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Genetic structure of Greater *Aquila clanga* and Lesser Spotted Eagle *A. pomarina* populations: Implications for phylogeography and conservation

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ABSTRACT

Greater *Aquila clanga* and Lesser Spotted Eagle *A. pomarina* are two closely related raptors of conservation concern. We used mitochondrial pseudo-control region to analyse (1) the population genetic structure in both species in a sympatric area of their ranges; (2) the phylogeography of *A. pomarina* using samples from the northern, central and southern parts of its distribution range. Rare *A. clanga* showed remarkably higher genetic diversity compared to the more abundant *A. pomarina*, whose population was not genetically differentiated within the Baltic region. Presence of the most common Baltic haplotype in Greece suggests that the Balkan Peninsula was the refugium for *A. pomarina* during the last ice age, northern regions being colonized rapidly after deglaciation 8000 ± 1500 years ago. The high diversity of *A. clanga* may be caused by differences in postglacial spreading (colonization by several founders and possibly from several glacial refugia, lack of population expansion) and/or by recent demographic events (high level of gene flow or decreased numbers).

INTRODUCTION

Greater Spotted Eagle *Aquila clanga* and Lesser Spotted Eagle *A. pomarina* are two closely related Eurasian raptor species which, according to genetic data, diverged approximately one million years ago (Seibold *et al.* 1996). The world population of *A. pomarina* consists of about 20,000 pairs (Meyburg 1996) whereas in *A. clanga* it includes only a few thousands of pairs (Meyburg *et al.* 2001). Therefore *A. clanga* is considered as a globally vulnerable species and *A. pomarina* as a species with unfavourable conservation status (Tucker & Heath 1994).

Distribution areas of the Spotted Eagles differ remarkably. *A. clanga* ranges from the Baltic Sea through Eurasia to the Pacific Ocean. *A. pomarina*, if we exclude the extremely rare Indian Lesser Spotted Eagle *A. pomarina hastata* that could even be a separate species (Parry *et al.* 2002), is distributed mainly in central and eastern parts of Europe and its range is one of the smallest among Palearctic raptors (Bergmanis 1999). During Pleistocene glaciation, large areas of present-day breeding grounds in Europe were covered by ice and permafrost, and both species must have been concentrated in refugia in southern peninsulas of the continent. The changes that glaciations made to the genetic structure of Spotted Eagle populations have remained unexplored. Moreover, investigation of genetic variability is important in the conservation of endangered species, enabling us better to ascertain the viability of and threats to populations (Haig & Avise 1996); loss of genetic diversity implies the greater probability of reduced fitness and population survival (Fleischer 1998).

Recently, a first description of genetic variation in Spotted Eagles, based on the analysis of mitochondrial pseudo-control region, has been published (Väli 2002). In the present work, we analyse the population genetic structures of two Spotted Eagle species in their sympatric area more thoroughly. We also investigate the phylogeographic pattern of *A. pomarina* in Europe by comparing the genetic data of three populations, in northern and southern limits as well as in a central region of its distribution range.

MATERIAL AND METHODS

Samples

We analysed mitochondrial pseudo-control region sequences of 80 *A. pomarina* from Estonia (collected in 1997–2002), 29 from eastern Lithuania (2001–2002) and four from north-eastern Greece (2001; Figure 1). Thirteen mitochondrial samples of *A. clanga* were collected during 1997–2001 in Estonia where this covers 40–65% of the local population (the 80 individuals of *A. pomarina* comprise about 15% of the population). Only one sequence per nest site was used in analysis. The two Spotted Eagle species hybridise in nature, but as the mitochondrial DNA is inherited maternally, its evolution is generally independent of interbreeding. Therefore our sample includes also eight *A. clanga* and one *A. pomarina* sequences from hybrid individuals collected in Estonia and these are treated as representatives of the female line.

DNA was obtained mostly from blood samples of nestlings, but feathers of a nestling enlarged the sample size in Lithuania and moulted feathers of adults formed the Greek sample. We checked the identity of sequences derived from two source types and we detected no difference in specific Ac6 sequences obtained from the nestling's blood sample and female's moulted feather. For DNA extraction from feathers, we used DNeasy Tissue kit (Qiagen) and manufacturer's directions. Extraction of DNA from blood has been described earlier, as well as amplification and sequencing of the pseudo-control region (Väli 2002). All mutations were checked by re-amplification and re-sequencing.

Figure 1. Map of the main distribution range of the *A. pomarina* in Europe and the localisations of sampled populations (1 – Estonia, 2 – Lithuania, 3 – Greece).

Data analyses

We included in our analysis 867 and 847 bases of pseudo-control region in *A. clanga* and *A. pomarina* respectively (bases 33–991 in alignment of genus *Aquila*; Väli 2002). Sequences were aligned using a Clustal W programme (Thompson *et al.* 1994) and improved by eye. A minimum spanning network was constructed manually according to the variable sites. Haplotypes are numbered as in Väli 2002 with continuing numeration for newly detected ones.

Populations were characterized by haplotype (h) and nucleotide diversity (π; Nei 1987) as well as by the number of polymorphic nucleotides per site (θ_s; Tajima 1996) using DNASP 3.53 software (Rozas & Rozas 1999). Diversity indices were compared by *t*-test. Tajima's *D* statistic (Tajima 1989) was computed by DNASP to investigate the selective neutrality and population expansion; to test the significance of *D* we checked its following to beta distribution. We also analysed the distribution of pairwise genetic distances according to sudden expansion model (mismatch distribution: Rogers 1995; Schneider & Excofficier 1999). Deviation of observed distribution from the expected one was checked by goodness-of-fit test with 1000

bootstrap replicates. Pairwise Φ_{st} statistic, with 1000 permutations for significance, and the number of migrants per generation (N_m; Nei 1987) were calculated for assessing the differentiation between populations. Analyses of population differentiation and mismatch distribution were performed by ARLEQUIN 2.000 (Schneider *et al.* 2000).

In coalescence time estimation we presumed that cytochrome b sequences of Spotted Eagles differ by 1.8% (Seibold *et al.* 1996) and the divergence rate for this gene is 1.6% per million years (Liebers & Helbig 2002). According to this, Spotted Eagles diverged about 1.125 million years ago. Kimura 2P distance (Kimura 1980) between species by 849-bp sequence (we excluded 17-bp species-specific insertion of *A. clanga*; Väli 2002) of pseudo-control region was 0.05085 (computed by MEGA 2.1; Kumar *et al.* 2001), which equals 43.17 substitutions per sequence since divergence time of the two species, i.e. one substitution per 26,057 years. This mutation rate per site per year (4.52×10^{-8}) is similar to that observed for avian hypervariable 1 domain in the control region (Liebers & Helbig 2002). We used only substitutions in molecular clock calibration and in coalescence time estimation and excluded three individuals from haplotype E9 that differed by 1-bp deletion in T-strech consisting of seven or eight nucleotides, because this deletion is likely generated by replication slippage and it occurs at higher rates than substitutions (e.g. Berg *et al.* 1995). For calculating the age of the specified root in network (coalescence time), we used the formula described by Saillard *et al.* (2000): $\rho = (n_1 l_1 + n_2 l_2 + \ldots + n_m l_m) / n$, where n is the number of sequences, m is the number of links leading from root to clades, and l is the number of mutations which separate a clade from a root. Similarly calculated $\sigma^2 = (n^2_1 l_1 + n^2_2 l_2 + \ldots + n^2_m l_m) / n^2$ serves as an estimator of variance and σ could be used as a standard deviation for time estimation. Additionally, value of $\rho / n\sigma^2$ shows the 'starlikeliness' of the network and at the same time indicates the efficiency of coalescence time estimation (Saillard *et al.* 2000).

RESULTS

Among 13 *A. clanga* sequences, a total of eight haplotypes were found (Figure 2). Twelve variable sites included eight transitions, two transversions and two one-bp indels. Two main haplotypes Ac1 and Ac3 (AF435094 and AF487438 in GenBank), consisting of four and three individuals, differed only by one transition. However, two other haplotypes (Ac7, Ac6) differed from nearest network neighbour even by five point-mutations and by two substitutions plus the 26 bp deletion, respectively.

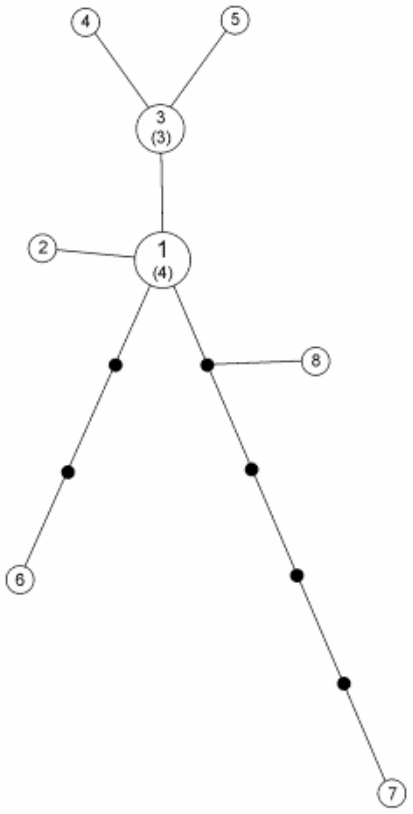
In 113 *A. pomarina*, 29 variable sites were detected (24 transitions, four transversions and a single one-bp indel) and 25 haplotypes distinguished (Figure 3). The most common haplotype (ELG1; AF115404) included 75.2% of individuals in Estonia and 69.0% in Lithuania, and both populations exhibited similar star-like distribution: an additional 15 haplotypes were found in Estonia and eight in Lithuania. From these, only one haplotype (EL2) was represented in both populations, others were present either in Estonia or Lithuania. After ELG1, the highest number of individuals was found in haplotype E9, which is characterized by a 1-bp deletion in T-strech of seven or eight nucleotides. Two Greek individuals belonged to the ELG1 haplotype whereas other birds differed by single substitution and formed two new haplotypes not found in the Baltic region. Due to its small size, the Greek sample was not used in further diversity analysis.

Figure 2. (A) Variable site matrix for the *A. clanga* population in Estonia based on the 867-bp sequence of the pseudo-control region. Site numbers correspond to positions in alignment of five *Aquila* species (Väli 2002), the 26-bp deletion in Ac6, bases 468–493, is not shown. (B) The minimum spanning network where circles represent haplotypes (sample sizes larger than one are given in brackets) and black nodes refer to substitutions or indels.

A.

Haplo-type	n	207	267	280	291	345	365	557	597	746	772	818	891
1	4	–	A	A	A	G	T	T	G	C	C	A	–
2	1	.	.	G	.	.	.	.	.	.	.	.	.
3	3	.	.	.	.	.	C	.	.	.	.	.	.
4	1	.	.	.	.	.	C	.	.	A	.	.	.
5	1	.	.	.	.	.	C	.	.	.	.	.	T
6	1	.	.	.	.	.	.	.	A	.	T	.	.
7	1	C	.	.	G	A	.	C	.	.	.	G	.
8	1	.	C	.	G	.	.	.	.	.	.	.	.

B.



Nucleotide and haplotype diversity was much higher in *A. clanga* than in *A. pomarina* (π: $P < 0.0001$, h: $P < 0.0001$; Table 1). In the latter species, the diversity was higher in Lithuania compared to Estonia (π: $P < 0.01$, h: $P < 0.0001$). However, the estimated number of migrants between two *A. pomarina* populations per generation was high ($N_m = 56.0$) and there was no differentiation between populations ($\Phi_{st} = 0.0088$; $P = 0.13$). Therefore, Estonian and Lithuanian samples were pooled for further analysis. Tajima's D showed the expansion of population size in *A. pomarina* ($D = -2.58$, $P < 0.001$) and this was also supported by distribution of pairwise genetic distances that did not deviate from the model of population expansion (sum of squared deviations = 0.0002, $P = 0.83$). For *A. clanga*, the Tajima's D value was negative as in *A. pomarina* but not significantly ($D = -1.48$, $P > 0.1$). However, also in *A. clanga* the observed mismatch distribution did not deviate from the expected model of sudden expansion (sum of squared deviations = 0.0134, $P = 0.48$).

Figure 3a. Variable sites in Lesser Spotted Eagle populations of Estonia (E), Lithuania (L) and Greece (G) based on the 847-bp sequence of the pseudo-control region.

Haplo-type	n	05 58	18 13	28 20	32 08	40 02	41 15	42 88	44 40	45 45	45 78	47 84	48 91	55 10	55 91	59 54	61 03	71 36	76 61	76 95	79 90	89 93	95 84	97 74
ELG1	84	C	C	A	A	G	T	C	T	T	T	A	T	T	G	T	G	T	T	C	T	A	G	G	T	T	T	T	C	A
EL2	2	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E3	2	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E4	1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E5	1	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E6	1	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E7	1	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E8	1	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E9	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.
E10	1	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E11	1	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E12	1	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
E13	1	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
E14	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.
E15	1	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E16	1	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.
L3	1	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L4	1	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L5	1	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L6	1	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L7	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L8	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
L9	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.
G2	1	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G3	1	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure 3b. The minimum spanning network for Baltic population (Greece excluded) where circles represent haplotypes (sample sizes larger than one are given in brackets) and black nodes refer to substitutions

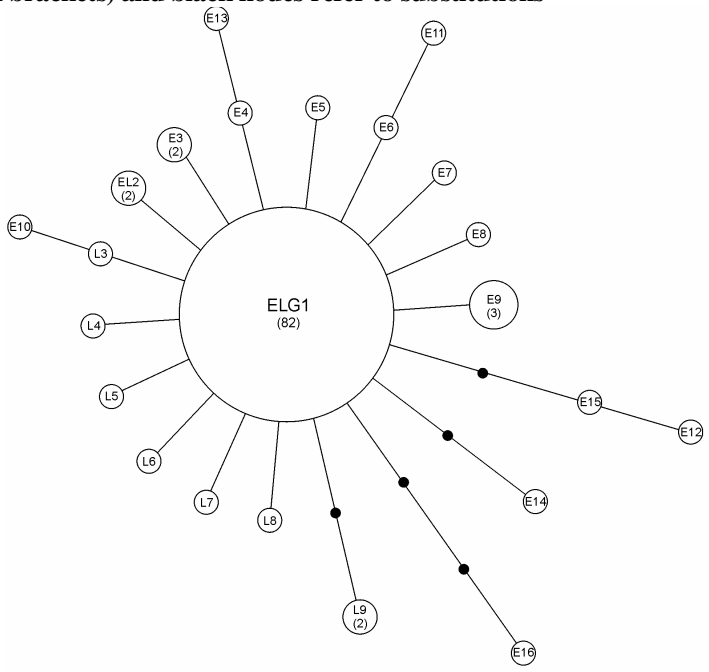


Table 1. Nucleotide diversity (π), haplotype diversity (h), number of polymorphic nucleotides per site (θ_s) and their standard deviations (SD) in three spotted eagle populations and for pooled Estonian and Lithuanian *A. pomarina* data. Note the over-representation of Estonian population in pooled data.

	n	π	h	θ_s
A. clanga, Estonia	13	0.00242 ± 0.00062	0.846 ± 0.076	0.00385 ± 0.00183
A. pomarina, Lithuania	29	0.00088 ± 0.00024	0.530 ± 0.112	0.00271 ± 0.00120
A. pomarina, Estonia	80	0.00074 ± 0.00019	0.341 ± 0.070	0.00477 ± 0.00158
A. pomarina, pooled	109	0.00076 ± 0.00015	0.393 ± 0.061	0.00584 ± 0.00177

Highly efficient ($\rho / n\sigma^2 = 0.85$) coalescence time estimation for the *A. pomarina* population resulted in the values of $\rho = 0.3113$ and $\sigma = 0.0589$ substitutions which equals 8000 ± 1500 years. For a recently expanded star-like population, this could be interpreted also as a time of expansion. It was more difficult to estimate coalescence time for *A. clanga*, because the sample was small and rooting of the network was complicated. Using the most common haplotype Ac1 as a root, we reached a coalescence time of 30 500 ± 10 500 years ago (ρ = 1.1538, $\sigma = 0.3997$). However, the efficiency index for calculation was much smaller (0.56) and this estimation should be taken with caution because it could be biased by the small sample or recent demographic events.

DISCUSSION

Endangered species are usually characterized by low genetic diversity (Haig & Avise 1996). High genetic structuring of the small Estonian *A. clanga* population was the most interesting finding of this study. However, it is easier first to explain the lower variation in the more abundant *A. pomarina*.

Star-like genetic structure, low differentiation between local populations, significantly negative Tajima's *D* value and the concordance between observed and expected distribution of pairwise genetic differences showed that the studied Baltic population of *A. pomarina* derived from a single glacial refugium and expanded rapidly after a recent bottleneck. The high estimated number of migrants between populations indicates probably the same and not the actual current gene flow. During the last glacial maximum the distribution range of the species had contracted to southern Europe. After the retreat of ice, vegetation spread rapidly over Europe enabling the northward expansion of animal species (Hewitt 1999). Spreading of broad-leaved forests that afford suitable nest sites and potential prey (e.g. Seddon *et al.* 2001) for *A. pomarina* started in the early Holocene and rapidly reached Estonia (Saarse & Veski 2001). This accords well with the calculated expansion time for *A. pomarina*.

Out of the three main well-studied European Pleistocene refugia – Iberia, Italy and the Balkans (Taberlet *et al.* 1998) – the current distribution range of *A. pomarina* includes only the last-named. The Balkan Peninsula is likely to be the source for the Baltic region because the main northern haplotype was present also in the Greek population. As most of today's East European animal

populations originate from the Balkan refugium (Hewitt 1999), the entire population of *A. pomarina* could have spread from there. The present, relatively small distribution range directly north from the Balkans, and use of a migration route via the Bosphorus (but not through Italy or Gibraltar), support this hypothesis. However, we cannot exclude the occurrence of other refugia during the last glacial maximum; potential candidates are Anatolia and the Caucasus, where local breeding populations still exist.

Is the recent northward expansion by *A. pomarina* still continuing? Although its numbers seem to have increased during the last century in Estonia (Lõhmus & Väli 2001), the species has not extended its range over the Baltic Sea. Thus there are no reliable data on present spreading of *A. pomarina* to the north. Instead, new areas have been settled in the eastern limit of distribution (e.g. Melnikov *et al.* 2001; V. Moseikin, pers. comm.) and an eastward expansion of range seems to be true.

The postglacial history of *A. clanga* in Europe should be generally similar to that of *A. pomarina,* i. e. a northern colonisation following the retreating unsuitable conditions, although from our data the expansion of population size is questionable. However, two reasons suggest that colonization could have occurred somewhat earlier than with *A. pomarina*: 1) *A. clanga* is distributed more to the east than *A. pomarina*, and northward spread in eastern regions was faster than in Central Europe (Hewitt 1999); 2) nest site requirements differ between the species and *A. clanga* is more linked to birches and pines that spread northwards earlier than broad-leaved trees (Walker 1995; Palmé *et al*. 2003). Even the present-day range limit of *A. clanga* lies further north than that of *A. pomarina* (Hagemeijer & Blair 1997) and, for example, it was recorded as a breeder between the 1880s and 1970s also in southern Finland (Solonen 1985). Even so, these few thousand years of earlier colonization cannot explain the large interspecific difference in genetic diversity noticed here.

Genetic diversity is higher in populations that originate from several glacial refugia (Hewitt 1999; Avise 2000). *A. clanga*, unlike *A. pomarina*, is seen regularly on migration and in winter also in Italy and Iberia (Cramp & Simmons 1980; Gutiérrez & Villa 2002). This raises the possibility that *A. clanga* used these, and probably also more eastern regions, as glacial refugia. Hence Estonia may form a hybrid zone between populations of *A. clanga* originating from at least two glacial refugia. This is supported by two haplotypes that exhibited a long genetic distance from others (Figure 2). Single substitution between main *A. clanga* haplotypes rather suggests the colonization by several founders from the same population or the recent divergence between two refugial populations. Finally, if the glacial range of *A. clanga* was relatively large due to the far northern range limit, the isolation between refugial populations was probably smaller or even lacking and this is reflected by higher heterogeneity.

In addition to the colonization history, genetic diversity is affected by recent demographic events (Avise 2000). From these, active gene flow between populations is one of the most important factors, especially in mobile birds. *A. clanga* individuals are distributed over a large area and exhibit a variety of mutations. Nowadays habitat loss enhances the dispersal of eagles, and their

integration into other populations should be relatively easy because competition for mates or for breeding territories is probably weak owing to sparse distribution. The two Spotted Eagle species hybridise in Estonia and most interbreeding *A. clanga* individuals are females (A. Lõhmus & Ü. Väli, unpubl. data), thus high diversity in maternally inherited mitochondrial DNA could reflect the female-biased immigration. In addition to the gene flow, decrease in population size affects the genetic structure by causing the loss of rare haplotypes and lowering the prevalence of common ones. This may also be the reason for high structuring of the *A. clanga* population.

The two factors described above (different origin of birds, demographic events) could act jointly in causing the high genetic diversity in *A. clanga*. Nevertheless, analysis of samples from a larger area is needed for understanding the status and structure of this rare species' population. Resolving the phylogeographic mystery is intriguing, but understanding of the current demographic situation and amount of gene flow between populations is needed for conservation. Our study shows that lack of genetic diversity is not the limiting factor for *A. clanga*.

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