Brown and Polar Bear Y Chromosomes Reveal Extensive Male-Biased Gene Flow within Brother Lineages

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Abstract

Brown and polar bears have become prominent examples in phylogeography, but previous phylogeographic studies relied largely on maternally inherited mitochondrial DNA (mtDNA) or were geographically restricted. The male-specific Y chromosome, a natural counterpart to mtDNA, has remained underexplored. Although this paternally inherited chromosome is indispensable for comprehensive analyses of phylogeographic patterns, technical difficulties and low variability have hampered its application in most mammals. We developed 13 novel Y-chromosomal sequence and microsatellite markers from the polar bear genome and screened these in a broad geographic sample of 130 brown and polar bears. We also analyzed a 390-kb-long Y-chromosomal scaffold using sequencing data from published male ursine genomes. Y chromosome evidence support the emerging understanding that brown and polar bears started to diverge no later than the Middle Pleistocene. Contrary to mtDNA patterns, we found 1) brown and polar bears to be reciprocally monophyletic sister (or rather brother) lineages, without signals of introgression, 2) male-biased gene flow across continents and on phylogeographic time scales, and 3) male dispersal that links the Alaskan ABC islands population to mainland brown bears. Due to female philopatry, mtDNA provides a highly structured estimate of population differentiation, while male-biased gene flow is a homogenizing force for nuclear genetic variation. Our findings highlight the importance of analyzing both maternally and paternally inherited loci for a comprehensive view of phylogeographic history, and that mtDNA-based phylogeographic studies of many mammals should be reevaluated. Recent advances in sequencing technology render the analysis of Y-chromosomal variation feasible, even in nonmodel organisms.

Key words: **Y chromosome, phylogeography, bears, introgression, SNP, microsatellite.**

Introduction

Phylogeography describes the origin of genetic variation among closely related lineages, tracing the geographic distribution of genetic variation through time and space (Avise 2000; Hewitt 2000). Historically, phylogenetic and phylogeographic research has relied heavily on mitochondrial DNA (mtDNA), with the brown bear (*Ursus arctos*) as an extensively studied example (Taberlet et al. 1998; Purvis 2005; Davison et al. 2011). Advantages of analyzing mtDNA include its high mutation rate, availability of markers, high copy number, lack of recombination, and its haploid nature. However, the typically maternal inheritance of mtDNA implies that signatures of male-mediated dispersal cannot be detected. An approach to further investigate phylogeographic patterns is to analyze independently and biparentally inherited autosomal loci in a multilocus framework. However, recombination hampers inferences of haplotypes over long genomic regions, limiting the resolution that is available from individual autosomal loci.

The only other haploid fraction of the mammalian genome is the male-specific Y chromosome. Due to its lack of recombination, except for the small pseudoautosomal regions, haplotypes can be inferred over extended genomic regions, providing a high-resolution view of patrilineal evolutionary history. Also, both mtDNA and the Y chromosome exhibit faster lineage sorting than nuclear loci, facilitating the detection of population structuring (Avise 2000). The male-specific section of the Y chromosome therefore provides an essential complement to data from maternally inherited mtDNA and biparentally inherited loci, giving insight into the history of uniquely male-inherited lineages. Y-linked variation allows the detection of potentially contrasting patterns of male and female gene flow (Chan et al. 2012). This is particularly relevant in many mammals, where males typically disperse much

farther than females (Pusey 1987). Along with other loci, Y-linked variation has therefore provided a backbone for our understanding of phylogeography in humans (Hughes and Rozen 2012; Wei et al. 2013), canids (Brown et al. 2011; Sacks et al. 2013), and domesticated animals (Meadows et al. 2006; Lippold et al. 2011).

Despite these qualities, very little data is available from mammalian nonprimate Y chromosomes, in part because it has been disregarded from many genome sequencing projects due to its repetitive nature (Willard 2003). In addition, other technical challenges, such as avoiding co-amplification of homologous X-chromosomal regions, have hampered the analysis of paternally inherited markers in natural populations (Greminger et al. 2010). The Y chromosome thus represents an understudied part of the mammalian genome, with a large potential to add valuable information to our understanding of phylogeography. In the era of genomics, it is now feasible to identify large regions on the Y chromosome and develop male-specific markers for studies of evolutionary history.

Brown and polar (*U. maritimus*) bears have been model species in phylogeography since the early 1990s (Cronin et al. 1991; Taberlet and Bouvet 1994; Kohn et al. 1995; Paetkau et al. 1998; Taberlet et al. 1998; Hewitt 2000; Waits et al. 2000), in part because these species are widely dispersing and provide the advantage of being distributed over large parts of the Northern hemisphere. Polar bears exhibit low levels of population differentiation at biparentally inherited and mitochondrial markers throughout their range (Paetkau et al. 1999; Cronin and MacNeil 2012; Miller et al. 2012; Campagna et al. 2013). Brown bears, in contrast, show considerable phylogeographic structuring at mitochondrial markers (Davison et al. 2011; Edwards et al. 2011; Hirata et al. 2013; Keis et al. 2013), and population structuring can also be discerned at biparentally inherited microsatellites (Paetkau et al. 1997; Tammeleht et al. 2010; Kopatz et al. 2012). Most mtDNA clades are confined to certain geographical regions and are not shared between continents, although one brown bear clade is widespread throughout Eurasia and extends into North America (Korsten et al. 2009; Davison et al. 2011). Surprisingly, all range-wide phylogeographic studies on brown bears have so far relied on mtDNA. Studies of autosomal markers were regionally restricted to either North America or Eurasia (Paetkau et al. 1997; Tammeleht et al. 2010; Kopatz et al. 2012; Cahill et al. 2013), and no phylogeographic study of Y chromosome markers in bears exists. However, analysis of male-specific markers is crucial to understand bear evolution in the light of their well-documented male-biased dispersal (McLellan and Hovey 2001; Zedrosser et al. 2007).

With regard to bear phylogeny, reliance on mtDNA alone has proven problematic. Polar bear mtDNA sequences are nested within the genetic diversity of brown bears, resulting in a paraphyletic matrilineal relationship (Cronin et al. 1991; Lindqvist et al. 2010). Although mtDNA is expected to attain reciprocal monophyly faster than nuclear loci (Avise 2000), recent studies utilizing autosomal markers have shown that extant brown and polar bears comprise distinct sister lineages at the species tree level, and that their divergence occurred earlier than previously estimated (Hailer et al. 2012; Miller et al. 2012; Cahill et al. 2013). Therefore, brown bear paraphyly for mtDNA is likely a consequence of past introgressive hybridization with polar bears (Edwards et al. 2011; Hailer et al. 2012; Miller et al. 2012; Hailer et al. 2013).

We mined a recently sequenced polar bear genome and developed 13 male-specific markers to sequence 5.3 kb of the Y chromosome and to analyze microsatellite variation in a broad geographic sample of 130 brown and polar bears from across Europe, Asia, and North America. We also analyzed a 390-kb-long genomic Y-chromosomal scaffold in available brown, polar, and American black bear genomes. These data allowed us to investigate 1) whether introgression between brown and polar bears can be detected at Y chromosome markers, 2) whether the male lineage shows less geographic structuring than the maternal lineage, and 3) the relative intraspecific clade depth of mtDNA and the Y chromosome.

Results

Y Chromosome Phylogeny and Lack of Introgression Signals

Male-specific sequence data revealed that brown and polar bears carry differentiated, species-specific Y chromosomes, each exhibiting a closely related group of haplotypes (fig. 2*A*). The clear separation and reciprocal monophyly of brown, polar, and American black bear (*U. americanus*) Y chromosomes was further supported by Bayesian phylogenetic analyses (fig. 2*B*), with high statistical support ($P > 0.95$) for all major nodes.

In 3,078 bp of Y chromosome sequence analyzed in 90 brown, 40 polar, and 4 black bears (fig. 1 and table 1), we found over 75% of the variable sites among species (3.1-kb data set, solid lines in fig. 2*A*). Only a small portion of sequence polymorphism was intraspecific. We encountered eight haplotypes, five within brown, two within polar, and one within black bears. These haplotypes were defined by a total of 21 segregating sites, 10 of which discriminate between brown and polar bears, 9 between brown and black bears, and 13 between polar and black bears. Brown and polar bears each showed one abundant haplotype that was dominant in all populations across their ranges. Haplotype BR1.1 was found in 94% of brown bears and PO1.1 in 90% of polar bears (fig. 2*A*). Two haplotypes found in brown bears from the ABC islands (BR5) and the Alaskan mainland (BR4) formed a joint lineage, indicative of a geographically informative clustering. Additional rare haplotypes in brown bears were found in two individuals from Kamchatka (BR2) and in one individual from the Ural Mountains (BR3). In polar bears, the rare haplotype PO2 was found in three individuals from Alaska and in one from Western Greenland (fig. 1). Results for four black bear males are described in the supplementary material, Supplementary Material online.

Increasing sequence length by ~70% (adding 2,216 bp, 5.3-kb data set, dotted lines in fig. 2*A*) for 63 individuals chosen to represent most populations (supplementary table S1, Supplementary Material online) increased the resolution

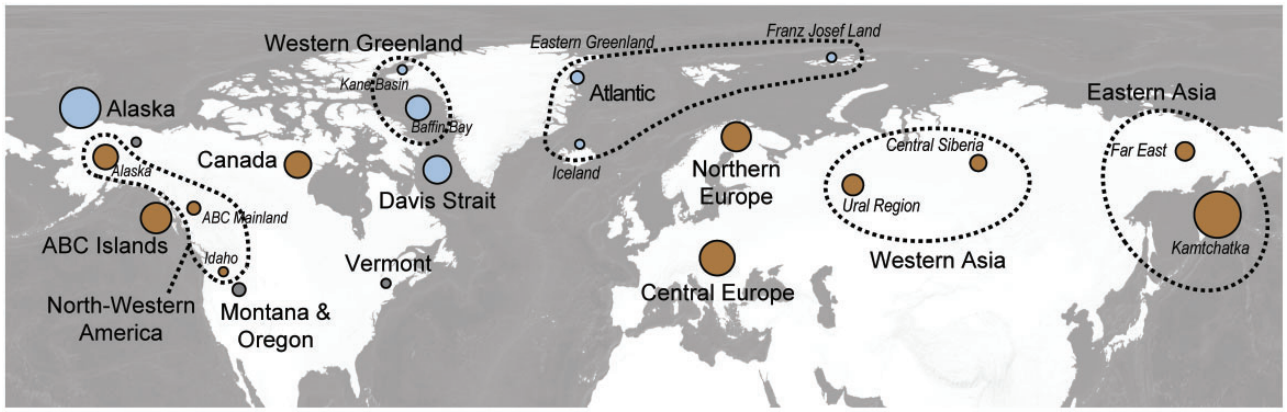

FIG. 1. Geographical distribution of analyzed bear samples. Circle area is proportional to the number of individuals. Some sampling localities (italics) were combined into groups (see table 1). Brown: brown bears; blue: polar bears; black: black bears.

Table 1. Sample Size (*n*), Number of Haplotypes (H), and Haplotype Diversity (HD) Based on the Combination of 3.1-kb Y-Chromosomal Sequence and Six Microsatellites.

Species and Population (abbreviation)	*n*	*H*	HD
Brown bear	**90**	**41**[a]	**0.96 ± 0.01**
Central Europe (C-EU)	**14**	**8**	**0.89 ± 0.06**
Northern Europe (N-EU)	**10**	**4**	**0.73 ± 0.12**
Western Asia (W-AS)	**8**	**7**	**0.96 ± 0.08**
Ural Region	**5**	**5**	
Central Siberia	**3**	**2**	
East Asia (E-AS)	**29**	**12**	**0.84 ± 0.05**
Far East	**4**	**4**	
Kamchatka	**25**	**9**	
North-West America (NW-A)	**10**	**6**	**0.84 ± 0.10**
Alaska	**7**	**4**	
ABC Mainland	**2**	**1**	
North-Western USA/Idaho	**1**	**1**	
ABC islands (ABC)	**11**	**5**	**0.82 ± 0.08**
Canada (CAN)	**8**	**2**	**0.25 ± 0.18**
Polar bear	**40**	**17**[a]	**0.83 ± 0.06**
Atlantic (ATL)	**4**	**3**	**0.83 ± 0.22**
Eastern Greenland	**2**	**1**	
Iceland	**1**	**1**	
Franz Josef Land	**1**	**1**	
Alaska (AK)	**19**	**7**	**0.72 ± 0.10**
Western Greenland (W-GR)	**8**	**5**	**0.79 ± 0.15**
Baffin Bay	**7**	**4**	
Kane Basin	**1**	**1**	
Davis Strait (DS)	**9**	**6**	**0.89 ± 0.09**
Black bear	**4**	**4**	**1.00 ± 0.18**
Alaska zoo, Oregon, Montana, Vermont	**4**	**4**	

[a]Sum of haplotypes across populations is larger than the number of haplotypes per species, due to haplotype sharing.

among species and revealed additional, rare haplotypes in brown bears (BR1.2, BR1.3), polar bears (PO1.2), and black bears (BL2). The general patterns were not substantially changed compared with the 3.1-kb data set, and still one single haplotype remained dominant across the distribution ranges in each species (BR1.1/PO1.1). Reflecting the few polymorphic sites found within species, nucleotide diversity ($\pi \pm SD$) was low in brown (0.00007 ± 0.00002) and polar bears (0.00003 ± 0.00002) (table 2).

Using a Bayesian approach, we estimated the timing of the split between brown and polar bear male lineages ($T_{MRCA\ (B/P)}$). This was based on 5,197 bp of Y-chromosomal sequence using the spectacled bear (*Tremarctos ornatus*) as outgroup. Assuming 6 Ma for the split from the spectacled bear (a calibration based on the fossil record; Wayne et al. 1991), we estimated a $T_{MRCA\ (B/P)}$ of ~1.12 Ma (fig. 2*B*). We also constrained the analysis to a pedigree based Y-specific mutation rate (3.0×10^{-8}/site/generation [Xue et al. 2009], rendering 3.0×10^{-9}/site/year with a generation time estimate for bears of 10 years) and obtained estimates of $T_{MRCA\ (B/P)}$ of ~0.43 Ma (supplementary table S2, Supplementary Material online). The absolute timing of the split, therefore, depended strongly on the calibration prior (i.e., divergence time of the outgroup or substitution rate). Additional calibration scenarios from previous studies are examined in the supplementary material, Supplementary Material online. Our data consistently recovered the brown/polar bear split to be ~80% of the age of the older split from the black bear lineage, indicating that the divergences among different ursine species occurred relatively shortly after each other. We note, however, that the design of our Y sequence fragments targeted regions exhibiting nucleotide differences between one polar and one brown bear individual, which could lead to an upward ascertainment bias with regard to the magnitude of the brown/polar bear divergence (discussed later). Nevertheless, all variable sites on the black bear branch (fig. 2*A*) were newly discovered in our sequencing data, confirming the divergence of the black bear lineage with respect to brown and polar bears.

The findings of species-specific groups of haplotypes and the lack of haplotype sharing among species (fig. 2*A*) revealed no signal of recent Y-chromosomal introgression. In contrast, analysis of a 642-bp fragment of the mtDNA control region of the same samples showed polar bears nested within the variation of all brown bears (fig. 2*C*), as expected for this locus.

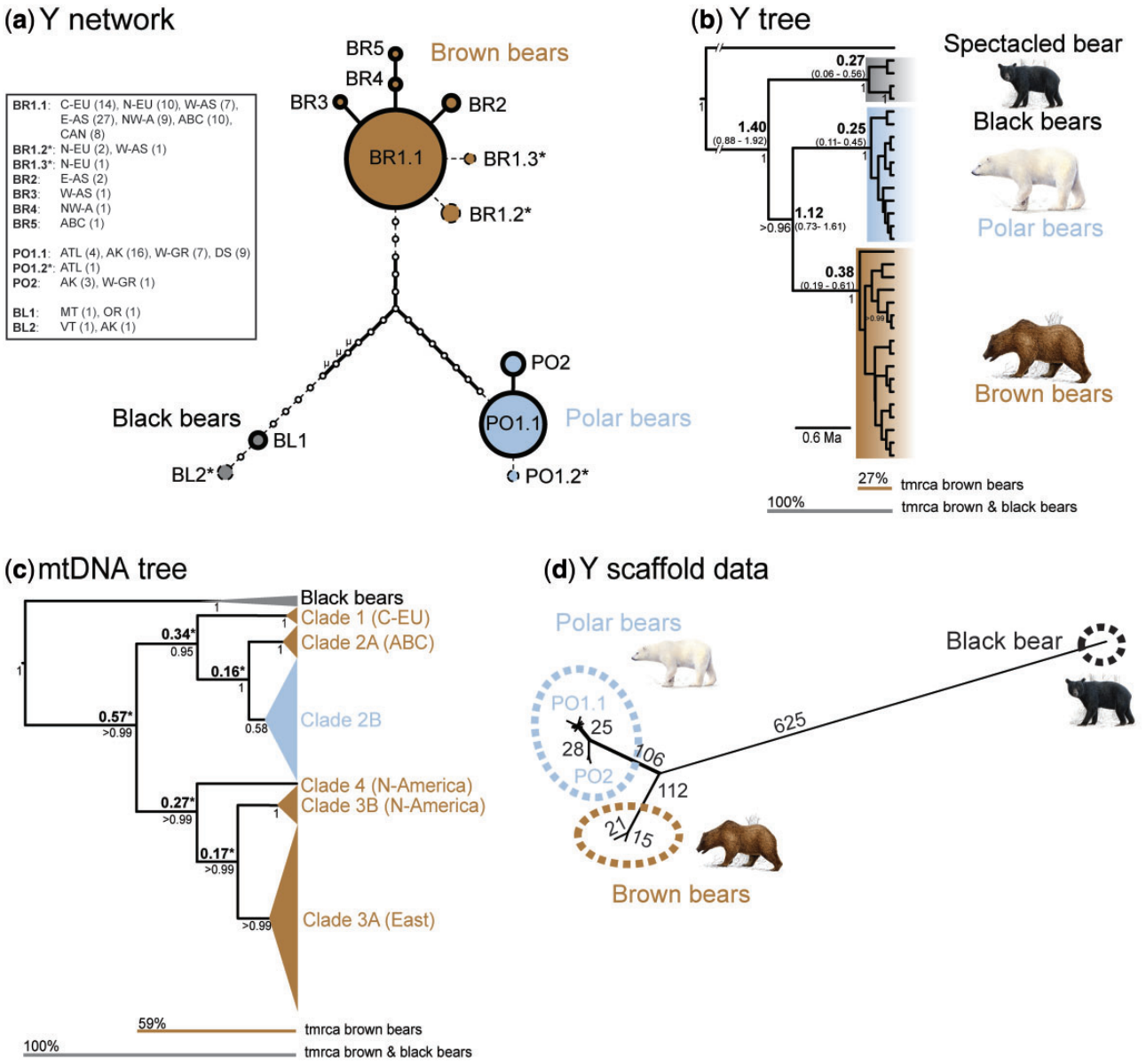

FIG. 2. Phylogenetic relationships of bears for Y-chromosomal and mitochondrial markers. (*A*) Parsimony network of Y chromosome sequences. Solid lines: variation in 3.1 kb; dashed lines: variation from additional 2.2 kb (total: 5.3 kb). Circle area is proportional to number of individuals; small, open circles: inferred, intermediate haplotypes; lines represent single mutational steps. Inset boxes: number of individuals per population. Asterisks: haplotypes found only in the 5.3-kb data set (individuals with these haplotypes have the respective common haplotypes in the 3.1-kb data set). Insertions/deletions of repeat units in microsatellite-like regions counted as number of repeat unit changes (μ). Population abbreviations as in table 1. (*B*) Maximum clade credibility tree of Y chromosomal sequence (5,197 bp), based on a divergence of the spectacled bear 6 Ma. Bold: median divergence in Ma (95% highest posterior density intervals in brackets). Numbers below nodes: posterior support >0.95. (*C*) Maximum clade credibility tree of mtDNA control region data. Sampling covers all major matrilineal brown bear clades (Davison et al. 2011) (collapsed into triangles), and polar bears (clade 2B) are nested within brown bear variation. Asterisks: divergence times obtained from complete mtDNA sequences (Hirata et al. 2013). Numbers below nodes: posterior probabilities. Below (*B*) and (*C*), brown bear clade depth (relative to the divergence from black bears) is indicated. (*D*) NeighborNet network based on a ~390 kb Y-chromosomal fragment from 12 polar bears, 2 brown bears, and 1 black bear. Numbers on branches denote numbers of variable sites. Within polar bears, two haplogroups were identified corresponding to the haplotypes PO1.1 and PO2 in figure 2*A*.

Y Chromosome Phylogeography of Bears

On the Y chromosome, we found a maximum of three variable sites separating different brown bear haplotypes (e.g., the difference between BR3 and BR5), but 14 substitutions between brown and black bears (5.3-kb dataset, not counting sites in microsatellite-like regions; see μ in fig. 2*A*). The intraspecific divergences relative to the outgroup obtained from Bayesian analyses amounted to 27% for the Y-chromosomal data and 59% for mtDNA control region data (fig. 2*B* and *C*). Similarly, estimates of mean (± SE) among-group genetic distances from mtDNA control region sequences showed that divergence between two major brown bear

Table 2. Summary Statistics Based on 5.3-kb Y-Chromosomal Sequence.

Species	*n*	*H*	f_H	*S*	$\pi \pm$ SD ($\times 10^{-4}$)	θ_W ($\times 10^{-4}$)	Tajima's *D*	*D**	*F**	F_S
Brown bear	44	6[a]	0.84	6	0.7 ± 0.2	2.6 ± 1.3	−1.94[b]	−3.01[b]	−3.13[b]	−4.659[b]
Polar bear	15	2[a]	0.93	1	0.3 ± 0.2	0.6 ± 0.6	−1.16	−1.42	−1.52	−0.649

NOTE.—Sample size (*n*), number of haplotypes (*H*), the frequency of the dominant haplotype (f_H), number of segregating sites (*S*), nucleotide diversity (π), Watterson's θ_W (per site), Tajima's *D*, Fu and Li's *D** and *F**, and Fu's F_S are given.

[a]Individuals with haplotypes BR4 and PO2 (fig. 2*A*) were only represented in the 3.1-kb data set (supplementary table S6, Supplementary Material online), hence these haplotypes are not counted here.

[b]$P < 0.05$.

mtDNA clades (1 and 3a) (0.036 ± 0.007) amounted to 57–60% of the mean distance between brown and black bears (0.064 ± 0.009 for clade 1, and 0.061 ± 0.009 for clade 3a). Thus, a considerable reduction in phylogeographic structuring of the patriline was detected in comparison to the established matrilineal pattern, where deeply separated mtDNA clades, most of which are region-specific, are found within brown bears.

This discrepancy in clade depth between the matri- and patriline was also obvious when analyzing a ~390-kb Y-chromosomal scaffold (scaffold number 297) from 14 published male bear genomes (Miller et al. 2012), along with the corresponding sequence from a male brown bear from northern Norway (supplementary table S3, Supplementary Material online). This alignment of 2 brown, 12 polar bears, and 1 black bear identified >1,000 high-quality variable sites, most of them distinguishing between the three bear species (fig. 2*D*). In this data set, the divergence between the two brown bear individuals (one from Norway and one from the ABC islands) was ~5% of the divergence of these to one black bear individual (36 substitutions between the two brown bears, 752–758 substitutions between brown and black bears), compared with ~20% between the divergence of all brown bears from the black bear based on whole mitochondrial sequences (Lindqvist et al. 2010).

The shallow clade depth on the brown bear Y chromosome could result from population expansion of one Y lineage that has replaced other clades. The pattern is also consistent with positive selection favoring a particular Y variant, and male-mediated gene flow spreading this variant across the range. To disentangle the effects of background selection, genetic hitchhiking, and recent population growth, we calculated four summary statistics to test for deviations from neutral expectations. In brown bears, all estimates were significant and negative (Tajima's $D = -1.94$, $P < 0.01$; Fu and Li's $D^* = -3.01$, $P < 0.05$; $F^* = -3.13$, $P < 0.05$; Fu's $F_S = -4.659$, $P < 0.01$; table 2), consistent with all three selective/demographic processes. The values calculated for polar bears were not significantly different from neutral expectations (Tajima's $D = -1.16$, $P > 0.1$; Fu and Li's $D^* = -1.42$, $P > 0.05$; $F^* = -1.52$, $P > 0.05$, Fu's $F_S = -0.649$, $P > 0.1$; table 2). Haplotype configuration tests (Innan et al. 2005) did not allow us to distinguish between signals of population stasis ($g = 0$), population growth ($g = 2$, $g = 10$), or selection in brown bears, because no tested scenario differed significantly from neutral expectations (cumulative $P > 0.05$ for all tests).

In addition to sequence data, we developed and analyzed six faster evolving male-specific microsatellites to obtain a high-resolution data set (fig. 3 and supplementary figs. S1–S4, Supplementary Material online). Although the overall Y-chromosomal haplotypic variability was high (table 1) and we observed a ratio of haplotypes to individuals of >40%, branches between haplotypes were short and defined by few mutational steps (fig. 3 and supplementary material, Supplementary Material online). Except for a group of three haplotypes found in Central European brown bears (fig. 1), and a group of 13 brown bears from eastern Asia (Kamchatka) exhibiting five differentiated haplotypes, all populations contained haplotypes that were distributed across the network (fig. 3*A*).

In polar bears, male-specific sequence data showed few rare mutations (fig. 2*A*), and even when combined with microsatellites, one haplotype was found to be abundant across much of the range (fig. 3*B*). From analysis of molecular variance (AMOVA), we obtained estimates of the proportion of variation among all populations of 0.28 for brown and 0.16 for polar bears (supplementary tables S4 and S5, Supplementary Material online). This is consistent with results from autosomal microsatellite markers which show stronger population differentiation in brown than in polar bears (Cronin and MacNeil 2012).

ABC Islands Brown Bears—Evidence for Male-Mediated Gene Flow from the Mainland

The Alaskan ABC islands are inhabited by brown bears that are unique in the close relatedness of their maternal lineage to polar bears. All polar and ABC islands brown bear samples included in our study show this expected relationship (fig. 2*C*). For the Y chromosome, we found five haplotypes among 11 ABC islands brown bears (fig. 3*A*), all clustering with brown rather than polar bears (fig. 2*A*). One haplotype was shared with individuals from Canada and another with individuals from northwest America and western Asia (fig. 1). Nonsignificant differentiation from brown bears on the adjacent North American mainland (ABC/NW-A: $\Phi_{ST} = 0.02$, $P > 0.05$; supplementary table S4, Supplementary Material online), but significant differentiation from all other populations further confirmed the connectivity by male-mediated gene flow. This gene flow is evidently substantial enough to maintain a high level of variability on the ABC islands: we found five haplotypes in 11 ABC islands individuals

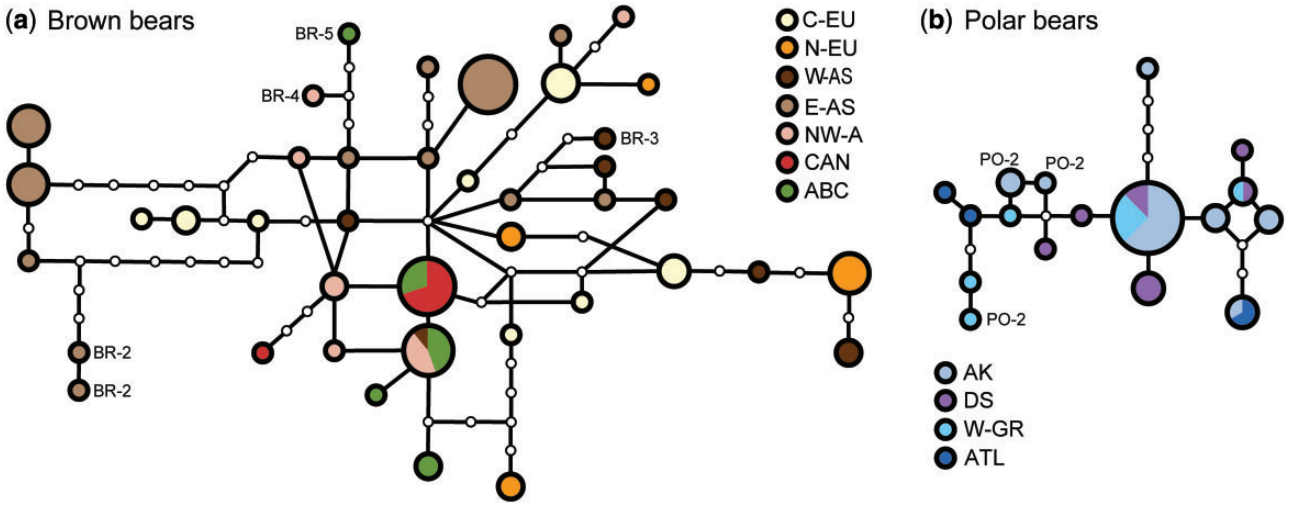

FIG. 3. Statistical parsimony networks of Y chromosome haplotypes, inferred from unweighted combination of 3.1-kb sequence data and six microsatellites, for (*A*) brown bears and (*B*) polar bears. Rare haplotype names as in figure 2*A*, population abbreviations as in table 1.

(haplotype diversity HD = 0.82; table 1), which is similarly high as the variability of all brown bears combined (HD = 0.96; table 1).

Discussion

Phylogeographic research has relied heavily on maternally inherited mtDNA, but male-biased dispersal in many mammals implies that mtDNA provides a highly structured (philopatric) estimate of population differentiation compared with paternally and biparentally inherited loci. Modern sequencing techniques now allow the generation of extensive genomic data, enabling large-scale identification and analysis of sequences from the male-specific Y chromosome (Bachtrog et al. 2011; Wei et al. 2013). This chromosome is especially interesting for evolutionary studies because it allows the inference of high-resolution haplotypes from long sequences, avoiding analytical challenges posed by interchromosomal recombination. Our analysis of newly developed Y-linked markers in comparison to results from maternally inherited mtDNA revealed a large impact of sex-biased gene flow on phylogeographic structuring and enabled us to examine phylogeny and introgression in brown and polar bears.

Speciation and Introgression

The Y chromosome phylogeny of brown and polar bear lineages resembles the topology of species trees reconstructed from biparentally inherited autosomal markers (Hailer et al. 2012; Miller et al. 2012; Cronin et al. 2013), where the species constitute distinct sister (or rather brother) lineages, with black bears clustering outside their variation (fig. 2*B*). This contrasts with the pattern obtained from maternally inherited mtDNA, where polar bears cluster within the variation of brown bears, rendering the latter paraphyletic (Cronin et al. 1991; Edwards et al. 2011) (fig. 2*C*).

The timing of the split between brown and polar bears has been the subject of recent debates, with inferred dates ranging from ~160,000 to ~5 million years (Lindqvist et al. 2010; Edwards et al. 2011; Hailer et al. 2012; Miller et al. 2012; Cahill et al. 2013; but see Ho et al. 2008 and Davison et al. 2011 for even younger estimates depending on the calibration method used). Compared with the mtDNA divergence estimate of ~160,000 years between polar and brown bears (Lindqvist et al. 2010; Edwards et al. 2011; Hirata et al. 2013), divergence times for the Y chromosome (>0.43 Ma, supplementary table S2, Supplementary Material online) are much older, confirming earlier suggestions that mtDNA has been introgressed (Hailer et al. 2012, 2013; Miller et al. 2012; Cahill et al. 2013). Compared with divergence times estimated from autosomal data, our 1.12 Ma estimate for brown/polar bear Y chromosomes (fig. 2*B*; scenario B in supplementary table S2, Supplementary Material online) is older than a divergence time estimate from introns of ~0.34-0.93 Ma (Hailer et al. 2012), but younger than the 4–5 Ma estimate by Miller et al. (2012) from genomic data. When based on a rate calibration from human Y chromosomes (scenario D in supplementary table S2, Supplementary Material online), our estimate of the Y chromosome divergence (0.43 Ma) falls into the Middle Pleistocene, resembling the estimate of Hailer et al. (2012). In summary, Y chromosome evidence support the emerging understanding of brown and polar bears as distinct evolutionary lineages that started to diverge no later than the Middle Pleistocene, at least several hundreds of thousands years ago.

Although incomplete lineage sorting can hamper definite conclusions, brown and polar bears likely carry introgressed alleles at mtDNA and autosomal loci (Hailer et al. 2012; Miller et al. 2012; Cahill et al. 2013). Current hybridization levels, however, appear to be low (Cronin and MacNeil 2012; Hailer et al. 2012). Our findings of species-specific groups of Y chromosome haplotypes and a lack of haplotype sharing among species revealed no signal of patrilineal introgression. Reduced introgression of Y chromosomes has been reported previously (e.g., Geraldes et al. 2008) and can arise from several mechanisms: random effects of lineage sorting, sex-biased hybridization, reduced hybrid fitness of the heterogametic sex

due to genomic incompatibilities (Haldane's rule), or lower introgression rates at markers exhibiting high intraspecific gene flow (Petit and Excoffier 2009).

Variability on the Y Chromosome

Most variable sites on the Y chromosome in bears were found among species, while only relatively little intraspecific sequence variation was encountered. The latter is compatible with the generally low intraspecific variability observed on mammalian Y chromosomes, including field voles, elephants, chamois, and humans (Hellborg and Ellegren 2004; Roca et al. 2005; Pérez et al. 2011; Wilson Sayres et al. 2014). Nakagome et al. (2008) compared Y, X, and mtDNA phylogenies and variability in bears based on single representations per species. They found a lower than expected Y-chromosomal substitution rate within *Ursinae* as compared with the deeper nodes of the tree, possibly mirroring our findings of low variability on the Y chromosomes of brown and polar bears. After applying a standard correction factor of four to account for the smaller effective population size of the Y chromosome (but see Chesser and Baker 1996), variability on the brown bear Y chromosome was ~10% of that on the autosomes (data from Hailer et al. 2012). As shown for other mammals (Hellborg and Ellegren 2004), this discrepancy between the Y chromosome and autosomes exists despite higher male than female mutation rates. Low intraspecific variability on the Y chromosome can be explained by its haploid and uniparental inheritance, reproductive skew among males, male-biased dispersal, demographic history, but also by selection or a combination of these (Chesser and Baker 1996; Charlesworth and Charlesworth 2000; Petit et al. 2002; Wilson Sayres et al. 2014).

In polar bears, Y-linked variability patterns did not deviate significantly from neutral expectations (table 2). In brown bears, the deviation was significant, with most of the applied tests showing an excess of rare mutations (table 2), consistent with population growth and/or positive selection. However, haplotype configuration tests did not necessitate a history of ongoing or recent positive selection on the Y chromosome in brown bears. Based on SNPs from the nuclear genome, Miller et al. (2012) found a long-term decline in brown bear effective population size, particularly since the Eemian interglacial. Genome-wide data thus do not indicate recent population growth, reinforcing the particular evolutionary history of the Y chromosome in brown bears.

Despite overall low levels of intraspecific variation on the Y chromosome, our analysis of long scaffold sequences (fig. 2*D*) illustrates that application of modern genomic techniques can nevertheless recover large numbers of polymorphic sites on the Y chromosome, enabling high-resolution inferences.

Phylogeographic Structuring

mtDNA control region data show pronounced phylogeographic structuring in brown bears, with 1) deeply separated clades and 2) clades which are geographically restricted (Davison et al. 2011) (fig. 2*C*). The Y chromosome is predicted to be a geographically informative marker that shows differences among populations, because of strong genetic drift in the patriline (Petit et al. 2002). However, we observed neither of the abovementioned signals at paternally inherited markers: no deep intraspecific divergences were found, and, over evolutionary time scales, male-biased gene flow has distributed genomic variation across and among continents. Compared with mitochondrial control region data, brown bear Y chromosomes showed shallow intraspecific divergences relative to the divergence from black bears, with few substitutions differentiating among Y-chromosomal haplotypes. Despite limited sample numbers, because to date only few male bear genomes have been sequenced, ascertainment bias-free scaffold data confirm the main conclusions from our sequence data. First, patrilineal genomic divergences within brown and polar bears were considerably shallower than for mtDNA. Second, the 390-kb data set recovered the same two groups of polar bear Y haplotypes that correspond to PO1.1 and PO2. Finally, brown bear sequences were separated from each other by small genetic distances. Although increased sampling and sequencing of longer fragments might recover additional clades, our conclusions are not impacted by a strong ascertainment bias (Brumfield et al. 2003). On deeper phylogenetic scales, however, we note that the divergence of the black bear Y chromosome was likely underestimated in our 3.1- and 5.3-kb data sets.

The observed discrepancy between the matri- and patriline can be due to effects of demography and selection on the Y chromosome. In addition, mtDNA can show signals of mutational saturation (Ingman and Gyllensten 2001) and purging of slightly deleterious mutations due to purifying selection (Subramanian et al. 2009), leading to a time dependency of evolutionary rates for mtDNA (Ho et al. 2008). Whole mtDNA data from Lindqvist et al. (2010) show, relative to the divergence from black bears, a shallower clade depth in brown bears compared with data from the control region. However, our analysis of longer sequences from Y scaffold data confirmed the weaker structuring of the patriline than the matriline. Whichever the mechanism(s), a reduced phylogeographic structuring on the Y compared with well differentiated mtDNA clades has also been found in other species, for example, shrews, chamois, and gibbons (Lawson Handley et al. 2006; Pérez et al. 2011; Chan et al. 2012).

Despite known uncertainties with regard to absolute ages, our Bayesian phylogenetic analyses suggested that the most basal divergence of brown bear Y haplotypes considerably predates the last glacial maximum, with plausible dates reaching into the Middle Pleistocene (95% highest posterior density: 0.19–0.61 Ma; fig. 2*B*). This suggests that one Y chromosome lineage (BR1.1) has been maintained for a long time and at a high frequency throughout Eurasia and North America. While selection may therefore have contributed to the shallow Y-chromosomal clade depth within brown bears, our data are also consistent with a purely demographic scenario, involving extensive male gene flow across large geographical distances. Indeed, analysis of a

390-kb-long Y-chromosomal fragment showed that two brown bears from populations as far away from each other as Norway and the Alaskan ABC islands carried highly similar Y chromosomes (fig. 2*D*). This pattern in brown bears covers even larger geographic areas (throughout Eurasia and North America) than analogous findings from humans, where the Y-chromosomal lineage of Genghis Khan, founder of the Mongol Empire, was spread across much of Asia (Zerjal et al. 2003).

Our discovery of distinct Y-chromosomal haplotypes on Kamchatka mirrors previous findings of distinct mtDNA lineages (Korsten et al. 2009), highlighting the complex biogeography of this peninsula. Besides this clear signal from Kamchatka, brown bear populations in general contained a mix of different Y chromosome lineages, with the most closely related lineages of a given haplotype being located in a different geographic region. This lack of pronounced patrilineal geographic structuring is an expected consequence of male-mediated gene flow and contrasts strongly with the picture from mtDNA, where populations tend to contain region-specific lineages (Davison et al. 2011).

In polar bears, we observed weak population structuring and no clear evidence of past phylogeographic barriers on the Y chromosome. This is similar to patterns from maternally and biparentally inherited markers (Paetkau et al. 1999; Cronin et al. 2006; Miller et al. 2012; Campagna et al. 2013), reflecting the large dispersal distances described for polar bears.

Male-Biased Gene Flow and the Alaskan ABC Islands Bears

We provide the first direct evidence for male-mediated gene flow between the mainland and the Alaskan ABC islands, which host a population of bears that has long been of interest to evolutionary biologists, due to the close matrilineal relationship to extant polar bears—the extant polar bear matriline is the sister lineage of the ABC clade (Cronin et al. 1991; Davison et al. 2011). The absence of mainland brown bear mtDNA haplotypes on the ABC islands, and vice versa, shows that female-mediated gene flow is effectively zero. However, nuclear microsatellites (Paetkau et al. 1998) and comparisons of autosomal versus X chromosome variation (Cahill et al. 2013) demonstrated that ABC bears are not isolated from continental brown bear populations, postulating that connectivity between the ABC islands and the mainland stems from male-mediated gene flow. We here show that male-mediated gene flow is connecting the ABC islands to the North American mainland, and that this gene flow is substantial enough to maintain appreciable genetic variability in this island population. Cahill et al. (2013) suggested an initial polar bear ancestry of ABC islands brown bears, followed by extensive male-biased immigration of mainland brown bears. Based on this scenario, the fact that we found no polar bear Y chromosomes on the ABC islands indicates a replacement of the original polar bear Y chromosomes.

Phylogeography: Insights from Matri- and Patrilineal Markers

Since its conception, the field of phylogeography has realized the importance of sampling several statistically independent loci (reviewed in Avise 2000), but problems related to discovering intraspecific variability on the Y chromosome (Hellborg and Ellegren 2004; Luo et al. 2007) have long hampered the application of patrilineal markers in nonmodel species. Nevertheless, some studies have revealed similar paternal and maternal structuring (Hellborg et al. 2005), while others recovered discordant signals (Boissinot and Boursot 1997; Roca et al. 2005; Pidancier et al. 2006; Pérez et al. 2011). Inference of the mechanism(s) that could have led to differences in genetic structuring between the matri- and patriline is generally not straightforward, because the effects of demography and selection are difficult to disentangle (Lawson Handley et al. 2006; Pidancier et al. 2006; Nakagome et al. 2008; Pérez et al. 2011), even in humans (Wilson Sayres et al. 2014). Regardless whether demography or selection are the ultimate cause, a weaker paternal than maternal structuring is indicative of gene flow among populations, implying that mtDNA alone in such cases overestimates population structuring.

Conclusions

Bears are a prominent and widely cited example in phylogeography, with range-wide signals of pronounced population structuring reported for brown bear mtDNA (Davison et al. 2011). We reexamined this paradigm using paternally inherited markers. In strong contrast to mtDNA data, shallow divergences and lack of pronounced geographic structuring of brown bear Y chromosomes were found. mtDNA-based inferences have thus overestimated phylogeographic structuring, due to extensive male gene flow on regional and range-wide scales. Nevertheless, various adaptive traits have been linked to mtDNA (Ballard and Rand 2005), and the mtDNA of an individual may have important consequences for its phenotype and local adaptation. Phylogeographic structuring of the brown bear matriline into regional assemblages could therefore be adaptively significant. Our findings highlight that evolutionary patterns inferred from mtDNA, despite its popularity, are not representative of the entire genome and that phylogeographic histories of many species may need to be reevaluated. Y-chromosomal data are essential in any phylogeographic analyses of mammals—even in presumably well-studied species such as bears.

Materials and Methods

Identification of Y-Chromosomal Markers

A whole genome sequence assembly of a male polar bear (Li et al. 2011) was used to identify putative Y-chromosomal scaffolds by searching for matches with the sequences of known Y-linked genes (SMCY, ZFY, SRY, UBEY, RMBY). We identified five scaffolds from ~19 to ~390 kb in length (scaffold numbers: *297*, *318*, *369*, *579*, *605*). These scaffolds were extracted and compared with the corresponding sequences in a male brown bear (accession numbers: CBZK010000001–

CBZK010000005) in order to identify genomic regions containing either variable sites or microsatellite motifs, respectively, between the two individuals. To decrease the possible ascertainment bias in the subsequent application of the markers in samples from different species and populations, we did not type these variable sites, but we designed and sequenced 11 polymerase chain reaction (PCR) fragments around them with lengths of at least 500 bp (529–1,216 bp). All variable sites on the black bear branch, and most variable sites within brown and polar bears, respectively, were newly discovered by this sequencing approach (supplementary table S6, Supplementary Material online). All but three variable sites between brown and polar bears, however, were known from the ascertainment panel. Y-chromosomal sequences for each haplotype can be accessed at the EMBL data archive (accession numbers: HG423284–HG423309). The scaffold sequences were then mined for di- and tetranucleotide microsatellites that exhibited at least five uninterrupted repeat units. Primers for nine microsatellite markers are shown in supplementary table S9, Supplementary Material online. Allele size data can be accessed at the DRYAD repository (http://doi.org/10.5061/dryad.3p21q).

PCR fragments obtained from brown, polar, and black bears were then evaluated for their male specificity. This assessment resulted in seven sequence fragments and nine microsatellite markers that were ultimately used (supplementary tables S1 and S6, Supplementary Material online). Male specificity was ensured throughout all experiments by consistently including female DNA controls. See supplementary tables S7–S9, Supplementary Material online, for details on PCR conditions, sequencing, and fragment analysis.

Sampling and DNA Extraction

Tissue and DNA samples from 90 male brown and 40 male polar bears were included in this study, covering large parts of their distribution ranges (fig. 1, table 1, and supplementary table S1, Supplementary Material online). For comparison, we also analyzed four American black bear samples, covering their two previously described mitochondrial clades (supplementary fig. S4, Supplementary Material online), and a male spectacled bear as outgroup for divergence time estimations. All tissue samples originated from animals legally hunted for purposes other than this study or from zoo individuals. Individuals with unknown sex were tested as in Bidon et al. (2013). DNA was extracted using a modified Puregene (Qiagen, Hilden, Germany) DNA salt extraction protocol or DNeasy Tissue kit (Qiagen).

Analysis of Y-Chromosomal Scaffold Sequences

Genomic sequence data was used from 12 male polar bears, 1 male brown bear, and 1 male black bear (Miller et al. 2012), plus 1 male brown bear from Northern Europe (supplementary table S3, Supplementary Material online). Short reads were mapped to a >390-kb-long putative Y-linked scaffold from a male polar bear (Li et al. 2011) (scaffold 297). Consensus sequences were determined for every individual using Geneious 6.1.6 (Biomatters, Auckland, New Zealand), calling "?" for regions without coverage and "N" for bases with a Phred quality score <20. Consensus sequences of the 15 individuals were aligned and single-nucleotide variants determined in regions with coverage for all individuals. All variants were manually checked in the alignment, and we excluded all sites that contained insertions/deletions or ambiguous bases. Additionally, variants within 5 nt of ambiguous sites (? and N, respectively), variants directly adjacent to each other, and variants in microsatellite regions were excluded, in order to account for sequencing and alignment errors.

Data Analysis

PCR products were sequenced or subjected to fragment analysis (microsatellites). Sequences were aligned and edited in Geneious 5.6.2 (Biomatters, Auckland, New Zealand) and allele sizes were determined using Genemapper 4.0 (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). To infer phylogenetic relationships among haplotypes, networks were estimated using statistical parsimony as implemented in TCS 1.21 (Clement et al. 2000), with the connection limit set to 0.95 for sequence data or fixed at 50 steps for microsatellite haplotypes. For the combined analysis of sequence and allele size polymorphisms, data from all Y-linked markers were combined into one compound haplotype per individual. A haplotype distance matrix was calculated from allele sizes with GenoDive 2.0b23 (Meirmans and Van Tienderen 2004), assuming a strictly stepwise mutation model, with single repeat unit changes counted as one mutational step. Analyses of polymorphic sites and other summary statistics, nucleotide diversity π, tests for signals of demography and selection (Tajima 1989; Fu and Li 1993; Fu 1997), and analysis of molecular variance (AMOVA) were done in DnaSP v5.10 (Librado and Rozas 2009) and Arlequin 3.5 (Excoffier and Lischer 2010). Haplotype configuration tests were performed in haploconfig and haplofreq (Innan et al. 2005), with theta values obtained from the number of segregating sites (Watterson's theta) and nucleotide diversity (π), respectively, and simulating different population expansion scenarios ($\theta = 1.38, 0.37$; growth rate $g = 0, 2, 10$; $a = 10{,}000$; $n = 44$; $s = 6$). Different weighting schemes were applied to sequence and microsatellite markers, as in Brown et al. (2011). Estimates of mean (±SE) among-group distances were obtained in MEGA5 (Tamura et al. 2011). SplitsTree4 (Huson and Bryant 2006) was used to calculate a NeighborNet network for the 390-kb-long data set. Bayesian phylogenetic analyses and divergence time estimations were performed in Beast v1.7.4 (Drummond et al. 2012).

Supplementary Material

Supplementary material, figures S1–S4, and tables S1–S9 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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