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Exceptional maternal lineage diversity in brown bears (*Ursus arctos*) from Turkey

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The genetic diversity and phylogeography of maternal lineages in *Ursus arctos* Linnaeus, 1758 (the brown bear) have been studied extensively over the last two decades; however, sampling has largely been limited to the northern Holarctic, and was possibly biased towards lineages that recolonized the vast expanses of the north as the Last Glacial Maximum (LGM) ended. Here we report the genetic diversity and phylogeography of *U. arctos* from Turkey based on 35 non-invasive samples, including five from captive individuals. Bayesian phylogenetic analyses based on a 269-bp fragment of the mitochondrial DNA control region revealed 14 novel haplotypes belonging to three major lineages. The most widespread lineage was found to be the Eastern clade 3a, whereas geographically more restricted Western and Middle Eastern lineages were reported for the first time in Turkey. A specimen from the Taurus mountain range carried a haplotype closely related to the presumably extinct bears in Lebanon. Moreover, we identify a unique new lineage that appears to have split early within the Middle Eastern clade. Despite limited sampling, our study reveals a high level of mitochondrial diversity in Turkish *U. arctos*, shows that the ranges of both European and Middle Eastern clades extend into Turkey, and identifies a new divergent lineage of possibly wider historical occurrence. Obtaining these results with 35 samples also demonstrates the value of proper sampling from regions that have not been significantly affected by the LGM.

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ADDITIONAL KEYWORDS: brown bear – genetic diversity – mtDNA control region – phylogeography – Turkey – *Ursus arctos syriacus*.

INTRODUCTION

The brown bear (*Ursus arctos* Linnaeus, 1758) is the largest member of Carnivora, and has a highly fragmented distribution across the Holarctic (Herrero, 1999; McLellan, Servheen & Huber, 2008). Declines in population over most of its range, increased conflict with people, and a need to make sound conservation decisions have led to numerous studies on conservation genetics, life-history traits, and behaviour in *U. arctos* (see Martin *et al*., 2010; Swenson, Taberlet & Bellemain, 2011; Deecke, 2012; Jasmine *et al*., 2012; Steyaert *et al*., 2012). A widespread range of modern populations and an increasing availability of ancient DNA samples (Barnes *et al*., 2002; Hofreiter *et al*., 2002, 2004; Miller, Waits & Joyce, 2006; Valdiosera *et al*., 2007, 2008; Calvignac *et al*., 2008; Bray *et al*., 2013) also make this species a useful model to study phylogeography in the Late Pleistocene–Holocene (Davison *et al*., 2011). The mitochondrial genetic diversity of *U. arctos* is well studied in Europe, Japan, and North America, where several divergent clades, including some that are now extinct, have been discovered (Randi *et al*., 1994; Taberlet & Bouvet, 1994; Kohn *et al*., 1995; Taberlet *et al*., 1995;

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Talbot & Shields, 1996; Masuda *et al*., 1998; Waits *et al*., 1998; Matsuhashi *et al*., 1999, 2001; Leonard, Wayne & Cooper, 2000; Calvignac *et al*., 2008; Calvignac, Hughes & Hänni, 2009; Korsten *et al*., 2009). A clear split between two main mitochondrial lineages (i.e. Eastern versus Western) in modern European *U. arctos* populations has for a long time been considered to reflect the general pattern of recolonization from peninsular refugia following the Last Glacial Maximum (LGM; Taberlet & Bouvet, 1994; Taberlet *et al*., 1998; Hewitt, 2000); however, this view has recently been challenged by findings based on fossil *U. arctos* DNA that instead indicated a more complex historical phylogeographic structure and apparent gene flow among populations during the LGM (Hofreiter *et al*., 2004; Valdiosera *et al*., 2007).

As opposed to the high number of genetic studies from Europe, Japan, and North America, studies from West or Inner Asia are lacking. The genetically divergent clades described from those regions (Miller *et al*., 2006; Calvignac *et al*., 2009) are based on few samples, whereas the recent sample-rich study by Murtskhvaladze, Gavashelishvili & Tarkhnishvili (2010) is restricted to Georgia in the Caucasus; however, given that *U. arctos* are believed to have evolved in Asia (Kurtén, 1968), and as recurrent glacial episodes made large expanses of the north inhospitable at the time (Hewitt, 2000), the study of the genetic make-up of *U. arctos* populations in the south of their range becomes necessary.

Ursus arctos still occur in reasonable numbers in northern and eastern Turkey, but as a result of human persecution, dam construction, or road networks, smaller and apparently disjunct populations exist in the west and the south (Turan, 1984; Can, 2001; Ambarlı, 2006). Countrywide numbers are estimated to be about 3500–4000 individuals, with a stable trend in the last decade (Bilgin, 2010; Ambarlı, 2015). Despite such a large population, until now only information from mitochondrial DNA (mtDNA; cytochrome *b* sequences) for two individuals from the extreme north-east of Turkey (Artvin), belonging to subclade 3a, had been published (Talbot & Shields, 1996).

Unlike bear populations in Europe, Turkish bears might not have experienced severe demographic bottlenecks, and hence might harbour yet unidentified genetic variation. The recent discovery of new lineages originating from Iran and Lebanon from a few captive or fossil specimens (Calvignac *et al*., 2009) supports this hypothesis. Here we report on the genetic diversity of *U. arctos* in Turkey by analyzing the mtDNA control region of 35 wild and captive Turkish bears along with several publicly available sequences. We aim to: (1) identify the distinct maternal lineages present in the country; (2) evaluate their relationship with known lineages; and (3) try to understand the factors that might have shaped the current phylogeographic pattern in Turkey.

MATERIAL AND METHODS

Sample collection

Hair ($N = 47$), scat ($N = 49$), and tissue ($N = 9$ old skin and $N = 6$ fresh tissue) samples of *U. arctos* were obtained from different parts of Turkey, mostly from the north-east, where the species is most numerous (for a list of samples and their origins, see Table 1). Hair samples were collected from 2009 until 2011, mainly from 16 natural rubbing trees (with barbed wire attached to improve effectiveness) growing between 1090 and 2200 m a.s.l., from six barbed wire hair traps with scent lures (Woods *et al*., 1999) at 1700–2130 m a.s.l. (H. Ambarlı, unpubl. data), and from various fences and additional rubbing trees around villages and agricultural fields in Yusufeli (Ambarlı, 2010). Scat samples were collected opportunistically between 2005 and 2011. In addition, fresh scat samples were obtained in 2011 from Konya Zoo ($N = 2$), Antalya Zoo ($N = 2$), Bursa Zoo ($N = 1$), and Karacabey Bear Sanctuary ($N = 5$) (H. Ambarlı, unpubl. data), although we did not know the exact origin (within Turkey) of these individuals. Private collectors provided old skin samples from specimens that were hunted during the years when bear hunting was legal in Turkey. A few fresh tissue samples from claws were also obtained from live captures under anaesthesia during fieldwork for an MSc study ($N = 1$) in 2005 and during fieldwork for a PhD study ($N = 7$) between 2010 and 2011 in Yusufeli district (Ambarlı, 2006, 2012).

The distances between sampling locations and microsatellite work (F.G. Çilingir, Ç.A. Pekşen, unpubl. data) with the same samples indicate that none of the samples come from the same individual.

DNA extraction

All samples were appropriately stored before processing for DNA extraction. Hair samples were preserved in dry paper envelopes, as suggested in Gagneux, Boesch & Woodruff (1997) and Woods *et al*. (1999). In order to prevent cross-contamination, only hair follicles of the same colour and length were used. For each extraction, 10–20 hair follicles were selected under the microscope (Poole, Mowat & Fear, 2001; Riddle *et al*., 2003; Lorenzini *et al*., 2004). DNA from the follicles were isolated with Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions with slight modifications at the lysis step: hair samples were incubated at 65 °C with 180 µL of ATL buffer, 20 µL of 0.15 M DDT, and 20 µL of 20 mg mL^{-1} Proteinase K, and they were vortexed regularly until the bulbs disappeared. Scat samples were preserved in 95% EtOH until the time of DNA extraction (Murphy *et al*., 2002; Beja-Pereira *et al*., 2009). DNA isolation from faeces

Table 1. mtDNA sequences used in the analysis

Sample ID	Accession no.	Location	Species	References
GE-1	GU057343	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-2	GU057345	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-3	GU057346	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-4	GU057347	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-5	GU057349	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-6	GU057351	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-7	GU057352	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-8	GU057353	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-9	GU057356	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-10	GU057357	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-11	GU057358	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-12	GU057359	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-13	GU057363	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-14	GU057366	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-15	GU057367	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-16	GU057368	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-17	GU057369	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-18	GU057371	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-19	GU057372	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-20	GU057373	Georgia, Greater Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-21	GU057374	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-22	GU057375	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
GE-23	GU057376	Georgia, Lesser Caucasus	*Ursus arctos*	Murtskhvaladze *et al*., 2010
SK-1	X75876	Slovakia	*Ursus arctos*	Taberlet & Bouvet 1994
GR-1	X75870	Greece	*Ursus arctos*	Taberlet & Bouvet 1994
BA-1	X75877	Bosnia	*Ursus arctos*	Taberlet & Bouvet 1994
HR-1	X75867	Croatia	*Ursus arctos*	Taberlet & Bouvet 1994
BG-1	X75864	Bulgaria	*Ursus arctos*	Taberlet & Bouvet 1994
FR-1	X75878	France	*Ursus arctos*	Taberlet & Bouvet 1994
SE-1	X75868	Sweden	*Ursus arctos*	Taberlet & Bouvet 1994
RO-1	X75872	Romania	*Ursus arctos*	Taberlet & Bouvet 1994
ES-1	X75865	Spain	*Ursus arctos*	Taberlet & Bouvet 1994
RO-2	X75873	Romania	*Ursus arctos*	Taberlet & Bouvet 1994
CN-1	X75863	Tibet	*Ursus arctos*	Taberlet & Bouvet 1994
AT-1	FN663157	Austria	*Ursus spelaeus*	Stiller *et al*., 2010
HR-2	HQ602653	Croatia	*Ursus arctos*	Kocijan *et al*., 2011
HR-3	HQ602652	Croatia	*Ursus arctos*	Kocijan *et al*., 2011
HR-4	HQ602651	Croatia	*Ursus arctos*	Kocijan *et al*., 2011
XX-Z1	FN292981	Unknown origin, Heidelberg Zoo	*Ursus arctos*	Calvignac *et al*., 2009
XX-Z2	FN292980	Unknown origin, Heidelberg Zoo	*Ursus arctos*	Calvignac *et al*., 2009
XX-Z3	FN292979	Unknown origin, Montpelier Zoo	*Ursus arctos*	Calvignac *et al*., 2009
XX-Z4	FN292978	Unknown origin, Montpelier Zoo	*Ursus arctos*	Calvignac *et al*., 2009
XX-Z5	FN292982	Unknown origin – Ostrava Zoo	*Ursus arctos*	Calvignac *et al*., 2009
IR-Z1	FN292977	Paris Zoo	*Ursus arctos*	Calvignac *et al*., 2009
IR-Z2	FN292976	Paris Zoo	*Ursus arctos*	Calvignac *et al*., 2009
IR-1	FN292974	Iran	*Ursus arctos*	Calvignac *et al*., 2009
IR-2	FN292975	Iran	*Ursus arctos*	Calvignac *et al*., 2009
SY-1	FN292973	Syria	*Ursus arctos*	Calvignac *et al*., 2009
LB-1	FN292972	Lebanon	*Ursus arctos*	Calvignac *et al*., 2009
LB-2	FN292971	Lebanon	*Ursus arctos*	Calvignac *et al*., 2009
LB-3	FN292970	Lebanon	*Ursus arctos*	Calvignac *et al*., 2009
RU-1	EU526794	Siberia, Russia	*Ursus arctos*	Korsten *et al*., 2009
US-1	EF198825	USA	*Ursus americanus*	Robinson *et al*., 2007
CN-2	AB010727	Tibet	*Ursus arctos*	Masuda *et al*., 1998

Table 1. *Continued*

Sample ID	Accession no.	Location	Species	References
MN-1	AB010728	Gobi	*Ursus arctos*	Masuda *et al*., 1998
MA-1	AM411399	Morocco	*Ursus arctos*	Calvignac *et al*., 2008
DZ-1	AM411400	Algeria	*Ursus arctos*	Calvignac *et al*., 2008
CN-3	DQ914407	Tibet	*Ursus arctos*	Miller *et al*., 2006
IR-3	DQ914408	Iran	*Ursus arctos*	Miller *et al*., 2006
PK-1	DQ914409	Pakistan	*Ursus arctos*	Miller *et al*., 2006
PK-2	DQ914410	Pakistan	*Ursus arctos*	Miller *et al*., 2006
XX-Z6	DQ914411	Unknown origin, Greek zoo	*Ursus arctos*	Miller *et al*., 2006
RO-3	L38270	Romania	*Ursus arctos*	Kohn *et al*., 1995
RO-4	L38272	Romania	*Ursus arctos*	Kohn *et al*., 1995
ES-2	EF488487	Spain	*Ursus arctos*	Valdiosera *et al*., 2007
ES-3	EF488503	Spain	*Ursus arctos*	Valdiosera *et al*., 2007
FR-2	EF488495	France	*Ursus arctos*	Valdiosera *et al*., 2007
ES-4	EF488504	Spain	*Ursus arctos*	Valdiosera *et al*., 2007
IT-1	EF488488	Italy	*Ursus arctos*	Valdiosera *et al*., 2007
ES-5	EF488490	Spain	*Ursus arctos*	Valdiosera *et al*., 2007
FR-3	EF488496	France	*Ursus arctos*	Valdiosera *et al*., 2007
FR-4	EF488492	France	*Ursus arctos*	Valdiosera *et al*., 2007
FR-5	EF488493	France	*Ursus arctos*	Valdiosera *et al*., 2007
FR-6	EF488491	France	*Ursus arctos*	Valdiosera *et al*., 2007
FR-7	EF488494	France	*Ursus arctos*	Valdiosera *et al*., 2007
FR-8	EF488505	France	*Ursus arctos*	Valdiosera *et al*., 2007
ES-6	EF488497	Spain	*Ursus arctos*	Valdiosera *et al*., 2007
DE-1	EF488501	Germany	*Ursus arctos*	Valdiosera *et al*., 2007
DE-2	EF488498	Germany	*Ursus arctos*	Valdiosera *et al*., 2007
DE-3	EF488499	Germany	*Ursus arctos*	Valdiosera *et al*., 2007
AT-2	AJ809334	Austria	*Ursus arctos*	Hofreiter *et al*., 2004
TR-1	KT438621	Yusufeli, Artvin – Turkey	*Ursus arctos*	This study
TR-2	KT438632	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-3	KT438643	Altıparmak, Artvin – Turkey	*Ursus arctos*	This study
TR-4	KT438644	Bıçakçılar, Artvin – Turkey	*Ursus arctos*	This study
TR-5	KT438645	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-Z1	KT438650	Hakkari/Sivas/Siirt – Turkey	*Ursus arctos*	This study
TR-6	KT438646	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-7	KT438647	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-8	KT438648	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-9	KT438649	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-10	KT438622	Yusufeli, Artvin – Turkey	*Ursus arctos*	This study
TR-11	KT438623	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-12	KT438624	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-13	KT438625	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-14	KT438626	Bıçakçılar, Artvin – Turkey	*Ursus arctos*	This study
TR-15	KT438627	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-16	KT438628	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-17	KT438629	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-18	KT438630	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-19	KT438631	Meydancık, Artvin – Turkey	*Ursus arctos*	This study
TR-20	KT438633	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-21	KT438634	Altıparmak, Artvin – Turkey	*Ursus arctos*	This study
TR-22	KT438635	Nallıhan, Ankara – Turkey	*Ursus arctos*	This study
TR-Z3	KT438652	Not known – Turkey	*Ursus arctos*	This study
TR-Z4	KT438653	Not known – Turkey	*Ursus arctos*	This study
TR-23	KT438636	Akseki, Antalya – Turkey	*Ursus arctos*	This study
TR-24	KT438637	Akseki, Antalya – Turkey	*Ursus arctos*	This study

Table 1. *Continued*

Sample ID	Accession no.	Location	Species	References
TR-Z6	KT438655	Uludağ, Bursa – Turkey	*Ursus arctos*	This study
TR-25	KT438638	Özgüven, Artvin – Turkey	*Ursus arctos*	This study
TR-26	KT438639	Karakışla, Artvin – Turkey	*Ursus arctos*	This study
TR-Z2	KT438651	Not known – Turkey	*Ursus arctos*	This study
TR-Z5	KT438654	Not known – Turkey	*Ursus arctos*	This study
TR-27	KT438640	Erikli, Artvin – Turkey	*Ursus arctos*	This study
TR-28	KT438641	Çoraklı, Artvin – Turkey	*Ursus arctos*	This study
TR-29	KT438642	Ortaköy, Artvin – Turkey	*Ursus arctos*	This study

Sample ID used in this paper, accession number in GenBank, location, species name, and references are provided. The first two letters of sample IDs are constructed from the location where the samples were taken, and abbreviations are country codes at the top-level domain. Samples with three-letter IDs correspond to samples taken from zoos (i.e. IR-Z1 etc.) Samples starting with XX have unknown origins.

was conducted with NORGEN™ Stool DNA Isolation Kit (Norgen Biotek Corp., ON, Canada), following the manufacturer's instructions.

Fresh tissue samples were preserved in 95% EtOH and Qiagen DNeasy™ Blood and Tissue Kit was used for DNA isolation, following the manufacturer's instructions. Old tissue samples were preserved in dry envelopes and ground in liquid nitrogen before extraction. Samples were then incubated overnight in L6 extraction buffer (Boom *et al*., 1990) in order to eliminate any inhibitors in the samples. We took 200 mg of old tissue extracts and followed the manufacturer's instructions for the NORGEN™ Stool DNA Isolation Kit.

Genomic DNA extracted from all types of sources was stored at –20 °C until DNA amplification via polymerase chain reaction.

All DNA extractions and polymerase chain reactions (PCRs) were conducted in separate dedicated sections in the wet lab. PCR reactions were prepared in the ultraviolet (UV) sterilization cabinet, and each lab instrument was UV-sterilized before and after carrying out the experiments.

DNA amplification and sequencing

Genomic DNA elutes (2–10 μL), and the primers 5′-CTCCACTATCAGCACCCAAAG-3′ (forward) and 5′-GGAGCGAGAAGAGGTACACGT-3′ (reverse) (Taberlet & Bouvet, 1994), were used for the amplification of a 269-bp fragment of the mtDNA control region. The PCR of the control region involved initial incubation at 93 °C for 3 min, followed by 45 cycles of 93 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, with a final 5-min extension at 72 °C.

For the samples giving weak outcomes with the primer set above, we performed a nested PCR. A 400-bp fragment of the mtDNA control region was amplified with L15774 in the cytochrome *b* gene region (Kocher *et al*., 1989) and H16498 in the control region (Shields & Kocher, 1991). The PCR product obtained from this primer set was used as a template to amplify the 269-bp frgment of the mtDNA control region, using the primer set described by Taberlet & Bouvet (1994). Negative controls were included into each sample set in order to monitor contamination. PCRs were performed twice for each sample and at least three times for the unique haplotypes. Finally, PCR products were purified with the Gene Mark Gel Extraction Kit (Hopegen Biotechnology, Dali City, Taiwan).

Sequencing reactions were performed with an ABI terminator 3.1 kit (Applied Biosystems Inc., Foster City, CA, USA) at Mclab (San Francisco, California, USA). PCR products were sequenced in both directions to increase accuracy. Electrophoresis and detection of fluorescently labelled nucleotides were performed with an automatic DNA sequencer (ABI 3730x1 Genetic Analyzer; Applied Biosystems). Mitochondrial DNA sequences representing distinct haplotypes were deposited in GenBank under the accession numbers shown in Table 1.

Data analysis

The alignment of mtDNA sequences was performed using the CLUSTAL W algorithm in MEGA 5.1 (Tamura *et al*., 2011). A 269-bp alignment was used to perform Bayesian phylogenetic analysis and network construction, and to calculate genetic diversity indices and genetic distances among clades. The estimation of a sequence evolution model for the mtDNA data set was performed in MEGA 5.1 (Tamura *et al*., 2011), based on the Bayesian information criterion (BIC; Schwarz, 1978). The best-fitting model for the data set was the Tamura three-parameter (T92) model (Tamura, 1992), with a Gamma-distributed site rate variation, $\Gamma = 0.19229$, governed by the shape parameter α

(ln L = −1310.52, BIC = 4869.29). As the T92 + G model was not available in BEAST, the second best-fitting model, the Hasegawa–Kishino–Yano (HKY) mutation model (Hasegawa, Kishino & Yano, 1985), with gamma-distributed site rate variation was used for Bayesian phylogenetic analysis (ln L = −1306.48, BIC = 4881.65). To calculate haplotype frequency and diversity (h) as well as nucleotide diversity (π) (Nei & Kumar, 2000), ARLEQUIN 3.5 was used (Excoffier & Lischer, 2010). Uncorrected p distances (Nei & Kumar, 2000) were calculated in MEGA 5.1 (Tamura *et al*., 2011) to define the boundaries of clades, subclades, and populations.

To evaluate the phylogenetic position of Turkish *U. arctos* populations within *U. arctos* of the Western Palaearctic, 63 mtDNA control region haplotypes corresponding to maternal lineages identified from Western and Eastern Europe, the Middle East, Inner Asia, and North Africa were downloaded from GenBank and combined with 35 Turkish haplotypes. American black bear [*Ursus americanus* (Pallas, 1780)] and cave bear (*Ursus spelaeus* Rosenmüller, 1794) sequences were used as out-groups (see Table 1).

BEAST 1.7.1 (Drummond *et al*., 2012) was used for the analysis of the phylogeny and divergence times. The data set was constructed using the BEAST assistance program BEAUTI 1.7.1. We set up the run allowing for the HKY mutation model with gamma-distributed site rate variation, using four discrete mutation classes, and a percentage of invariant sites (HKY + G + I). We employed two analyses: one with 21 ancestral mtDNA control region sequences that were only 193 bp long, and another from the same region with sequences that were 269 bp long. For the shorter sequences, we used the carbon dates of specimens as the sampling date. The longer data set contained only contemporary samples, except for *U. spelaeus*.

A relaxed molecular clock (Drummond *et al*., 2006) was estimated using a lognormal prior with two parameters: the mean rate and a standard deviation. For the mean rate, we used a narrow normal-distributed hyperprior with a mean of 0.39 mutations per million years and a standard deviation of 0.08 per million years based on the results of Ho *et al*. (2008). We also used a normal-distributed hyperprior for the standard deviation parameter of the lognormal distribution with a mean of 0.08 and a standard deviation of the same magnitude. Priors for the mutation model were default. We reduced the default upper bounds for the tree height by a factor of 10. We adjusted the Markov chain Monte Carlo run parameters so that 200 million steps were executed, and so that a total of 10 000 trees were sampled. Trial runs were performed with 50 million steps. The 200- and the 50-million-step runs revealed almost identical branching patterns, suggesting that the 200-million-step run converged. The effective sample sizes evaluated from the logfiles in TRACER 1.5 (Rambaut & Drummond, 2009) corroborated convergence. We report the majority consensus tree of the 269-bp data set, generated with sumtrees.py (Sukumaran & Holder, 2010), and the timings from the maximum posterior of the data set with the dated samples (TREEANNOTATOR 1.7.1; Drummond *et al*., 2012).

To understand evolutionary relationships and probable ancestral connections among haplotypes, a median-joining network was constructed with NETWORK 4.6.1.0 (Bandelt, Forster & Röhl, 1999) using only sequences of 269 bp in length to avoid any loss of information. Any sequences shorter than 269 bp were removed from this analysis.

We followed the nomenclature of Leonard *et al*. (2000), Calvignac *et al*. (2009), and Davison *et al*. (2011) in the labelling of observed lineages, with the exception of the 'Iran' clade of Calvignac *et al*. (2009), which we renamed 'clade 7', as we found that it is not restricted to Iran.

RESULTS

We obtained mtDNA control region sequences of 265–271 bp in length (with variance in length resulting from the indels at the pyrimidine tract) from a total of 35 samples. Among those 35 bear sequences, 14 different haplotypes were identified. When jointly analysed with additional published haplotypes, both the Bayesian phylogenetic tree (Fig. 1) and the median-joining network (Fig. 2) show that Anatolian *U. arctos* haplotypes cluster into three major, highly divergent maternal lineages, namely clade 1, clade 3, and clade 7. These are further divided into five distinct subclades, three of which are already known, one known but presumed extinct, and one is a novel lineage, highly divergent from its sister subclade (Fig. 1).

Two individuals from western and south-western Turkey (TR-24 and TR-Z6, respectively) provided distinct haplotypes that are firmly placed within subclade 1b. These haplotypes belong to the 'Western' lineage (Taberlet & Bouvet, 1994), and are the easternmost – and so far the sole Asian – records representing that particular subclade. One sample (TR-23) from the Central Taurus Mountains clustered with three ancient samples from Lebanon reported in Calvignac *et al*. (2009), therefore showing that subclade 1d is not extinct as previously assumed. We named this lineage subclade 1d, as ancient haplotypes from France (Valdiosera *et al*., 2007) were already grouped as subclade 1c by Davison *et al*. (2011). This lineage splits off early from sister subclades 1a and 1b with a high posterior probability value of 0.99.

Seven individuals from Turkey, five from Iran, and one captive individual of an unknown origin at a Greek zoo (Miller *et al*., 2006) fell into clade 7 (formerly known

Figure 1. Majority-rule consensus tree. The majority consensus tree of the 269-bp mtDNA control region data set listed in Table 1. Values at nodes are posterior probabilities. TMRCA calculations, with 95% confidence intervals, belonging to particular nodes are indicated with arrows. HDP and YBP refer to highest posterior density and years before present, respectively.

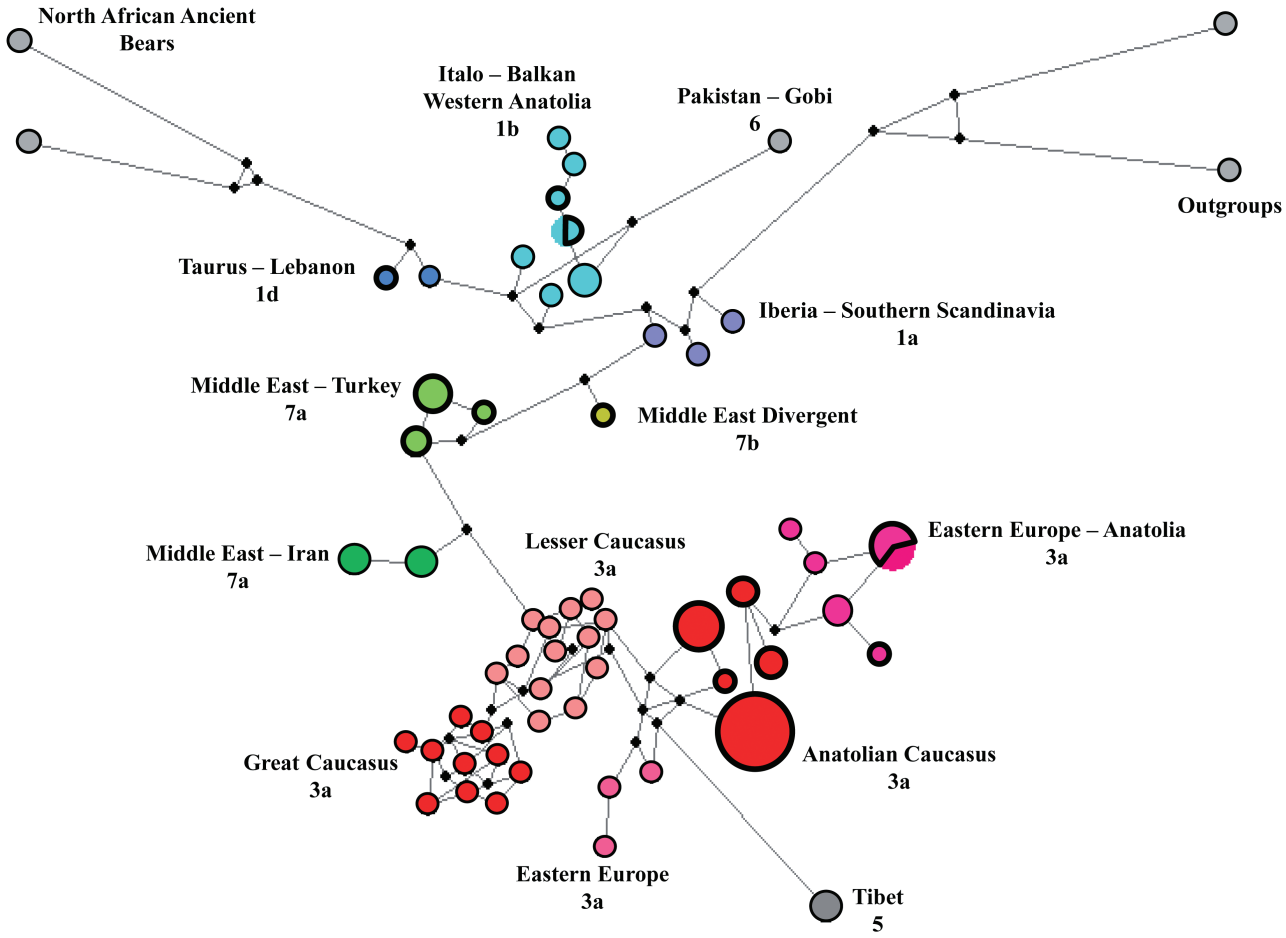

Figure 2. Median-joining network. Median-joining network showing the evolutionary relationships and probable ancestral connections among haplotypes from the Western Palaearctic based on the 269-bp sequence of mtDNA control region. Lengths of the lines connecting different haplotype groups are proportional to the number of mutational positions. The size of each circle is proportional to the number of individuals carrying that particular haplotype (see Table 1). The proportion of haplotypes from Turkey is framed with bold lines, i.e. a circle fully enclosed by a bold line represents Turkish-only haplotypes.

as the 'Iran' clade). Haplotype and nucleotide diversity within the group are estimated to be 0.92 ± 0.05 and 0.064 ± 0.034, respectively. This group consists of two geographically separate and divergent subclades, supported by a posterior probability value of 0.95, and with a mean genetic difference of 4.3%. The phylogenetic placement of this clade among or within other clades is weakly supported, however, and hence is not yet resolved. All Iranian and six Turkish specimens jointly formed subclade 7a. Iranian samples are represented by five already published haplotypes, two of which are ancient (Calvignac *et al*., 2009) and three are modern DNA sequences (Miller *et al*., 2006; Calvignac *et al*., 2009). Of the Turkish samples, four were obtained from north-eastern Turkey (Artvin), whereas the origins of another two captive specimens (TR-Z2 and TR-Z5) are not clear; these latter specimens yielded two distinct haplotypes that are separate from the rest of the members of Turkish subclade 7a. Subclade 7b is a highly divergent branch within clade 7, and is supported by a posterior probability value of 0.95. This novel subclade is formed by two distinct haplotypes, one of which (TR-25) is from north-eastern Turkey, whereas the others (XX-Z6) were previously published but not associated with any major lineage (Miller *et al*., 2006).

Haplotypes belonging to subclade 3a form the remaining majority of our samples. Haplotype and nucleotide diversity within this group is 0.85 ± 0.04 and 0.05 ± 0.03, respectively. The Bayesian consensus tree (Fig. 1) indicates a close relationship between Anatolian and Georgian (Caucasus) bear populations. Anatolian haplotypes, however, seem to be geographically

structured into two distinct populations. The first is found exclusively in Eastern Turkey, and includes 23 individuals representing seven distinct haplotypes from Artvin. The clustering of one ancient (SY-1) and two modern (XX-Z4 and XX-Z3) samples (Calvignac *et al*., 2009) along with samples in this subgroup, however, indicate that their range extends further south into Syria. A second subgroup, represented by four individuals with two distinct haplotypes (one from northern Turkey, one from eastern Turkey, and two from zoos with unknown origin), has a more western distribution. Moreover, these haplotypes cluster together with a sample from Romania (RO-1; Taberlet & Bouvet, 1994) as well as with three additional zoo samples (XX-Z2, XX-Z1, and XX-Z5) of unknown origin (Calvignac *et al*., 2009).

The median-joining network of mtDNA haplotypes (Fig. 2) supports the partitioning of Anatolian haplotypes into divergent clusters, as does the Bayesian tree. The central positioning of Anatolian haplotypes within subclade 3a, connected to Caucasian (Lesser and Greater) haplotypes at one end, and Eastern European/Siberian haplotypes at the other, is clearly evident; however, the presence of several hypothetical nodes within subclade 3a suggests that the inclusion of missing haplotypes are needed to fully resolve the phylogeny within this part of the network.

Bayesian analyses indicated that subclade 1d (Taurus–Levant) formed a monophyletic group that appeared to diverge from Western European groups (i.e. subclades 1a and 1b) about 77 000 years ago (95% highest posterior density, HPD: 120 732–45 991 years ago), whereas the most recent common ancestor (MRCA) of subclades 1a and 1b lived about 57 000 years ago (95% HPD: 83 988–39 965 years ago). The time to the most recent common ancestor (TMRCA) of modern sequences belonging to subclades 7a and 7b was calculated to be around 50 000 years (95% HPD: 19 684–96 239 years). In contrast, the estimated timing for the split of the Iranian and Turkish branches of subclade 7a was more recent, *c*. 21 000 years ago (95% HPD: 44 100–6807 years ago). Similarly, the major split within subclade 3a (Holarctic) – excluding two groups that split earlier (Eastern Europe/Siberia and an early branch of the Lesser Caucasus) – was 28 000–17 000 years ago (95% HPD: 48 482–13 899 years ago; 30 726–7854 years ago; Fig. 1).

DISCUSSION

We found a high level of diversity within Turkish bears, despite the limited number of samples available to us. In addition to the already reported occurrence of clade 3 (Talbot & Shields, 1996), new haplotypes that belong to clade 1, essentially a European lineage, and clade 7, a Middle Eastern lineage previously only reported from Iran, were detected for the first time in Turkey. Our findings extend the boundaries of both clade 1 ('West European') and clade 7 ('Iranian') by several hundred kilometres eastwards and westwards, respectively, into Turkey.

The most unexpected finding was that three Turkish haplotypes belonged to clade 1, which was until recently known to be restricted to Europe (Leonard *et al*., 2000; Miller *et al*., 2006). Calvignac *et al*. (2009) identified a divergent but related haplotype from ancient samples originating in Lebanon, with which a sample from the Taurus Mountains (TR-23) formed a divergent subclade (Fig. 1). The Taurus Mountains extend on an east–west axis along southern Turkey, and are linked to the coastal mountains along Syria and Lebanon via the Amanos chain (Fig. 3). It is therefore conceivable that the Taurus and Levant populations were connected in the not-so-distant past; however, whether this connectivity is still functional or whether any viable populations are left in Syria and Lebanon is questionable (Herrero, 1999; Hajjar, 2011). Two other haplotypes (TR-24 and TR-26) from southern and north-western Turkey, respectively, are closely related to bears from the West Balkans (subclade 1b), particularly to those from Croatia (Taberlet & Bouvet, 1994; Kocijan *et al*., 2011; Figs 1, 2). Moreover, subclades 1b and 1d, which both occur near Akseki on the Taurus Mountains, constitute the only known case of sympatry of separate extant subclades within this lineage.

Most clade-7 haplotypes of known origin in Turkey are restricted to the extreme north-east of the country, where they are found only south of the River Çoruh; however, two captive specimens (TR-Z2 and TR-Z5) in the same subclade have slightly different haplotypes, a possible indication of a geographical origin other than north-eastern Turkey, and hence a wider range. The remaining two haplotypes in this clade belong to a wild-living specimen originating from Artvin and a captive bear from a Greek zoo (Miller *et al*., 2006). Greek bears have so far all been designated to clade 1 (Taberlet & Bouvet, 1994; Korsten *et al*., 2009), but whether this captive specimen was captured in Greece is unknown.

Clade-3 haplotypes are absent from the Taurus Mountains, although a nearby ancient Syrian specimen (Calvignac *et al*., 2009) suggests their historical or yet undetected presence in southern Turkey. Turkish subclade-3a haplotypes show weak geographical structure, and appear intermediate between those from the Caucasus (Murtskhvaladze *et al*., 2010) and those from Eastern Europe and Siberia (Taberlet & Bouvet, 1994; Kohn *et al*., 1995; Korsten *et al*., 2009). The particular Romanian haplotype (RO-1) that clusters with samples from western Turkey show some divergence from other Romanian subclade-3a haplotypes, pointing to relatively recent gene exchange between Anatolia and the Balkans. In contrast, almost all Greater and Lesser

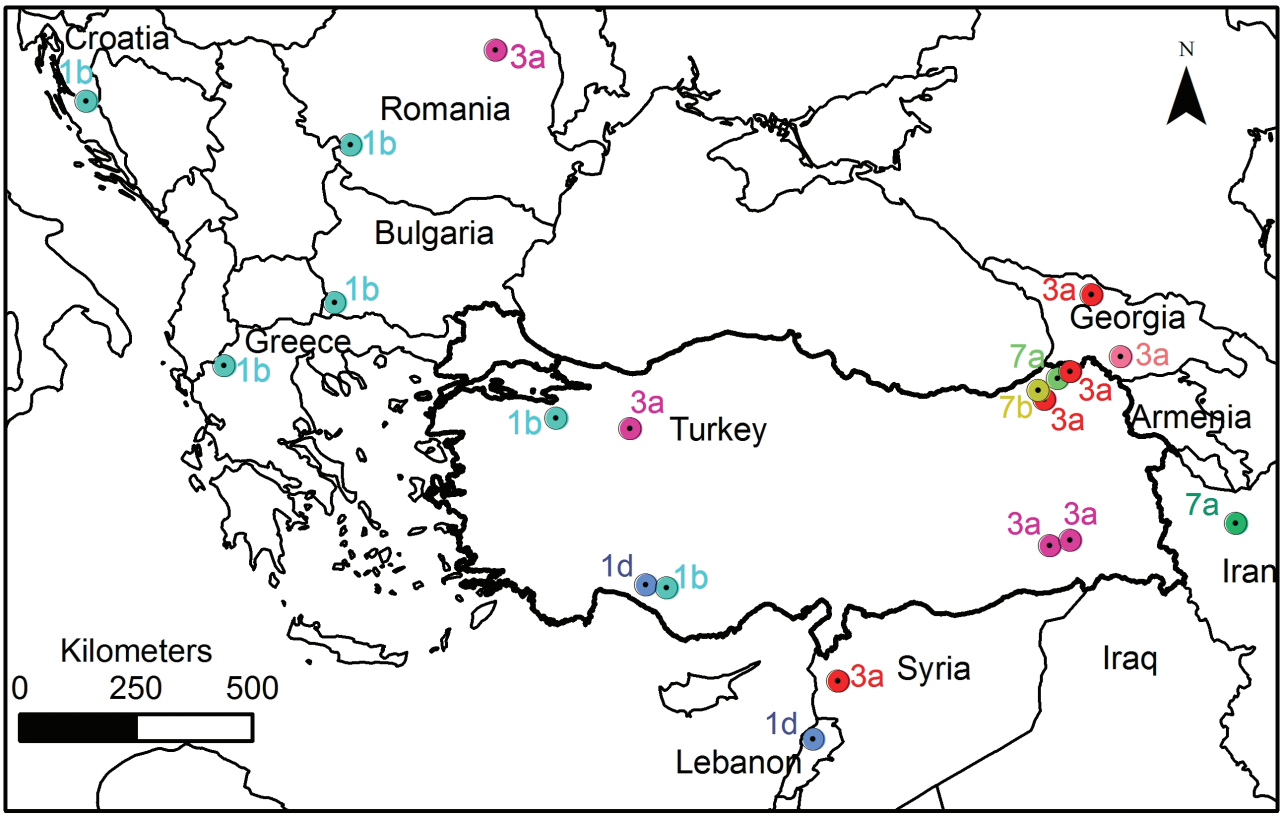

Figure 3. Map of the region with sample localities and clade designation (only specimens with known origins are shown; clade 1, tones of blue; clade 3, tones of red; clade 7, tones of green).

Caucasus (i.e. Georgian) bears cluster separately from Turkish subclade-3a specimens.

The presence of three major lineages with overlapping distributions in Anatolia provides insight into the historical processes that led to their current distributions. We have shown that specimens of clade 3 occur sympatrically with bears of clades 1 and 7 in north-western and north-eastern Turkey, respectively (Fig. 3). These represent additional cases of clade overlap in western Eurasia after the well-known zone of sympatry in the East Carpathians (Kohn *et al*., 1995; Zachos *et al*., 2008). In addition, Lebanese bears should not be considered genetically isolated from Western European bears any more, as suggested by Calvignac *et al*. (2009), because members of subclades 1b and 1d are found in western and southern Turkey, thus forming a link between populations of this major lineage from the Balkans and those from the Levant until about 6700 years ago, when the Bosphorus Strait was breached and formed an impassable barrier (Okay *et al*., 2011). Therefore, our data suggest a complex but weak phylogeographic structure in Turkey, where the admixture of maternal lineages is not uncommon. Such a structure is thought to have existed in Europe until a few thousand years ago (Hofreiter *et al*., 2002; Valdiosera *et al*., 2007; Davison *et al*., 2011). This might have evolved into today's considerable geographic differentiation through the loss of genetic diversity and lineage sorting as a result of human-mediated stochastic events (Valdiosera *et al*., 2007, 2008).

The wide confidence intervals on our TMRCA estimates do not allow for the straightforward association of splits in the evolution of *U. arctos* with particular climatic periods. Especially where subclades 7a or 1d are concerned, small sample sizes and short sequences call for careful interpretation; however, our estimates are in line with recent such estimates made by others. We found the split of subclade 1d (Taurus–Levant) from Western European *U. arctos* (1a and 1b), for example, to have occurred about 77 000 years ago, during the Marine Isotope Stage 5a (MIS 5a). This finding is congruent with a suggestion of *c*. 65 000 years ago by Calvignac *et al*. (2009). Similarly, TMRCA for subclades 1a and 1b (57 000 years ago) falls within the time ranges suggested by Ho *et al*. (2008), Calvignac *et al*. (2009), or Davison *et al*. (2011), whereas the TMRCA for subclades 7a (Middle East–Iran/Turkey) and 7b (Middle East–divergent) is *c*. 50 000 years ago. These

latter two divergence dates fall within the early part of MIS 3. In contrast, the estimated time for the split into separate Turkish and Iranian populations within subclade 7a is *c*. 21 000 years ago. Similarly, the local (i.e. Anatolian and Caucasian) lineages of subclade 3a appear to have diverged into western, eastern, and northern local lineages during the period spanning 28 000–17 000 years ago (Fig. 1). These dates roughly coincide with the LGM, and are in agreement with the findings of Murtskhvaladze *et al*. (2010) for the bears of the Caucasus.

Pollen records during either period (i.e. 70 000–50 000 years ago and 20 000–18 000 years ago, respectively) indicate an extreme decline in oak (*Quercus* spp.), beech (*Fagus* spp.), and other woody taxa, whereas pollen from typical steppe flora (*Artemisia* spp., Graminae, and Chenopodiaceace) increase substantially over the same period, suggesting that treeless desert–steppe vegetation has become dominant during those periods in western Asia, whereas deciduous oak and beech could only be found in more favourable habitats (Allen *et al*., 1999; Wick, Lemcke & Sturm, 2003; Allen, 2009). Hard mast has been shown to be important in the diet of bears from temperate environments at lower latitudes (Bojarska & Selva, 2012). Therefore, it is likely that rapid vegetation change and a decline in mast-producing trees would affect *U. arctos* populations by restricting them to fragments of suitable habitat in southern Europe and western Asia, leading to lineage formation that created the distinct subclades of today.

The exclusive specificity of Turkish and Georgian haplotypes to their respective countries of origin is surprising, given the lack of any significant barriers, the presence of contiguous suitable habitat, and relatively dense sampling at both sites. Only a single subclade-3a specimen (GE-12) from Georgia clustered with neighboring Turkish samples (Fig. 2). Similarly, clade 7 has not been reported from Georgia or elsewhere in the Caucasus (Calvignac *et al*., 2009; Murtskhvaladze *et al*., 2010); however, strong female philopatry (Randi *et al*., 1994; Waits *et al*., 1998; Støen *et al*., 2005) and saturated populations (Ambarlı, 2006, 2012) impeding incursions from outside may explain the observed exclusivity. In the case of clade 7, this may also signify a recent entry into north-eastern Turkey from further south. Additional sampling from eastern Turkey, the Caucasus, and Iran is necessary to understand the exact distribution of this latter clade.

Validity of the 'Syrian bear'

Bears from the Middle East and the Caucasus have generally been considered to belong to a distinct taxon (*Ursus arctos syriacus* Hemprich & Ehrenberg, 1828), characterized by a small body, relatively small molars, and a 'blond' coat (Kurtén, 1968; Cowan, 1972; Pasitschniak-Arts, 1993; Chestin & Mikeshina, 1998; Garshelis & McLellan, 2011). On the other hand, based on either morphological or molecular evidence, several authors (e.g. Calvignac *et al*., 2009; Kitchener, 2010) have recently questioned the legitimacy of this taxon. There is no single, clear concept on the rank of subspecies (Haig *et al*., 2006), but some degree of geographical separation leading to reduced gene flow is usually considered necessary. Our study revealed that the so-called 'Syrian bears' in Turkey are made up of at least three divergent clades that are sometimes further divided into deep subclades (see Results). These separate lineages often occur in sympatry and lack any apparent correlation with particular morphological traits, such as pale coat colour or small size. Therefore, there appears to be neither a clear geographical separation nor evidence for isolating mechanisms between different genetic lineages, in line with the findings of Chestin & Mikeshina (1998) for the Caucasus.

Moreover, hunting records and recent fieldwork show that, unlike the accepted description for *U. a. syriacus*, adult bears in Turkey commonly weigh upwards of 150 kg, are up to 2 m in length, and often display dark coat coloration (Ambarlı, 2006; Ambarlı, Kuşdili & Bilgin, 2010). Therefore, even though the potential conservation benefits of distinct taxonomic names are recognized (Kitchener, 2010), there is simply not enough morphological or DNA evidence to delineate the bears of the region as a single distinct subspecies. Alternatively, the original description for the 'Syrian bear' may apply only to populations of *U. arctos* characterized by mitochondrial haplotypes of subclade 1d, now restricted to Syria, Lebanon, and southern Turkey.

There are a number of captive bears registered as 'Syrian bears' in the European Brown Bear Studbook, although not all have known origins to substantiate this label (D. van Bendegem, pers. comm.). Nevertheless, samples from some of those bears have been used in previous publications to represent *U. a. syriacus* in analyses (e.g. Calvignac *et al*., 2009). Through the mating of close relatives, apparently a widespread practice in the past in most zoos, many such samples have potentially the same maternal founder. A quick inquiry with zoo studbook keepers revealed that every single zoo sample in Calvignac *et al*. (2009) was maternally related within two generations to at least one other individual in the sample, bringing the effective sample size from seven down to three or four. Captive individuals can be useful, and sometimes provide the easiest option to obtain DNA, but ancestries need to be carefully checked to avoid redundancy in the analyses, and results that are largely based on specimens with unknown origins must be treated cautiously.

Conservation implications

It has been suggested that population declines during the Holocene in Europe or North Africa have led to a significant loss of genetic diversity, including the complete extinction of lineages (Valdiosera *et al*., 2007, 2008; Calvignac *et al*., 2008; Davison *et al*., 2011, but see Bray *et al*., 2013). The continued presence of diverse maternal lineages in Turkey implies that the bear populations here did not go through severe population bottlenecks historically. Nevertheless, *U. arctos* experienced severe persecution all over Turkey in the past, including poisoning in the Mediterranean region, although numbers now appear to have recovered, especially in the north and east of the country. A decline in human-caused mortality and disturbance following large-scale emigration of villagers from rural areas and the ban on bear hunting are probably the main reasons for this recovery (Ambarlı & Bilgin, 2008).

Populations in the south and west of the country are still precariously small and isolated, however. As these are the only extant populations known to represent clade 1 outside Europe, including the ancient lineage of subclade 1d, a better knowledge of their status and their effective conservation are of particular urgency. The Taurus Mountain populations are closely related to ancient Lebanese bears (Calvignac *et al*., 2009). Probably related bears from Syria were presumed extinct until recently, when tracks of an individual were observed in 2004 and 2011 (Hajjar, 2011); however, given the history of political instability in the region, the long-term viability of *U. arctos* there remains doubtful. At any rate, genetically related populations in southern Turkey may act as a source for reintroduction or augmentation, playing the same role that Slovenian and Croatian populations played for repopulating suitable sites in Italy, Austria, and France (Randi *et al*., 1994; Clark, Huber & Servheen, 2002).

The unexpected finding of a Middle Eastern (clade-7) haplotype in a Greek captive specimen needs to be further explored. Unfortunately, the origin of this specimen is totally obscure (L. Waits, pers. comm.; Y. Mertzanis, pers. comm.). Given the large geographical distance between northern Greece and Artvin, the locality of the only other specimen in this lineage, and the otherwise exclusively Asian nature of clade 7, it is likely that this captive specimen is not native to south-east Europe, but was imported from further east, perhaps as a 'dancing bear', and ended up in a zoo. A less likely but intriguing explanation is that it is of local origin and represents a remnant of the Middle Eastern lineage that might have extended all the way west into the Balkans in the past.

Finally, our study demonstrates the importance of sampling properly from the whole range of a species to best understand its diversity and phylogeography. Davison *et al*. (2011) report that modern mtDNA sequences have been characterized from less than half of the countries in which the species currently occurs. Moreover, sampling has so far largely focused on Europe and North America (Davison *et al*., 2011; Swenson *et al*., 2011). Until this study, the only published sample from Turkey belonged to two specimens from the extreme north-east of the country that represented a single haplotype of the subclade-3a lineage (Talbot & Shields, 1996). Despite its obvious unrepresentative nature, both in a geographical and a statistical sense, this finding had until now been used to represent the whole of Turkey (e.g. Miller *et al*., 2006; Calvignac *et al*., 2009; Davison *et al*., 2011). Even with limited sampling, our study changes this picture considerably, by adding two previously unreported major clades for the country, as well as revealing some regional structure within the subclade-3a lineage. Additional sampling and analysis of nuclear DNA variation would further improve our understanding of *U. arctos* diversity and help identify appropriate units for conservation and management.

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