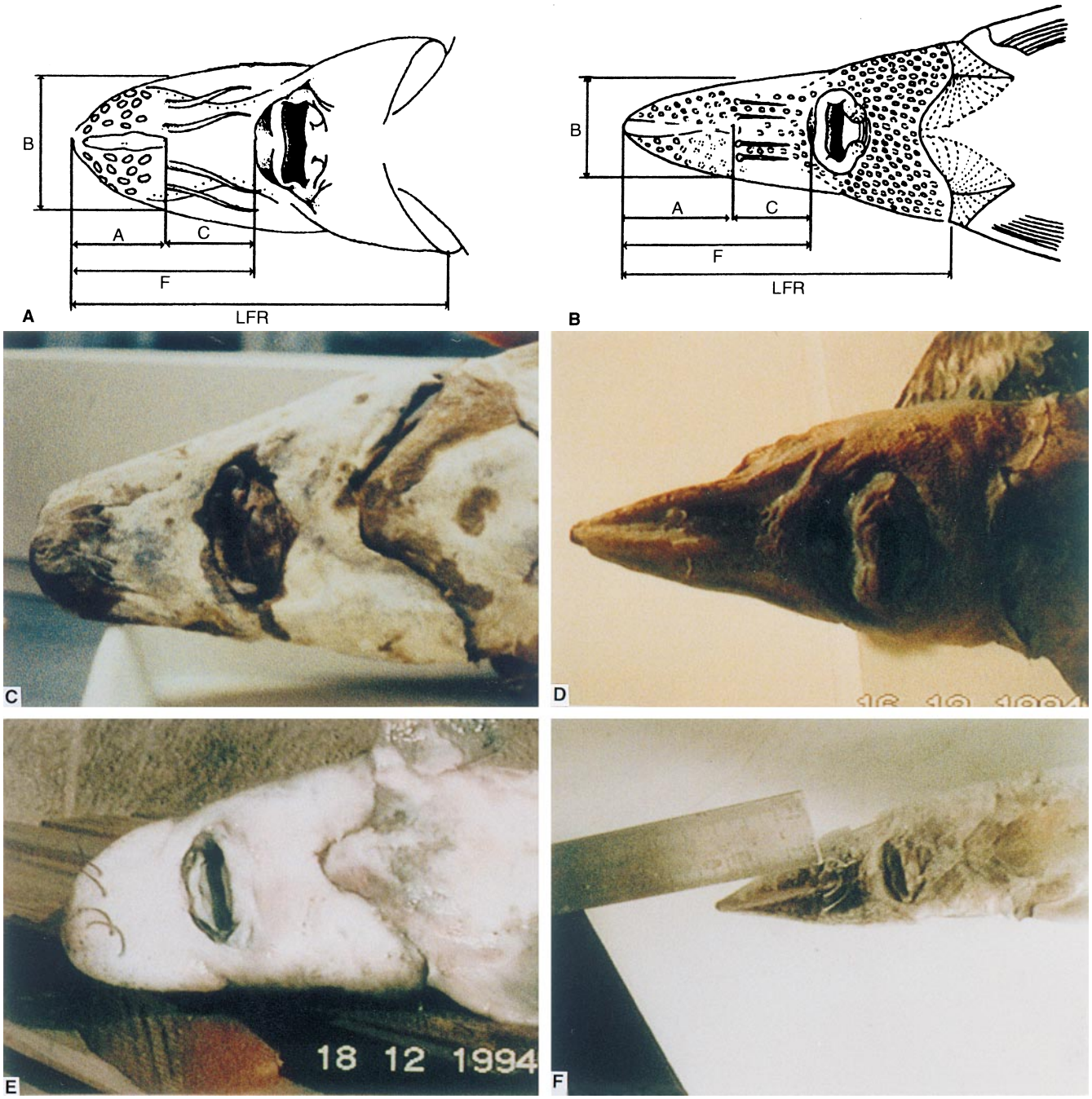

Fig. 2 *Acipenser* spp. Archetypes of *A. naccarii* (**A**) and *A. sturio* (**B**) identified according to Svetovidov (1989); **C** EBD-8174 specimen of *A. naccarii from Doñana;* **D** AEC specimen of *A. sturio* from Aguilar and Eslava Museum; **E** live *A. naccarii* specimen from Sierra Nevada Fishery; **F** *A. sturio* specimen kept in refrigerator since 1989 in Bordeaux by Dr E. Rochard (CEMAGREF, Division of Aquaculture and Fish, Bordeaux, Gaxinet Cedex) (*A* distance from tip of snout to barbels; *B* width of snout at base of barbels; *C* distance between base of barbels and cartilaginous arch of mouth; *F* distance between tip of snout and cartilaginous arch of mouth; *LFR* length from snout to frenulum)

PSN-1). In contrast, C-A values were all <0.0 in the remaining specimens, who thus formed a second group. In the first group of specimens, the snouts were wide ($6.14 < B < 11.6$) and short ($1.10 <$ F:B < 1.90); in the second group they were narrow ($1.03 < B < 7$) and long ($1.83 <$ F:B < 3.12). According to Sokolov (1989) and Svetovidov (1989), specimens of the first group morphologically correspond to *A. naccarii* and those of the second to *A. sturio* (archetypes illustrated in Fig. 2). Therefore, two groups were defined, "*naccarii*" and "*sturio*", to which each specimen was assigned according to a code – 1 (*naccarii*) and 2 (*sturio*). A Student's *t*-test of the slope of the regression lines revealed no allometric relationships for standard values of alpha (Table 2). In

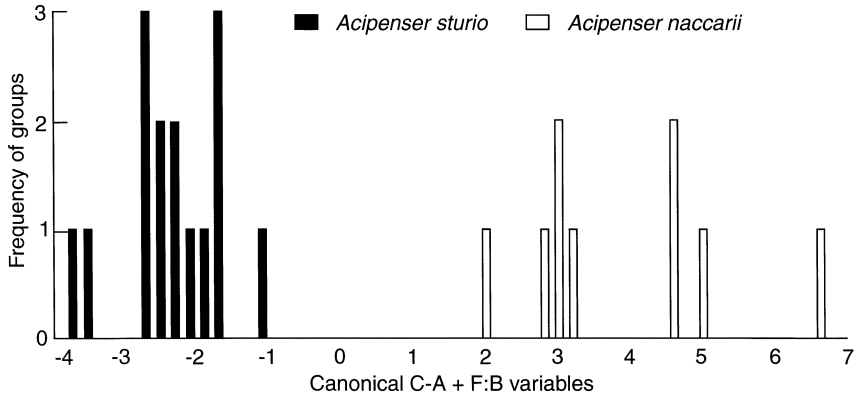

Fig. 3 *Acipenser* spp. Histogram of canonical variables separating *naccarii* group (positive values), from *sturio* group (negative values), on plane defined for the two groups and using distances calculated by Mahalanobis method (*C-A, F:B* as in legend to Table 1)

the stepwise discriminant-analysis, the first discriminatory variable of the groups was C-A ($F = 112.53$; $df = 1.22$; lambda = 0.163); the second was F:B, the ratio between the distance from the tip of the cartilaginous arch to the mouth and the width of the snout to the height of the barbels ($F = 103.43$; $df = 1.21$; lambda = 0.092), both had a tolerance of 0.820. With these data, we defined two planes that differentiated the two groups with 100% reliability in the assignation of specimens. The *naccarii* group is defined by the equation 5.68 (C-A) + 4.79 F:B − 9.14 = 0, the *sturio* group by 15.04 F:B − 8.27 (C-A) − 21.02 = 0, with a canonical correlation of 0.953. Calculation of the distances calculated by the Mahalanobis method and representation of the two groups on the plane defined by the two canonical variables (Fig. 3), using the equation 2.25 (C-A) − 1.65 F/B + 2.69 = 0, groups the specimens assigned to *naccarii* on the positive side and those to *sturio* on the negative side of the graph (Fig. 3).

Thus, morphometric data from sturgeon specimens preserved in different Spanish and Portuguese museums revealed that two specimens from the Guadalquivir river (EBD-8173 and EBD-8174), one specimen from the Tagus river (MUC-1) and one from the Mondego river (MUC46B), correspond to *Acipenser naccarii*. In addition, the five males (CM1 to CM5) from the Guadalquivir river (included in Table 1 of the pioneer work by Classen 1944) also belong to *A. naccarii*. *A. naccarii* and *A. sturio* differ fundamentally in that the barbels of *A. naccarii* are closer to the tip of the snout, which is short and wide, while the barbels of *A. sturio* are closer to the mouth or equidistant between the mouth and the tip of the snout, which is long and narrow (Fig. 2). The SDA (Fig. 3) clearly confirms that the relative position of the barbels (C-A) is the principal trait differentiating the two species, followed by the ratio of the distance from the tip of the cartilaginous arch to the mouth and the snout width to the height of the barbels (F:B). Therefore, discriminant-analysis separates specimens with barbels nearer to the tip of the snout than to the mouth and with a roundish snout (*A. naccarii*) from specimens with a pointed snout and barbels halfway between the mouth and the tip of the snout or closer to the mouth (*A. sturio*). These results assigned the specimens captured in the Guadalquivir by Classen (1944) and Hernando (1975) to the *naccarii* group.

Genetic analysis

To verify the above conclusions, we searched for a molecular marker which could differentiate between the two sturgeon species. Using samples with confirmed classification of *Acipenser naccarii* (Specimens PSN-1 and PSN-2) and *A. sturio* (Specimens CEM-1 and CEM-2; Table 3), we identified a satellite-DNA family that is present in the genome of *A. naccarii* but absent from the genome of *A. sturio*. Satellite-DNA sequences evolve rapidly, being relatively homogeneous within a species but differing between even closely related species (Singer 1982). Satellite-DNA sequences are, therefore, a valuable tool for establishing evolutionary relationships between related species (Garrido-Ramos et al. 1995). Moreover, satellite-DNA sequences often display such dramatic contrast between closely related species that new satellite-DNA families can appear or disappear within a short evolutionary time. Some satellite-DNA families are therefore species-specific, while others occur in a complete group of species (Miklos 1985). In addition to phylogenetic purposes, satellite-DNA could also be valuable for determining the specific status of a given specimen.

Cloning and characterisation of a satellite-DNA family from Acipenser naccarii

A brilliant band of ~170 base pairs (bp), visible when *Hind*III-restricted DNA of *Acipenser naccarii* (Specimen PSN-1) was fractionated in low-melting agarose gel, was isolated and cloned. Eleven of the positive clones probed with an aliquot of the isolated band were selected. Southern-blot hybridisation of the cloned sequences to restricted DNA from *A. naccarii* (PSN-1) revealed that these sequences are part of a repeated DNA family composed of 171 bp repeats organised in tandem

Fig. 4 *Acipenser naccarii.* Southern-blot hybridisation of plasmid insert of Clone pHAn19 (containing one *Hind*III satellite-DNA repeat) to DNA digested with *Hind*III (*A*), *Hae*III (*B*), *Bam*HI (*C*), *Dra*I (*D*) and *Eco*RI (*E*) (*arrowhead* indicates monomeric units of 171 bp generated with *Hae*III and *Hind*III)

(Fig. 4). No hybridisation was detected with restricted DNA of the two specimens of *A. sturio* from Bordeaux.

Fig. 5 shows the nucleotide sequence of the 11 cloned *Hind*III repeats with a consensus length of 171 bp. Pairwise comparison between the 11 cloned sequences indicated $<7\%$ sequence divergence. The sequences are AT(adenine-thymine)-rich (67%) and present numerous short stretches of consecutive adenines and thymines, which is a common feature of other satellite-DNAs.

Use of satellite-DNA family of Acipenser naccarii for species identification in Guadalquivir River

Southern-blot hybridisation analysis revealed that the *Hind*III satellite-DNA family is absent from the *Acipenser sturio* genome. The presence or absence of the *Hind*III satellite-DNA sequences thus differentiates the two sturgeon species. To analyse the presence or absence of this satellite-DNA in certain specimens captured in the Guadalquivir river, DNA from these specimens were dotted on a membrane and hybridised with a cloned unit of the satellite-DNA. The results are summarised in Table 3. Hybridisation was detected in DNA from live specimens of *A. naccarii* and the two specimens from the Doñana Museum. No hybridisation was detected in the remaining samples (Table 3). To test the quality of the "ancient DNA", samples were also hybridised with ribosomal DNA as a positive control and a repeated

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pAl1     CTTTTTTAAT CTTTTGGGGC ATTGAAATTA TGAAAAAATA AAATTGGCCA AAATTATTAT TTTTT*GACA GGACCGTACC AGACCACTTT
         |||||| ||  |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||| |||| |||||| ||| ||||||||||   90
C.S.     CTTTTTCAAA CTTTTGGGGC ATTGAAATTA TGAAAAAATA AAATTGGCCA AAATTATTAT TTTTTTGACA GGACCGGACC AGACCACTTT
pHAn3    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn5    ---------T ---------- ---------- ---------- ---------- ---------- -----*---- ------T--- ----------
pHAn18   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn25   ---------- ---------- ---------- ---C------ ---------- ---------- *--------- ---------- ----------
pHAn31   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn40   ---------G ---------- ---------- ---------- ---------- ---------- ---------- ---------- --T-------
pHAn49   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn19   ---------- AGC-C----- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn29   ---------- AGC-C----- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn43   ---------- AGC-C----- ---------- ---------- ---------- ---------- ---------- ---------- ----------
pHAn52   ---------- AGC-C----- ---------- ---------- --T------- ---------- -G--------- ---------- ----------
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pAl1     TTCAAAAAAG GGGGCTGTCT AAATTTTGGT AGTTCTGAAG ATCATAAAAT TGTGTTTTCT TGACAGGAAC GAACCTGTAA G
         |||||||||| |||| ||||| |||||||||| |||||||||| |||| ||||| |||||||||| |||||||||| |||||||||| |          171
         TTCAAAAAAG GGGGATGTCT AAATTTTGGT AGTTCTGAAG ATCAAAAAAT TGTGTTTTCT TGACAGGAAC GAACCTGTAA G
         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
         ---------- ----C----- ---------- ---------- ----T----- ---------- -------G-- ---------- -
         ---------- ---------- ---------- ---------- ---------- G--------- ---------- ---------- -
         ---------- ----C----- ---------- T--------- ---------- ---------- ---------- ---------- -
         ---------- ---*------ ---------- ---------- ---------- ---------- ---------- ---------- -
         ---------- ----G----- ---------- ---------- ---------- ---------- ---------- A--------- -
         ---------- ---------- ---------- ---------- -------C-- ---------- -C--------- ---------- -
         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
         ---------- ---------- ---------- ---------- ---------- ---------- ---------- A--------- -
         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
         ---------- ---------- ---------- --G------- ---------- ---------- ---------- ---------- -
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Fig. 5 *Acipenser naccarii.* Nucleotide sequence of 11 cloned *Hind*III repeats from live specimens (*C.S.* consensus sequence) Only base changes differing from consensus sequence are shown for individual clones. Note divergent region between 11 to 15 base positions that distinguishes two types of repeat units. pAl1 clone is sequence of a monomeric unit obtained from Specimen EBD-8173 preserved in ethanol at Biological Station of Doñana since 1975 (*asterisks* indicate deletions)

DNA sequence from *Pagrus auriga* (Sparidae) as a negative control. The samples all hybridised with the positive control but not with the negative one. Therefore, dot-blotting hybridisation supports morphological results, confirming that Specimens EBD-8173 and EBD-8174 captured in the Guadalquivir river do not correspond to *A. sturio*.

To verify the suggestion that these two museum specimens are *Acipenser naccarii*, as indicated by morphological data, we cloned and sequenced one monomeric unit of this *Hind*III satellite in Specimen EBD-8173 (Clone pAl1; Fig 5). The sequence revealed a high level of sequence homogeneity (96.5%) with the consensus sequence obtained from the 11 clones isolated from one live specimen of *A. naccarii* (Specimen PSN-1). This sequence homology is even higher than that obtained between some pair-wise comparisons of the 11 clones from PSN-1 (93% homology), suggesting that the Museum Specimen EBD-8173 captured in the Guadalquivir river corresponds to *A. naccarii*.

In conclusion, morphometric and genetic data confirm the species' keys of Sokolov (1989) and Svetovidov (1989) and demonstrate that both *Acipenser naccarii* and *A. sturio* have been captured in some of the most important rivers of the Iberian Peninsula including the Guadalquivir river. *A. naccarii* is, therefore, demonstrated to be an autochthonous species of the Iberian Peninsula and not only endemic to the Adriatic Sea.

Acknowledgements We wish to express our gratitude to Dr A. Inacio, Aquario Vasco da Gama (Dafundo, Lisboa); Dr L. Sousa, Museu Augusto Nobre (Universidade do Porto); Dr M.A.V. Ferreira, Museu da Historia Natural (Universidade do Coimbra); Dr J. Cabot, Estación Biológica de Doñana (C.S.I.C.), Sevilla; Dr M. Rochard, Cemagref, Bordeaux; Dr J. Dorda, Museo Nacional de Ciencias Naturales de Madrid; Dr J.M. Pleguezuelos, Departamento de Biología Animal y Ecología de la Universidad de Granada; and Dr A.M. García-Rojas, Museo de Aguilar y Eslava en Cabra (Córdoba), for their advice and for providing us with samples. Also, we would like to thank Dr F. Fernández-Palacín (Universidad de Cádiz) for his help with the statistical analysis, and the British biologist C. Coope for her revision of the English language of the manuscript. Finally, we would like to thank all three referees for their useful suggestions in their review of our work.

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