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Phylogeography and local endemism of the native Mediterranean brine shrimp *Artemia salina* (Branchiopoda: Anostraca)

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Abstract

There has been a recent appreciation of the ecological impacts of zooplanktonic species invasions. The North American brine shrimp *Artemia franciscana* is one such alien invader in hyper-saline water ecosystems at a global scale. It has been shown to outcompete native *Artemia* species, leading to their local extinction. We used partial sequences of the mitochondrial Cytochrome *c* Oxidase Subunit 1 (COI or *cox1*) gene to investigate the genetic diversity and phylogeography of *A. salina*, an extreme halophilic sexual brine shrimp, over its known distribution range (Mediterranean Basin and South Africa) and to assess the extent of local endemism, the degree of population structure and the potential impact of traditional human saltpan management on this species. We also examined the phylogenetic relationships in the genus *Artemia* using COI sequences. Our results show extensive regional endemism and indicate an early Pleistocene expansion of *A. salina* in the Mediterranean Basin. Subsequent population isolation in a mosaic of Pleistocene refugia is suggested, with two or three refugia located in the Iberian Peninsula. Two instances of long-distance colonization were also observed. Surprisingly, given its strong phylogeographical structure, *A. salina* showed a signature of correlation between geographical and genetic distance. Owing to strong '*priority effects*', extensive population differentiation is retained, despite dispersal via migrant birds and human management of saltpans. The foreseeable expansion of *A. franciscana* is likely to be followed by substantial loss of genetic diversity in Mediterranean *A. salina*. Large genetic divergences between Mediterranean and South African *A. salina* suggest that the latter deserves species status.

Keywords: COI, conservation genetics, long distance dispersal, multiple refugia, Nested Clade Phylogeographical Analysis, population structure, priority effect

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Introduction

Lakes and ponds are isolated ecosystems which, in addition to the actively dispersed insects and amphibians, include a wide array of passively dispersed invertebrates, both planktonic and benthic (e.g. copepods, ostracods, rotifers and branchiopods), which form important part of the biomass and food webs of these systems. These aquatic invertebrates produce diapausing resistant stages in their life cycle which allow them to survive '*in situ*' during adverse periods or drought. There has been a recent realization that accidental (e.g. by transport of ballast water of ships) or intentional (e.g. through aquaculture, fisheries or pet trade) introductions are leading to nonindigenous species invasions in aquatic habitats. Invasive events are occurring at an alarming rate and often lead to drastic changes in ecosystem functioning and species extinctions (Cristescu *et al*. 2001; Bollens *et al*. 2002; Frisch *et al*. 2006; Mergeay *et al*.

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2006). The documentation of species invasions amongst passively dispersed invertebrates has been made easier by the use of polymerase chain reaction (PCR)-based genetic analyses, given the abundance of cryptic species amongst these organisms (Gómez *et al*. 2002; Adamowicz & Purvis 2005; Mergeay *et al*. 2005; Adamowicz *et al*. 2007). However, conservation and management strategies have largely ignored biodiversity losses in passively dispersed aquatic invertebrates, which comprise small 'uncharismatic' species (Hamer & Brendonck 1997; Belk 1998; Muñoz 2007).

Population structure and phylogeographical analyses in passively dispersed continental aquatic invertebrates have indicated that, despite their high colonization ability via diapausing stages, these organisms often show an unexpectedly high degree of genetic differentiation and local endemism which reflects a highly reduced level of gene flow among established populations (Hebert 1998; Hebert *et al*. 2003b; Penton *et al*. 2004; De Gelas & De Meester 2005; Paland *et al*. 2005; Ishida & Taylor 2007; see reviews in De Meester 1996, 2002). Given this combination of high colonization ability and reduced rates of ongoing gene flow, two types of phylogeographical scenario have been identified in continental zooplankton. In the first, relatively old populations, in or around Pleistocene refugia, show a deep genetic structure and strong interpopulation divergence leading to a high degree of local endemism (Gómez *et al*. 2000, 2007a; Zierold *et al*. 2007). In the second contrasting scenario, little or no geographical structure and reduced genetic diversity is found, indicative of recent and rapid range expansion into a newly available area, either after natural colonization — often postglacial — or following human introduction (Weider *et al*. 1999; Mergeay *et al*. 2005; Ishida & Taylor 2007). A combination of both scenarios can be found in the same species depending of the age of its habitat across the range (Ishida & Taylor 2007).

The brine shrimp genus *Artemia* (Crustacea, Branchiopoda, Anostraca) comprises seven recognized sexual species and several parthenogenetic lineages with different ploidy levels (Gajardo *et al*. 2002; Baxevanis *et al*. 2006). Brine shrimps are extreme halophilic organisms and can withstand a wide salinity range, from 45 g/L to up to 370 g/L (Browne & Hoopes 1990), including different types of ionic composition (see Van Stappen 2002 for a review). They inhabit patchy hypersaline aquatic habitats such as salt lakes and saltworks (salines or saltpans) worldwide in both inland and coastal localities, except Antarctica (Triantaphyllidis *et al*. 1998). These anostracans also constitute an important food source for aquatic birds (Sánchez *et al*. 2007). Most *Artemia* strains can reproduce both by ovoviviparity (producing immediately hatching eggs) and oviparity (producing diapausing cysts). The reproductive mode is often ovoviviparity when environmental conditions are favourable. Under adverse conditions, they produce resistant, diapausing cysts (i.e. encysted embryos) (Criel 2002). These resistant forms accumulate in saltpan or lake sediments forming diapausing egg banks, allowing the persistence of populations during unfavourable periods (Cáceres 1997); they are also the stage responsible for dispersal via waterfowl (Green *et al*. 2005; Sánchez *et al*. 2007).

Artemia salina (Linnaeus, 1758) is the only sexual brine shrimp species endemic to the Mediterranean Basin, and it is also found in a disjunct area in South Africa. This South African population was found to be reproductively compatible with Mediterranean isolates, but there were some significant morphological and life-history differences between both groups (Amat *et al*. 1995a). In addition, the sexual North American species *A. franciscana*, widely used as live food in aquaculture, has recently been introduced to the Mediterranean Basin and has quickly replaced native *Artemia* species in many localities (Amat *et al*. 2005b, 2007). Phylogeographical surveys in hypersaline anostracans are lacking, but analyses on freshwater sexual anostracan species indicate a high degree of endemism with a large proportion of genetic diversity being held by local populations (Ketmaier *et al*. 2003, 2005). Additionally, *A. salina* is associated with current or past commercially exploited saltpans (Barigozzi 1974; Abatzopoulos *et al*. 2002; Amat *et al*. 2005a).The effect of at least several millennia of traditional human management of these ecosystems (Kurlansky 2003) on the population genetic structure of this organism is currently unknown. Therefore, an analysis of the genetic diversity of *A. salina* would be expected to lead to two very different scenarios for the loss of genetic diversity arising from the ongoing *A. franciscana* invasion: (i) if human management was responsible for a recent expansion of *A. salina* in the Mediterranean area during historical times, or had significantly impacted population structure in this species, then the biodiversity loss associated to the expansion of *A. franciscana* would be reduced; and (ii) in contrast, if *A. salina* still holds substantial endemism in its local populations despite traditional human management, then *A. franciscana* invasion, together with habitat loss, could entail a rapid and significant loss of genetic diversity in this organism. For these reasons, an assessment of the genetic divergence, population structure and local endemism in *A. salina* is urgently required.

Here, we describe the geographical distribution of mitochondrial genetic diversity of the sexual brine shrimp *A. salina* in order to infer its evolutionary and biogeographical history (including the potential effects of human traditional management of saltpans). We investigate sequence variation using phylogenetic and phylogeographical methods in a large set of samples covering the entire native distribution of this anostracan. Our results provide one of the most complete surveys on the phylogeography of an anostracan, showing the high level of endemism and strong genetic structure in their isolated populations. This work also provides important information for management policies

Table 1 Populations of *A. salina* sampled in this study; *n*, number of samples; π (standard deviation), nucleotide diversity; *H* (standard deviation), gene diversity; *, haplotypes shared between Doñana area and Ebro Delta; †, haplotypes shared between Tunisia and Libya; ¶, haplotypes shared between Portuguesas saltpan and Sardinian saltpans; [1], collectively known as the Santa Pola saltpans. [2], collectively known as the Sanlúcar saltpans. In the column 'Source' we indicate the source of the samples as CB, samples obtained from the cyst bank of the Instituto de Acuicultura de Torre de la Sal; AD, adults sampled in saltpans; BE, cysts collected from bird excreta

ID (*n*)	Site	Latitude	Longitude	π	*H*	No. of haplotypes (Hap. codes)	Source
EBR (10)	Spain: Tarragona: Ebro Delta, La Trinitat salterns	40°34′N	00°41′E	0.0018 (0.0014)	0.71 (0.11)	4 (AS05*,27*,31,32)	BE
CAM (8)	Spain: Mallorca: Salobrar de Campos salterns	39°21′N	03°00′E	0.0004 (0.0006)	0.25 (0.18)	2 (AS39,40)	CB
BRAS (13)	Spain: Alicante: Bras del Port[1] salterns	38°11′N	00°36′W	0.0034 (0.0022)	0.89 (0.07)	8 (AS01,04,09,10, 11,12,13,14)	AD
BON (13)	Spain: Alicante: Bonmatí[1] salterns	38°10′N	00°37′W	0.0023 (0.0016)	0.65 (0.11)	4 (AS01,04,25,26)	BE
MAT (14)	Spain: Alicante: La Mata Lagoon	38°02′N	00°41′W	0.0011 (0.0010)	0.39 (0.16)	4 (AS01,02,03,04)	BE
ROS (7)	Spain: Córdoba: Los Rosales salterns	37°53′N	04°46′W	0.0000 (0.0000)	0.00 (0.00)	1 (AS33)	CB
SCA (7)	Spain: Jaén: San Carlos salterns	37°52′N	03°40′W	0.0000 (0.0000)	0.00 (0.00)	1 (AS34)	CB
PIN (13)	Spain: Murcia: San Pedro del Pinatar salterns	37°49′N	00°45′W	0.0039 (0.0025)	0.79 (0.11)	7 (AS01,15,16,17, 18,19,20)	AD
MAL (7)	Spain: Granada: La Malahá salterns	37°06′N	03°43′W	0.0014 (0.0012)	0.71 (0.18)	4 (AS35,36,37,38)	CB
DON (13)	Spain: Huelva: Doñana National Park salterns	36°52′N	06°21′W	0.0059 (0.0036)	0.75 (0.07)	4 (AS05*,06,07,08)	AD
POR (12)	Spain: Huelva: Portuguesas[2] salterns	36°52′N	06°20′W	0.0118 (0.0067)	0.88 (0.06)	6 (AS06,07,27*,28,29¶,30)	AD
ROC (11)	Spain: Huelva: El Rocío[2] salterns	36°48′N	06°20′W	0.0035 (0.0023)	0.61 (0.10)	3 (AS06,07,27*)	BE
CER (16)	Spain: Almería: Cerrillos salterns	36°41′N	02°40′W	0.0010 (0.0009)	0.35 (0.15)	4 (AS21,22,23,24)	AD
SAH (8)	Tunisia: Sahline: COTUSAL salterns	35°45′N	10°45′E	0.0029 (0.0021)	0.86 (0.11)	5 (AS47,52†,53†,54,55)	CB
ADH (8)	Tunisia: Sebkha El Adhibet: Mines Maghreb salterns	33°06′N	11°19′E	0.0039 (0.0027)	0.96 (0.08)	7 (AS45,46†,47, 48,49,50,51)	CB
JAD (8)	Morocco: Oualidia: Souzama salterns	32°53′N	08°50′W	0.0016 (0.0013)	0.75 (0.14)	4 (AS41,42,43,44)	CB
GAR (8)	Algeria: Aïn Beïda: Garaet et Tarf salt lake	35°40′N	07°09′E	0.0017 (0.0014)	0.71 (0.12)	3 (AS56,57,58)	CB
WAD (9)	Egypt: Wadi el Natrun salt lake	30°24′N	30°18′E	0.0006 (0.0007)	0.39 (0.16)	2 (AS59,60)	CB
LIB (8)	Libya: Lake Mandara	26°41′N	13°18′E	0.0023 (0.0018)	0.75 (0.14)	4 (AS46†,52†,53†,61)	CB
CYP (8)	Cyprus: Larnaca salt lake	34°53′N	33°37′E	0.0000 (0.0000)	0.00 (0.00)	1 (AS62)	CB
SGI (7)	Italy: Sardinia: Sta. Gilla salterns	39°12′N	08°59′E	0.0107 (0.0066)	0.80 (0.13)	4 (AS29¶,63,64,65)	CB
MOL (8)	Italy: Sardinia: Molentargius lagoon	39°12′N	09°02′E	0.0091 (0.0056)	0.86 (0.11)	5 (AS29¶,64,66,67,68)	CB
MES (8)	Italy: Sicily: Maria Estela salterns	37°59′N	12°31′E	0.0004 (0.0006)	0.25 (0.18)	2 (AS69,70)	CB
VEL (8)	South Africa: Saldanha Bay: Veldrif salterns	33°22′S	19°02′E	0.0091 (0.0056)	0.64 (0.18)	4 (AS71,72,73,74)	CB

with wider implications for the conservation biology of hypersaline habitats in the Mediterranean Basin.

Materials and methods

Samples and study area

We sampled 24 localities where *Artemia salina* had been previously described, covering its whole geographical distribution (Table 1). Most samples were obtained as dry cysts from the 'cyst-bank' of the Instituto de Acuicultura de Torre de la Sal (CSIC, Castellón, Spain) (see Table 1 for details). Additional samples were from adults collected in the field or cysts extracted from bird excreta, hatched and reared to adulthood in the laboratory as part of a morphological study (Table 1). Cysts and adults were preserved in 100% ethanol until needed for genetic study. We sequenced 7–16 individuals from each population.

DNA extraction, PCR and sequencing

For DNA isolation of adult specimens we used modified digestion temperature (56 °C) and RNAse addition (25 mg/mL) for the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol published by Palumbi (1996) and Bossier *et al*. (2004). For DNA isolation of cysts, we used an alkaline lysis protocol optimized for zooplanktonic diapausing eggs (Montero-Pau *et al*. in press).

We used specific *Artemia* primers designed in the same position as primers LCO1490/HCO2198 (Folmer *et al*. 1994) to amplify a fragment of the mitochondrial Cytochrome *c* Oxidase Subunit I (COI) gene. These new primers, 1/

2COI_Fol-F (5′-ATT CTA CGA ATC ACA AGG ATA TTG G-3′) and 1/2COI_Fol-R (5′-TAC ACT TCA GGA TGG CCA AAA AAT CA-3′), provided successful amplification and good quality sequences. A fragment of 710 base pairs (bp) was amplified by PCR under the following conditions: a cycle of 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C, with a final step of 5 min at 72 °C. PCR products were purified and subsequently sequenced using the BigDye Terminator Sequencing Ready Reaction version 3.1 kit (Applied Biosystems) following manufacturer instructions, on an ABI 3130xl and ABI 3730xl automated sequencer. Nucleotide sequences were edited by hand and aligned using SEQUENCHER™ version 4.5 (Gene Codes Corp., © 1991–2005) and further revised by eye. All sequences were deposited in GenBank (Accession Nos DQ426827–DQ426858 and EU543444–EU543485).

Genetic analyses

The *A. salina* sequences obtained were collapsed into haplotypes using COLLAPSE version 1.2 prior to genetic analyses (Posada 2005). For interspecific phylogenetic analyses, we downloaded all available *Artemia* COI sequences from GenBank to add to our *A. salina* dataset with three different objectives: (i) to assess whether our *A. salina* samples formed a monophyletic unit; (ii) to investigate the phylogenetic relationships of the geographically divergent *A. salina* population from South Africa (Veldrif, VEL); and (iii) to explore the usefulness of the COI gene for DNA barcoding purposes in *Artemia*. We used MODELGENERATOR version 0.63 (Keane *et al*. 2006) to select the best model of sequence evolution and used the model and estimated parameters in PHYML version 2.4.4 (Guindon & Gascuel 2003) for phylogenetic reconstructions by Maximum Likelihood (ML). Neighbour-joining (NJ) trees were reconstructed in MEGA version 4 (Tamura *et al*. 2007), using evolutionary distances computed with the Maximum Composite Likelihood method using the Tamura–Nei substitution model (Tamura *et al*. 2007). The robustness of the branches for both ML and NJ analyses was assessed with 1000 bootstrap replications.

Similarly, the phylogenetic relationships between Mediterranean *A. salina* haplotypes were reconstructed using the ML approach with PHYML incorporating the parameters and evolutionary model selected with MODELGENERATOR. In addition, we performed a Nested Clade Phylogeographical Analysis (NCPA) of haplotype data (see Templeton 2007). We constructed a haplotype network using TCS version 1.21 (Clement *et al*. 2000), which follows the statistical parsimony algorithm described in Templeton *et al*. (1992). Next, we built a nested design following the guidelines provided by Templeton *et al*. (1995). Finally, we tested for the existence of geographical associations of the haplotype clades in the nested design using GEODIS version 2.5 (1 000 000 permutations; Posada *et al*. 2000). The evolutionary and phylogeographical patterns responsible for the geographical distribution of the clades giving significant results were identified following the inference key (updated 11th November 2005) provided by these authors with GEODIS. The haplotypes from the South African population were excluded from the NCPA as they showed more than 40 substitutions with respect to Mediterranean haplotypes, well above the 95% confidence criterion.

Average genetic distances between *Artemia* taxa, and between the *A. salina* haplotype higher level clades, obtained in the nested design, were computed by MEGA version 4.0 (Tamura *et al*. 2007), using uncorrected *p*-distances and Kimura-2 parameter distances (K-2P) in order to compare our results with previous analyses reported in another zooplanktonic organisms. Estimates of population genetic diversity in *A. salina* (π, nucleotide diversity; *H*, gene diversity, ϕ_{ST} values, genetic variability distribution, and haplotype frequencies) were performed using ARLEQUIN 2.000 (Schneider *et al*. 2000).

Isolation-by-distance (IBD) analysis between *A. salina* populations was carried out using IBDWS version 3.14 (Jensen *et al*. 2005), which performs a Mantel test and carries out Reduced Major Axis (RMA) regression analysis. To visualize the relationship between genetic and geographical distances, pairwise population ϕ_{ST} values were plotted against geographical distance (Log geographical distance).

Results

Phylogenetic relationships in Artemia

After excluding 15 published *Artemia* sequences downloaded from the GenBank database, which were 561 bp long, and trimming longer sequences we obtained a 603 bp long alignment for all 98 COI sequences with no indels (alignment available from the authors). The evolutionary model selected by MODELGENERATOR was TrN + I + G (I = 0.62; G = 4.20) with a transition/transversion ratio = 5.76 and base frequencies A = 20.06%, C = 24.95%, G = 20.96% and T = 34.02%. Both methods of phylogenetic reconstruction (ML and NJ) resulted in similar topologies and supported the monophyly of all species, including all of our *A. salina* haplotypes, with minimum support values of 73% and 91%, depending on the method of analysis used (Fig. 2). Support was also found for a monophyletic cluster formed by *A. tibetiana*, *A. urmiana*, an undescribed bisexual population from Kazakhstan, and a diploid parthenogenetic lineage, with *A. urmiana* as a sister taxa to the parthenogenetic lineage and the bisexual population from Kazakhstan. Strong support was found for a sister status of Mediterranean *A. salina* and the group formed by the sequences from the South African population (VEL). However, South African and Mediterranean lineages were highly divergent, with

Table 2 Genetic pairwise distances of the COI mitochondrial gene between: (a) seven *Artemia* taxa and the *Artemia* population from Veldrif (VEL); (b) high-level clades of *A. salina* of the NCPA. The lower matrix shows the *p*-distances values and the upper matrix shows the K-2P correction values

(a)

	A. 'Veldrif'	*A. franciscana*	*A. tibetiana*	*A. urmiana*	*A. sinica*	*A. persimilis*	*A. salina*	*Diploid parth.*
A. 'Veldrif'	—	0.225	0.255	0.243	0.226	0.265	0.115	0.240
A. franciscana	0.189	—	0.185	0.192	0.184	0.227	0.241	0.200
A. tibetiana	0.209	0.160	—	0.041	0.180	0.238	0.248	0.044
A. urmiana	0.201	0.165	0.040	—	0.190	0.241	0.237	0.021
A. sinica	0.188	0.159	0.155	0.162	—	0.187	0.247	0.183
A. persimilis	0.218	0.193	0.199	0.201	0.163	—	0.248	0.228
A. salina	0.104	0.199	0.203	0.197	0.201	0.206	—	0.247
Diploid parth.	0.199	0.171	0.043	0.021	0.156	0.192	0.202	—

(b)

	3-1	3-2	3-3	3-4	3-5	3-6	3-7
3-1	—	0.026	0.025	0.027	0.017	0.022	0.023
3-2	0.025	—	0.012	0.018	0.018	0.015	0.012
3-3	0.025	0.012	—	0.016	0.018	0.013	0.011
3-4	0.027	0.018	0.016	—	0.023	0.018	0.017
3-5	0.017	0.018	0.017	0.022	—	0.014	0.016
3-6	0.022	0.015	0.013	0.018	0.014	—	0.013
3-7	0.023	0.012	0.011	0.016	0.016	0.013	—

distances of 10.4% (*p*-distance; see Table 2a). The haplotypes from Mexico and Cuba (which were named *A.* sp. in their GenBank record) form a monophyletic grouping within the diversity of *A. franciscana*.

Pairwise genetic distances between the seven *Artemia* species and the South African population (VEL) of *A. salina* are shown on Table 2a. The *p*-distances ranged from 2.1%, between the diploid parthenogenetic strain and *A. urmiana*, to a 21.8%, between *A. persimilis* and *A. salina* from South Africa.

Haplotype and nucleotide diversity in A. salina

The length of the *A. salina* alignment was 617 bp, with the 232 individuals sequenced yielding 74 haplotypes. No insertions, deletions, stop codon or ambiguities were present. There were 115 variable sites, 88 of them parsimony informative. The alignment had a total of 107 synonymous and nine nonsynonymous substitutions.

Most haplotypes were found within single populations, with 12 haplotypes shared among two or more populations. The average for each population was 3.87 haplotypes, with 9.67 individuals sequenced on average for each population (see Table 1). The ROS, SCA and CYP populations were found to be fixed for a single haplotype. The relative frequencies and accession number of each haplotype are listed in Appendix I.

The values of π and *H*, as well as details of the haplotypes present in each population, are given in Table 1. The π-values ranged from 0.0000 to 0.0118. The highest value was found in POR, but SGI, MOL and VEL also showed high π-values compared with the rest of the populations. In general, *H*-values were high, with a maximum in 0.96 and an average of 0.58.

Most pairwise ϕ_{ST} comparisons between *A. salina* populations were highly significant (Table 3). Non-significant ϕ_{ST} values involved two groups of populations within a reduced geographical area (Table 3 and Fig. 1), one formed by MAT, BRAS and BON in eastern Spain, and another formed by DON, ROC and POR in the Doñana area of southern Spain. A third group involved the two Tunisian (SAH and ADH) and the Libyan (LIB) populations. Additionally, several pairs of distant populations showed nonsignificant ϕ_{ST}: two southern Spanish localities (DON and POR) were not significantly different from EBR (northern Spain), a locality more than 700 km apart where *A. salina* has only been identified recently (see discussion); and one of these southern Spanish populations (POR) also showed nonsignificant ϕ_{ST} values when compared to the two Sardinian populations (SGI and MOL).

Mitochondrial phylogeography of Mediterranean A. salina

We found a total of 70 Mediterranean *A. salina* haplotypes with a maximum divergence between haplotypes of 3.4% and 4.5%, *p*-distance and K-2P, respectively (results not

Table 3 Pairwise ϕ_{ST} values obtained with a Tamura & Nei evolutionary model (similar values were obtained with K-2P model). Values in bold indicate NO statistical significance ($P > 0.01$)

	MAT	DON	BRAS	PIN	CER	BON	ROC	POR	EBR	ROS	SCA	MAL	CAM	JAD	ADH	SAH	GAR	WAD	LIB	CYP	SGI	MOL	MES
DON	0.795																						
BRAS	**−0.005**	0.741																					
PIN	0.094	0.723	0.077																				
CER	0.929	0.875	0.871	0.868																			
BON	**0.031**	0.761	**−0.028**	0.112	0.897																		
ROC	0.875	**−0.004**	0.816	0.798	0.924	0.840																	
POR	0.699	**0.049**	0.653	0.637	0.798	0.667	**0.164**																
EBR	0.899	**0.096**	0.822	0.803	0.942	0.855	0.324	**0.166**															
ROS	0.781	0.773	0.541	0.557	0.953	0.621	0.876	0.644	0.921														
SCA	0.593	0.744	0.286	0.379	0.945	0.346	0.860	0.607	0.909	1.000													
MAL	0.795	0.637	0.620	0.638	0.916	0.688	0.781	0.494	0.814	0.878	0.828												
CAM	0.841	0.804	0.662	0.647	0.952	0.731	0.890	0.691	0.927	0.958	0.938	0.892											
JAD	0.940	0.785	0.882	0.868	0.966	0.906	0.871	0.659	0.901	0.961	0.957	0.926	0.960										
ADH	0.869	0.526	0.793	0.777	0.925	0.821	0.681	0.363	0.673	0.873	0.856	0.761	0.886	0.821									
SAH	0.900	0.612	0.827	0.813	0.942	0.854	0.751	0.450	0.762	0.914	0.902	0.827	0.919	0.847	**0.096**								
GAR	0.934	0.763	0.872	0.856	0.958	0.899	0.858	0.620	0.888	0.955	0.950	0.915	0.954	0.924	0.828	0.871							
WAD	0.957	0.734	0.903	0.889	0.972	0.927	0.840	0.589	0.900	0.983	0.981	0.941	0.978	0.949	0.830	0.882	0.949						
LIB	0.914	0.646	0.842	0.829	0.950	0.870	0.780	0.487	0.800	0.934	0.925	0.859	0.936	0.877	0.189	**0.001**	0.891	0.906					
CYP	0.948	0.713	0.865	0.842	0.970	0.903	0.850	0.526	0.900	1.000	1.000	0.950	0.988	0.957	0.780	0.851	0.954	0.969	0.894				
SGI	0.813	0.473	0.756	0.739	0.868	0.777	**0.608**	0.158	0.588	0.772	0.749	0.664	0.801	0.751	0.511	0.586	0.715	0.746	0.625	0.662			
MOL	0.775	0.179	0.711	0.691	0.853	0.734	**0.368**	−0.028	0.317	0.735	0.703	0.584	0.772	0.731	0.416	0.518	0.687	0.693	0.565	0.626	**0.088**		
MES	0.963	0.782	0.911	0.898	0.972	0.935	0.887	0.524	0.924	0.991	0.990	0.953	0.984	0.958	0.857	0.899	0.948	0.975	0.922	0.986	0.405	0.582	
VEL	0.985	0.972	0.979	0.978	0.986	0.982	0.977	0.958	0.979	0.981	0.981	0.977	0.981	0.976	0.973	0.975	0.978	0.981	0.977	0.981	0.962	0.963	0.980

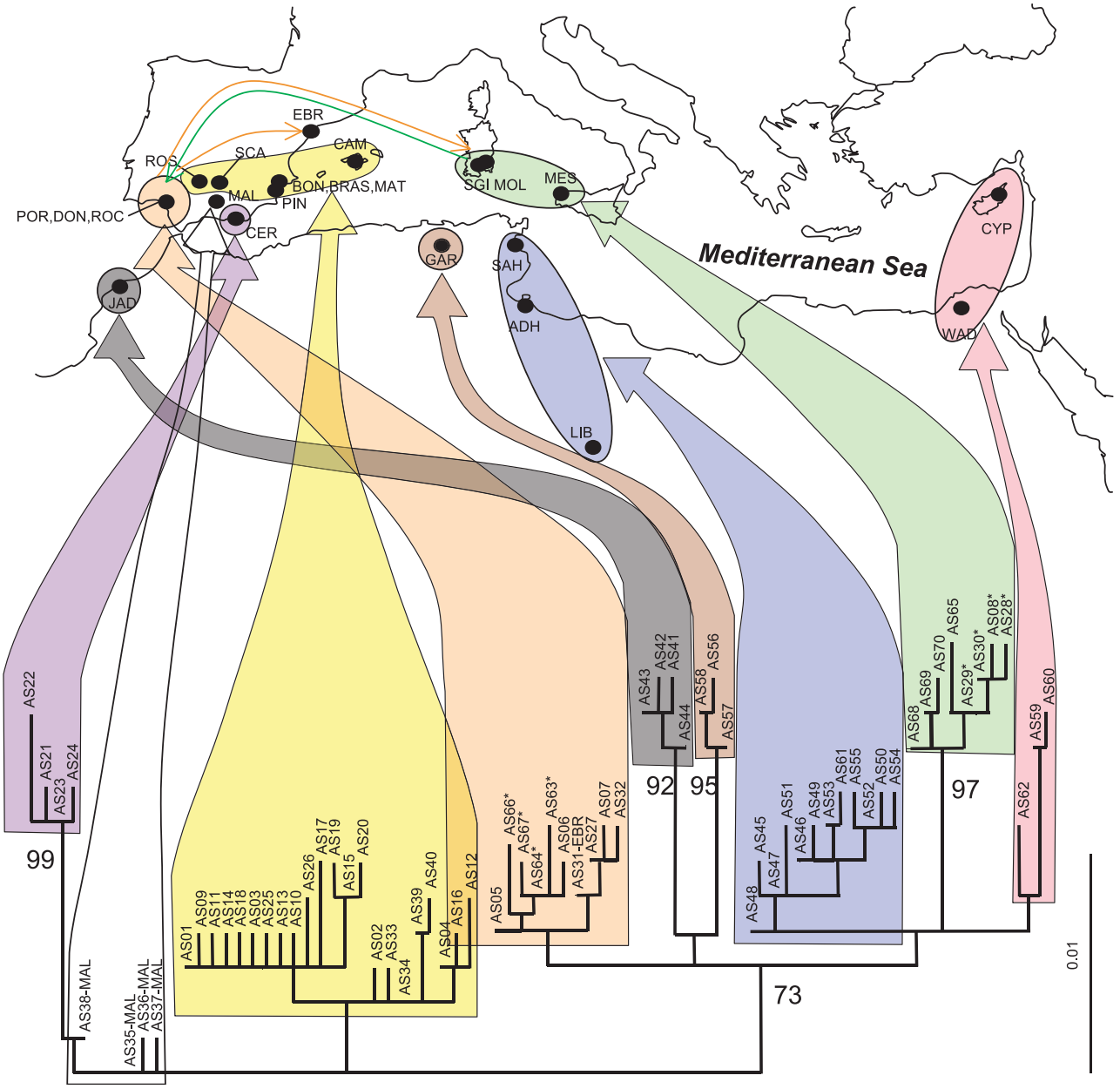

Fig. 1 Map of the Mediterranean Basin showing the location of the 23 Mediterranean *Artemia salina* populations sampled (see Table 1 for population codes and details). Note that POR, DON, ROC and BON, BRAS, MAT are different populations, but due to the scale of the figure they appear as single points. The figure also shows the phylogenetic relationships between Mediterranean *A. salina* COI haplotypes overlaid on their geographical distribution and potential Pleistocene refugia (areas joining sampling locations with same colour coding as clades). Thin lines indicate long-distance migration events identified in our analysis. The haplotypes marked with asterisks are found in these distant areas. The tree is a midpoint rooted maximum likelihood (ML) tree (see text for details) with branch support obtained by 1000 bootstrap pseudo-replicates (numbers next to branches). Only selected bootstrap values over 70% are shown.

shown). The haplotype network provided by TCS was used to construct a nested design (Fig. 3), which was structured into 22 first-level clades, 14 second-level clades and seven third-level clades. In addition, three second-level clades involved unsampled ancestral haplotypes. Out of the 26 clades with geographical and genetic information used in GEODIS, 13 showed statistically significant geographical – genetic associations, including the total cladogram (Table 4). The inference for the total cladogram is a global — Circummediterranean — range expansion with subsequent long-distance colonization and fragmentation for clade 3-2 (the only one in which the *Dn* value significantly reversed from the *Dc* value) which involves the two most Eastern populations (Egypt and Cyprus). Three clades (1-8, 2-8 and

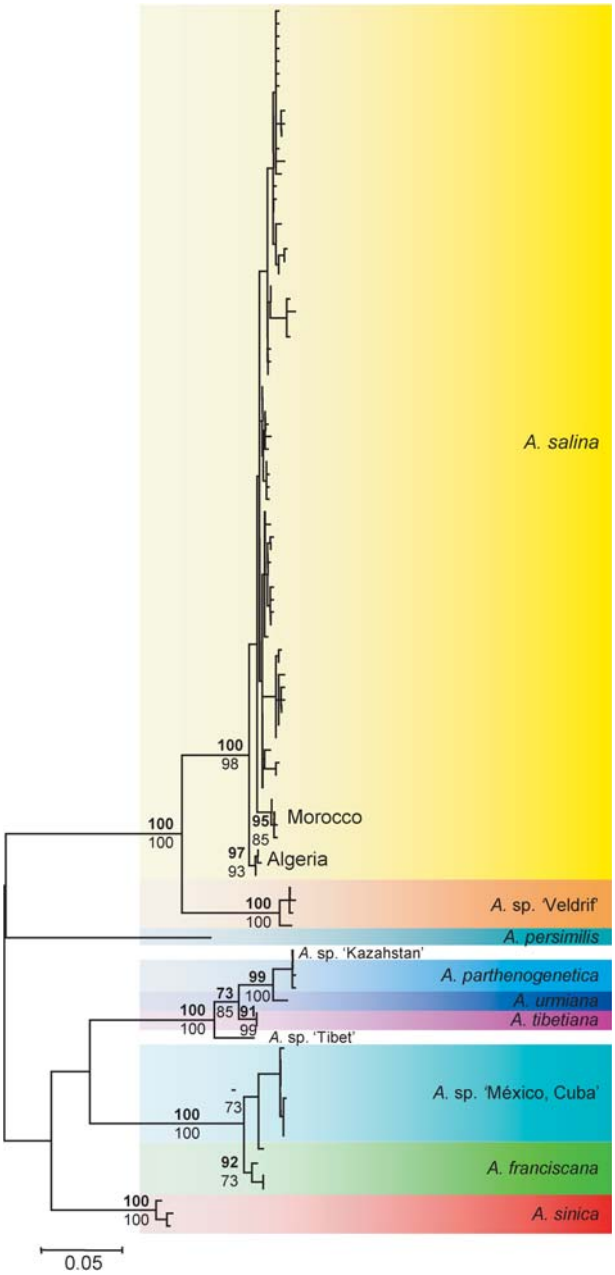

Fig. 2 Phylogram of the genus *Artemia* using Maximum Likelihood (ML, below branches) and neighbour-joining (NJ, above branches) methods. See text for details. Selected bootstrap support values over 70% are shown. Undetermined *Artemia* taxa are indicated as *A.* sp. followed by their geographical origin as reported in the GenBank database.

3-7) indicate more recent episodes of range expansion. All of them involve Spanish populations and four haplotypes from the Sardinian populations (AS63, AS64, AS66 and AS67). In three other clades (2-5, 3-2 and 3-6), allopatric fragmentation was inferred. They are formed by unique private haplotypes found in single populations either from islands (Mallorca and Cyprus) and the mainland, or clades separated by the Mediterranean Sea (Spain, Morocco and Egypt). Restricted gene flow with IBD was inferred in two clades (2-6, and 3-5) formed by populations more than 300 km apart (DON and EBR, or MAT and SCA). Finally, clade 2-1 showed evidence of long-distance colonization involving populations from southwest Spain (Doñana area, DON; ROC; POR) and Italian islands (Sardinia, MOL; SGI; Sicily, MES).

The phylogenetic and geographical information is represented in Fig. 1, identifying the geographical distribution of the main clades in the Mediterranean and those geographical areas with unique haplotypes obtained with our data set. Only the most divergent lineages show high phylogenetic bootstrap values (3rd level clades 3-1 and 3-2, as well as haplotypes from populations JAD, GAR and clade 2-1), as is usual in intraspecific phylogenetic trees.

The average pairwise distances values between haplotypes from third level Mediterranean clades ranged between 1.1 and 2.7% for both *p*-distance and K-2P (see Table 2b). The maximum value was found between clades 3-1 (the CER population from SE Spain) and 3-4 [involving the Algerian, Sicilian, Sardinian and southern Spain (DON, ROC, POR) populations].

IBD tested in the Mediterranean haplotype data set showed two groups of points, but a significant positive correlation of genetic and geographical distances ($Z = 560.7279$, $r = 0.50$, one-sided $P \leq 0.0001$ from 30 000 randomizations) and a value of $R^2 = 0.246$ for RMA regression analysis were obtained (Fig. 4).

Discussion

Phylogeography and local endemism of Artemia salina *in the Mediterranean Basin*

Artemia salina populations hold substantial haplotype diversity and are highly structured genetically with an important component of regional and local endemism. Our results strongly suggest that gene flow is not an important factor between *A. salina* populations. Population structure, instead, reflects a phylogeographical pattern of persistent founder events ('priority effects'), as found in many other passively dispersing continental aquatic invertebrates (see De Meester *et al.* 2002). Overlaying this strong genetic structure, we found some evidence of range expansions and a few long-distance dispersal events, with possible cases of colonization by immigrants derived from populations hundreds of km apart.

To obtain an approximate time frame for the oldest expansion event supporting the inference by the NCPA (i.e. the initial range expansion of *A. salina* in the Mediterranean), we estimated divergence times using the reported COI molecular clock for snapping shrimps (1.4% sequence divergence per million year; Knowlton & Weigt 1998).

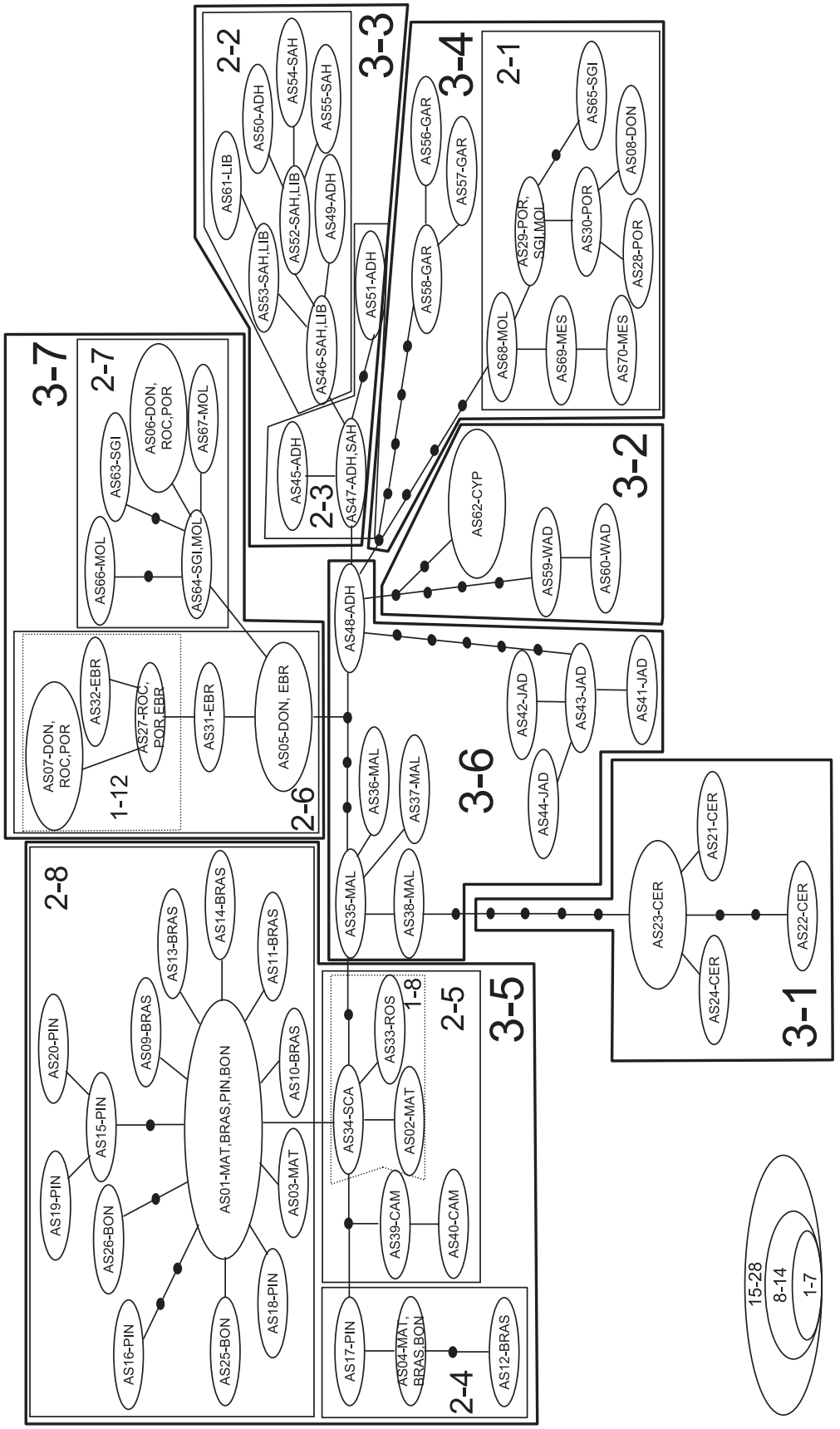

Fig. 3 Statistical parsimony network showing the nested design used in NCPA. Only second (continuous thin line), third (continuous thick line) and statistically informative 1stlevel clades (discontinuous thin line) are shown. Sizes of ellipses are proportional to the number of individuals included (ellipses with numbers bottom left). The populations where each haplotype was found are shown next to the haplotype code. Lines linking haplotypes indicate single substitutions. Small black circles indicate missing haplotypes.

Table 4 NCPA inferences as described in the GeoDis key of 11 November 2005. Different steps and conclusions are indicated in the chain-of-inference column. The alternative steps offered by the GeoDis key are included in parentheses. Locality acronyms are explained in Table 1

Clade	Chain of inference	Populations involved	Demographic event inferred
1-8	1,19,20,2,11YES (12NO)	ROS,SCA,MAT	Range Expansion (Contiguous Range Expansion)
1-12	1,2,11,17NO	DON,ROC,POR,EBR	Inconclusive Outcome
2-1	1,2,3,5,6,13YES	MES,SGI,MOL,DON,POR	Long Distance Colonization possibly coupled with subsequent Fragmentation
2-2	1,2,11,17NO	SAH,ADH,LIB	Inconclusive Outcome
2-5	1,19NO	ROS,SCA,MAT,CAM	Allopatric Fragmentation
2-6	1,2,3,4NO	DON,ROC,POR,EBR	Restricted Gene Flow with Isolation by Distance
2-8	1,2,11YES (12,13,14NO)	MAT,BRAS,BON,PIN	Range Expansion (Long Distance Colonization and/or Past Fragmentation)
3-2	1,19NO	WAD,CYP	Allopatric Fragmentation
3-4	1,19,20,2 INTERIOR STATUS CAN NOT BE DETERMINED	MES,SGI,MOL,DON,POR,GAR	Inconclusive Outcome
3-5	1,2,3,4NO	MAT,BRAS,BON,PIN,ROS,SCA,CAM	Restricted Gene Flow with Isolation by Distance
3-6	1,19,20,2,3,4,9NO	ADH,MAL,JAD	Allopatric Fragmentation
3-7	1,2,11YES (12NO)	DON,ROC,POR,EBR,MOL,SGI	Range Expansion (Contiguous Range Expansion)
Total Cladogram	1,2,11YES (12,13YES)	ALL LOCALITIES	Range Expansion (Long Distance Colonization possibly coupled with subsequent Fragmentation)

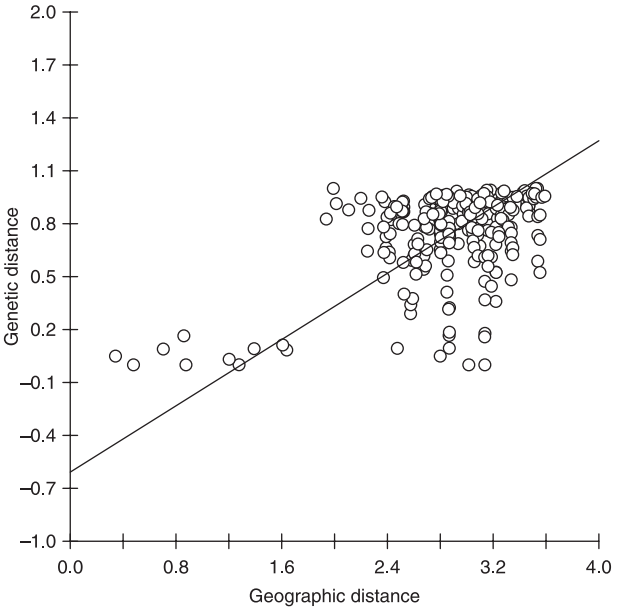

Fig. 4 Data plot of the genetic distance (ϕ_{ST}) vs. geographical distance (Log geographical distance in km) of the Mediterranean *Artemia salina* populations, showing the RMA regression line ($R^2 = 0.246$).

Although this calibration assumes a fixed rate of variation in shrimps, it is useful enough for our purposes. According to this, the split between the seven Mediterranean *A. salina* main clades (see Table 2b and Fig. 3) would have occurred during the Early Pleistocene, well before the last glacial maximum, between 0.78 and 1.93 million years ago. Gómez *et al*. (2000) found a pattern of regional endemism for the salt lake rotifer *Brachionus plicatilis* on the Iberian Peninsula, suggesting two glacial refugia (with an average pairwise sequence divergence of 2.8%). Divergences found by De Gelas & De Meester (2005) among groups in Europe for the freshwater cladoceran *Daphnia magna* were lower, ranging from 0.73% to 1.74%. Similar divergences were found by Penton *et al*. (2004) in North American populations of *D. obtusa*. Divergences in the Mediterranean 3rd-level clades of *A. salina* range from 1.1% to 2.7%, which could be tentatively dated as Pleistocene at both local (Iberian Peninsula) and regional (Mediterranean Basin) scales.

According to (i) the level of genetic differentiation between clades; (ii) supported monophyly of the clades; (iii) geographical isolation; (iv) presence of unique haplotypes; and (v) inference of allopatric fragmentation in the NCPA, our results suggest that *A. salina* populations survived during several glaciations in at least six suitable areas around the Mediterranean Sea (CER, JAD, GAR, Italian islands, CYP-WAD and Doñana area). The clades found in four of these areas show high support in the phylogenetic

analyses. CYP-WAD is geographically isolated, has high support for monophyly respect to the rest of lineages, and allopatric fragmentation is inferred by NCPA; and the Doñana area shows unique haplotypes very differentiated from the Italian lineage after inferring long-distance colonization coupled with fragmentation by NCPA (see Table 4, Figs 1 and 3). The precise location of such long-term refugia is difficult to ascertain. Most likely an area, instead of a particular saltpan or lake, can be considered as the 'refugia', given that particular lakes may have a short life span. Given this caveat, we propose that at least two refugia were located in the Iberian Peninsula (see Figs 1 and 3). These areas contain geographically differentiated clades, indicating long-term survival within the Iberian Peninsula which we propose are located in or around the following areas: (i) clade 3-1, southeastern Area (Almería), which is very isolated genetically from the other Iberian populations; and (ii) clade 3-7, southwestern region (Doñana area). This clade displays two significant historical signatures. One of them, a range expansion from the Iberian Peninsula towards Sardinia, is considered below in detail. The other is a signature of restricted gene flow with IBD between the Ebro (northeastern) and Doñana areas, which share two haplotypes. After Doñana, the Ebro Delta is the most important locality for migratory waterfowl in the Iberian Peninsula (Martí & Del Moral 2002). Several direct movements of ringed birds between both areas have been documented (Spanish Ringing Office, unpublished data), making dispersal of *A. salina* cysts between them particularly likely. Since *A. salina* has not previously been detected in the Ebro Delta population (Amat *et al*. 1995b), this could reflect the establishment of a new population after long-distance transport from the Doñana area. However, the high haplotype richness and the presence of unique haplotypes in the Ebro Delta population indicate that this population could be older, although a larger sample size might reveal these haplotypes to be present in the Doñana or surrounding areas. An additional refugium may possibly have been located in the Iberian Peninsula, harbouring the ancestors of clade 3-5 — interior and eastern coastal Iberian region (Levante Spanish coast and Balearic Islands) — which is supported by the high genetic differentiation of clade 3-5 from the other 3rd level clades. Sub-clades nested in clade 3-5 (clades 1-8, 2-5 and 2-8) have been inferred by NCPA to have undergone (i) a 'contiguous range expansion' in clade 1-8; (ii) 'allopatric fragmentation' between internal populations (SCA, ROS) and the geographically isolated populations MAT and CAM; and (iii) 'range expansion with long-distance colonization' for the Eastern Spanish coast populations, MAT, BRAS, BON and PIN. These inferences are concordant with a colonization route from interior saltpans towards the eastern coast of the Iberian Peninsula, arriving at the Balearic Islands (see Table 4 and Fig. 3 for details). The PIN population is the only Spanish eastern coastal population with significant ϕ_{ST} values compared to the others. The same occurs with the CAM population in the Balearics. Hence, the hypothetic colonization route is supported by 'allopatric fragmentation' and 'long-distance colonization' when the populations CAM and PIN are involved. A pattern of refugia within refugia has also been reported in Iberia for other organisms (Gómez & Lunt 2007), including salt lake invertebrates (Gómez *et al*. 2000, 2007a), and appears to be a common pattern in other Mediterranean peninsulas (Schmitt *et al*. 2006; Canestrelli *et al*. 2007).

Migratory birds are the main natural dispersal vectors of *A. salina* between patchy habitats (Figuerola *et al*. 2005; Sánchez *et al*. 2007). When suitable habitats are in expansion, therefore, colonization will not necessarily follow a pattern involving occupation of adjacent populations (McMaster *et al*. 2007). Previously colonized populations should be very resilient to the effects of new migrants due to monopolization effects (De Meester *et al*. 2002), but 'empty habitats' could be colonized by distant populations depending on the nature of bird movements. We found at least two examples of long-distance colonization, and one case of bidirectional exchange of migrants between areas. One of these bidirectional events was between the two clades involving Sardinia and southwestern Spain (Doñana area) populations. A relatively old episode of bidirectional migration between these areas followed by isolation was inferred from NCPA. This genetic structure coincided with a structured geographical distribution, but 'long-distance colonization' and 'range expansion' events were found between Doñana and Sardinian populations. Surprisingly, two 3rd-level clades were found in both geographical areas (clades 2-1, and clade 3-7 shown in Fig. 3). As the network shows, the mitochondrial lineage from clade 2-1 (with basal haplotypes in Sardinia and related haplotypes in Sicily) may have undergone long-distance dispersal and colonization in the past from Sardinia towards the Doñana area. Similarly, the lineage from clade 3-7 (with basal haplotypes in Doñana and Ebro Delta populations) could have suffered a range expansion from the Iberian Peninsula towards Sardinia. The presence of new tip haplotypes in each putatively colonized area indicates that the colonization event is unlikely to be recent (i.e. due to human management in the last few millennia). In any case, the colonization from Doñana to Ebro Delta appears to be more recent than that involving Doñana and Sardinia.

Our results rule out the existence of two geographically differentiated (eastern and western) *A. salina* clades in the Mediterranean Basin as recently proposed by Baxevanis *et al*. (2006), based on data from a small number of populations. Our work demonstrates that Mediterranean *A. salina* is subdivided into seven or eight main clades with mostly allopatric distributions, reduced gene flow and long-distance dispersal with fragmentation.

Given the strong genetic structure, the strong priority effects and subsequent monopolization likely to influence *A. salina* populations, migration–drift equilibrium as a result of restricted gene flow appears unlikely in the Mediterranean populations of *A. salina*. Therefore, the significant correlation between genetic and geographical distance (i.e. IBD pattern) could instead reflect a pattern of past sequential colonization as found in humans (Ramachandran *et al*. 2005), and also suggested to be responsible for the patterns found in several marine and freshwater aquatic invertebrates (Gómez *et al*. 2007a, b; Mills *et al*. 2007).

There is an ongoing debate on the usefulness and shortcomings of NCPA (Garrick *et al*. 2008; Petit 2008; Templeton 2008). The method has recently attracted criticism (Knowles & Maddison 2002; Panchal & Beaumont 2007; Petit 2008). In response to these criticisms the robustness of inferences using this approach has been further tested (Templeton 2004), and its use has been defended given its usefulness by generating specific testable hypothesis (Garrick *et al*. 2008). NCPA, indeed, offers a unique ability to explore patterns relating to complex historical scenarios. The high rate of false positives found by Panchal & Beaumont (2007) for some of the inferences could have been generated by the specific parameters of the simulations tested which are far from those found in natural scenarios. We therefore agree with Garrick *et al*. (2008) that NCPA is a good starting point 'to attempt distinguishing processes and events that shaped spatial-genetic structuring throughout complex evolutionary histories of natural populations', especially when cross-validated 'via assessment of multiple independent loci, complementary analyses, and/or prior expectations in conjunction with complementary analyses and strong prior expectations'. One consequence of the application of the interpretive key of NCPA to our dataset is noteworthy. In *Artemia*, as in other passively dispersed organisms in which migratory birds are the vectors of dispersal, a clear-cut distinction between 'contiguous range expansion' and 'long-distance colonization' is difficult to make, as their habitats are naturally isolated and often far from each other. So, the inference key provided by Posada and Templeton probably does not consider the high potential of dispersal and patchy nature of the habitats of these organisms (Posada *et al*. 2002). We suggest that in organisms that inhabit patchy and isolated habitats and are passively dispersed, the NCPA resulting inferences 'range expansion' and 'contiguous range expansion' could be considered to represent the same phylogeographical and evolutionary event at different but continuous scales. For instance, our results indicate 'range expansion' at first, second and third clade levels, and at the total cladogram level (see results section for details). The differences in geographical distances between the haplotypes involved in these clades are large, but the inference is the same. Another case is represented by an inference of 'contiguous range expansion' in clades whose haplotypes are separated by the sea (clade 3-7 found in southwestern Spain and Italian islands), where obviously no populations exist in between.

Phylogenetic relationships of Artemia

Inter-specific phylogenetic relationships in *Artemia* show that all the taxa included are monophyletic. In addition, a well-supported grouping of *A. tibetiana*, *A. urmiana* and diploid parthenogens was found (see Fig. 2), in agreement with previous work indicating that diploid parthenogenetic lineages are closely related to *A. urmiana* (Baxevanis *et al*. 2006). However, a surprising result was the near identical sequences of the *A.* sp. 'Kazakhstan' bisexual isolate (not analyzed by Baxevanis *et al*. 2006) and the diploid parthenogens. We suggest that in view of these results, the taxonomic status of the central Asian species should be re-evaluated. The Mexican and Cuban sequences can be considered conspecific with *A. franciscana* (Tizol-Correa 2006), suggesting that *A. franciscana* has also a strong phylogeographical structure with a similar depth to that found in *A. salina*, but more exhaustive analyses are needed in future studies. Several approaches have been proposed to identify different *Artemia* species, such as morphological parameters (Hontoria & Amat 1992; Zhou *et al*. 2003), or mitochondrial DNA restriction fragment length polymorphisms (Bossier *et al*. 2004). But, as has been suggested for most animal groups (Hebert *et al*. 2003a), our results support the use of COI as a valuable 'DNA bar-coding' tool in *Artemia*. Given the high interspecific diversity, it could also be used to assess contamination of *Artemia* cultures (see Campos-Ramos *et al*. 2003) using high throughput techniques such as TaqMan© Real Time PCR already used in other organisms (Fox *et al*. 2005; Walsh *et al*. 2005).

A remarkable finding of our work is the large divergence between the South African and the Mediterranean *A. salina* sequences. The phylogram shows that the South African haplotypes form a well-supported sister lineage to the Mediterranean ones, indicating that this population has diverged strongly from Mediterranean *A. salina* populations (see Fig. 2). The average sequence divergence between populations of both areas (see Table 2a) is within the range of the interspecific divergences found in COI for crustaceans (Costa *et al*. 2007), and it is much larger than the divergence between other sexual established *Artemia* species such as *A. tibetiana*, *A. sinica*, *A. urmiana* and the diploid parthenogens (Baxevanis *et al*. 2006). Although the VEL population showed reproductive compatibility with a Mediterranean population, clear morphological and life-history differences could be identified between South African and Mediterranean isolates (Amat *et al*. 1995a). Reproductive compatibilities have also been reported between other sexual *Artemia* species such as *A. sinica*, *A. urmiana* and *A.* sp. 'Kazakhstan'

(Triantaphyllidis *et al*. 1998). Therefore, the taxonomic status of the South African *A. salina* should be re-evaluated.

Consequences for conservation and population management

Saltpans are known to have existed and been managed by humans for at least 2000 years in the Mediterranean for evaporative salt production (Consejería de Medio Ambiente 2004), although others are far more recent. Our results on the structure and the age of the clades in *A. salina* suggest that traditional human management of saltpans has not played a significant role in the genetic structure of its populations, which we would have expected to result in strong signals of recent expansions or gene flow. Even in those cases of long-distance dispersal and colonization, the high haplotype diversity and number of unique haplotypes appears to rule out recent episodes of anthropogenic-derived dispersal. In addition, and given the level of differentiation and genetic divergence between geographical isolates, our results indicate that *A. salina* was either pre-existent in the natural saltpans or lagoons that later became saltworks, or colonized these from nearby natural populations. Therefore, traditional forms of saltpan management seem to have aided in the maintenance of the genetic diversity of *A. salina* populations; or at least, not to have resulted in severe losses of genetic diversity. In contrast to this low impact of traditional salt management, many saltpans are currently being abandoned or transformed into intensive aquaculture projects due to decreased economic returns of salt production (Kurlansky 2003; Amat *et al*. 2005a). After salt production is discontinued, saltpans usually become unsuitable for *Artemia* eventually, owing to conversion for aquaculture facilities or tourist development (Alonso-Villalobos *et al*. 2001), siltation (Aguilera & Gracia 2004) or due to a reduction in salinity due to lack of marine water influx or the impact of irrigation in surrounding areas (e.g. at CER; M. Paracuellos, personal communication). In addition, due to its value as fish food, the North American species *A. franciscana* has been introduced to different areas of the Mediterranean and has already colonized saltpans from Portugal, Spain, France, Italy and Morocco, causing the local extinction of native *Artemia* species (Amat *et al*. 2005a, 2007).

Our results provide evidence that native *Artemia* can be dispersed over long distances by birds. The invasive *A. franciscana* uses the same dispersal vector (Green *et al*. 2005). Both *A. salina* and *A. franciscana* were detected in the Ebro Delta population for the first time in 2004 (Amat *et al*. 2005a), whereas *A. franciscana* appears to have replaced *A. salina* in the main flamingo breeding site in Spain, Fuente de Piedra lagoon (Malaga), around the same time (Amat *et al*. 2007). Thus, further studies regarding its dispersal and establishment capacity must be carried out.

The high diversity of saline environments in the Iberian Peninsula is reflected in an exceptional endemic genetic diversity as has been documented in studies of salt lake fauna, such as rotifers (Gómez *et al*. 2000, 2007a), and aquatic coleopterans living in saline streams (Abellán *et al*. 2007). The evidence we provide on the high genetic diversity for *A. salina* in the Iberian Peninsula indicates that there is a risk of irreversible loss of genetic diversity caused by the extinction of local populations. The presence of the diapausing eggs or cysts in the *Artemia* life cycle could be exploited to enable the preservation of genetic diversity. The establishment of long-term cyst banks for all known populations could be an initial step for the conservation of *A. salina* diversity. These cysts would allow the possibility to restore lost populations at some time in the future if propagule banks are sampled timely.

Conclusions

Artemia salina populations hold substantial genetic diversity, with a strong phylogeographical structure and high regional endemism in the Mediterranean Basin and Iberian Peninsula. Our analyses point to an episode of population expansion in this area, possibly during the early Pleistocene, followed by more localized expansions through recolonization routes. These expansions sometimes involved long-distance colonization or isolation and genetic fragmentation.

Given the strong genetic structure, migration–drift equilibrium as a result of restricted gene flow in equilibrium conditions appears to be unlikely in the Mediterranean populations of *A. salina*. The signature of correlation between genetic and geographical distance could instead reflect a pattern of sequential colonization due to an ancient founder effect.

Our results indicate that the *A. salina* population from South Africa is highly differentiated genetically from the Mediterranean populations and might deserve specific status.

Finally, our results indicate that traditional human saltpan management used to be compatible with the maintenance of population genetic diversity in this organism. However, recent changes of saltpan use, such as aquaculture, which promotes the introduction of the alien species *A. franciscana*, as well as habitat loss, pose serious threats for the genetic diversity of *A. salina* populations in the Mediterranean area. Due to these threats, *A. salina* requires urgent measures to conserve its remaining genetic diversity throughout its range.

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This work is part of Joaquín Muñoz's doctoral thesis on *Artemia* species of the Mediterranean Basin. Africa Gómez is interested in the population structure and phylogeography of zooplankton and aquatic organisms with alternative reproductive modes. Andy Green has a broad interest in the ecology of Mediterranean wetlands, especially the functional role of waterbirds. Jordi Figuerola has a special interest on the role of bird on the dispersal of aquatic organisms and pathogens. Francisco Amat is interested in the ecology and biogeography of *Artemia*, with special attention to the consequences of biodiversity losses in the western Mediterranean. Ciro Rico has a longstanding interest in the use of molecular markers to study paternity, population structure and mechanisms of speciation in fish and other vertebrates.

Appendix I Summary of the relative frequencies of haplotypes, number of individuals per population and GenBank Accesion no

	A. salina populations																								
Haplotype	MAT (n = 14)	DON (n = 13)	BRAS (n = 13)	PIN (n = 13)	CER (n = 16)	BON (n = 13)	ROC (n = 11)	POR (n = 12)	EBR (n = 10)	ROS (n = 7)	SCA (n = 7)	MAL (n = 7)	CAM (n = 8)	JAD (n = 8)	ADH (n = 8)	SAH (n = 8)	GAR (n = 8)	WAD (n = 9)	LIB (n = 8)	CYP (n = 8)	SGI (n = 7)	MOL (n = 8)	MES (n = 8)	VEL (n = 8)	GenBank Acc. Num.
AS01	0.7860	—	0.3080	0.4620	—	0.5380	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426827
AS02	0.0714	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426828
AS03	0.0714	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426829
AS04	0.0714	—	0.1540	—	—	0.3080	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426830
AS05	—	0.2310	—	—	—	—	—	—	0.5000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426831
AS06	—	0.3080	—	—	—	—	0.3640	0.2500	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426832
AS07	—	0.3850	—	—	—	—	0.5450	0.2500	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426833
AS08	—	0.0769	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426834
AS09	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426835
AS10	—	—	0.1540	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426836
AS11	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426837
AS12	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426838
AS13	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426839
AS14	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426840
AS15	—	—	—	0.1540	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426841
AS16	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426842
AS17	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426843
AS10	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426844
AS10	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426845
AS20	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426846
AS20	—	—	—	—	0.0620	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426847
AS20	—	—	—	—	0.0620	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426848
AS20	—	—	—	—	0.8120	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426849
AS20	—	—	—	—	0.0620	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426850
AS20	—	—	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426851
AS20	—	—	—	—	—	0.0760	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426852
AS20	—	—	—	—	—	—	0.0900	0.1670	0.1000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426853
AS20	—	—	—	—	—	—	—	0.0830	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426854
AS20	—	—	—	—	—	—	—	0.0830	—	—	—	—	—	—	—	—	—	—	—	—	0.4290	0.2500	—	—	DQ426855
AS30	—	—	—	—	—	—	—	0.1670	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426856
AS30	—	—	—	—	—	—	—	—	0.3000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426857
AS30	—	—	—	—	—	—	—	—	0.1000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	DQ426858
AS30	—	—	—	—	—	—	—	—	—	1.0000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	EU543444
AS30	—	—	—	—	—	—	—	—	—	—	1.0000	—	—	—	—	—	—	—	—	—	—	—	—	—	EU543445
AS30	—	—	—	—	—	—	—	—	—	—	—	0.5710	—	—	—	—	—	—	—	—	—	—	—	—	EU543446
AS30	—	—	—	—	—	—	—	—	—	—	—	0.1430	—	—	—	—	—	—	—	—	—	—	—	—	EU543447
AS30	—	—	—	—	—	—	—	—	—	—	—	0.1430	—	—	—	—	—	—	—	—	—	—	—	—	EU543448
AS30	—	—	—	—	—	—	—	—	—	—	—	0.1430	—	—	—	—	—	—	—	—	—	—	—	—	EU543449
AS30	—	—	—	—	—	—	—	—	—	—	—	—	0.8750	—	—	—	—	—	—	—	—	—	—	—	EU543450
AS40	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	—	—	EU543451
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	—	EU543452
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2500	—	—	—	—	—	—	—	—	—	—	EU543453
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	0.5000	—	—	—	—	—	—	—	—	—	—	EU543454
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	—	EU543455

Appendix I *Continued*

	A. salina populations																								
Haplotype	MAT (n = 14)	DON (n = 13)	BRAS (n = 13)	PIN (n = 13)	CER (n = 16)	BON (n = 13)	ROC (n = 11)	POR (n = 12)	EBR (n = 10)	ROS (n = 7)	SCA (n = 7)	MAL (n = 7)	CAM (n = 8)	JAD (n = 8)	ADH (n = 8)	SAH (n = 8)	GAR (n = 8)	WAD (n = 9)	LIB (n = 8)	CYP (n = 8)	SGI (n = 7)	MOL (n = 8)	MES (n = 8)	VEL (n = 8)	GenBank Acc. Num.
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	EU543456
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2500	—	—	—	0.1250	—	—	—	—	—	EU543457
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	0.2500	—	—	—	—	—	—	—	—	EU543458
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	EU543459
AS40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	EU543460
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	EU543461
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	—	EU543462
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.3750	—	—	0.5000	—	—	—	—	—	EU543463
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	0.2500	—	—	—	—	—	EU543464
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	EU543465
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	—	—	—	EU543466
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2500	—	—	—	—	—	—	—	EU543467
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.5000	—	—	—	—	—	—	—	EU543468
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2500	—	—	—	—	—	—	—	EU543469
AS50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.7780	—	—	—	—	—	—	EU543470
AS60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2220	—	—	—	—	—	—	EU543471
AS60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	—	—	—	EU543472
AS60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0000	—	—	—	—	EU543473
AS63	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1430	—	—	—	EU543474
AS64	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1430	0.3750	—	—	EU543475
AS65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.2860	—	—	—	EU543476
AS66	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	EU543477
AS67	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	EU543478
AS68	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	—	EU543479
AS69	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.8750	—	EU543480
AS70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	—	EU54348
AS71	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.6250	EU54348
AS72	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	EU54348
AS73	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	EU54348
AS74	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1250	EU54348