Mammal Study 33: 43–49 (2008)

Construction of three-year genetic profile of Japanese wild boars in Wakayama prefecture, to estimate gene flow from crossbred Inobuta into wild boar populations

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Abstract. To estimate the degree of crossbreeding between Japanese wild boars and crossbred Inobuta in Wakayama prefecture, we examined haplotypes of mitochondrial DNA (mtDNA) and genotypes of the nuclear glucosephosphate isomerase-processed pseudogene (*GPIP*) in tissue samples obtained from 176 wild boars over a 3-year period. Five different haplotypes (J10, J15, J21, J22 and J23) and 3 *GPIP* alleles (*GPIP1*, *GPIP3a* and *GPIP3b*) were detected. These genetic profiles were classified as Japanese wild boar lineage without the genetic markers typical of Inobuta. The present genetic profile of wild boars, consisting of mtDNA haplotypes and *GPIP* genotypes, is a useful tool for studying the genetic structure of the local feral population.

Key words: *GPIP*, haplotype, Inobuta, mtDNA, wild boar.

The wild boar (*Sus scrofa*) is the ancestral species of domestic pigs and is wildly distributed in Europe and Asia. Two kinds of wild boar inhabit Japan: the Ryukyu wild boar (*S. s. riukiuanus*) on the islands of Ryukyu; and the Japanese wild boar (*S. s. leucomystax*) on the islands of Honshu, Kyushu and Shikoku. The Ryukyu wild boar differs from the Japanese wild boar in morphological and genetic features (Endo et al. 1998, 2000, 2002; Hongo et al. 2002). In recent decades, numbers of Japanese wild boars have increased in several prefectures in Kyushu, Shikoku, and western regions of Honshu (Japan Integrated Biodiversity Information System: http://www.biodic.go.jp/site_map/site_map.html). The increasing numbers of wild boars have caused serious problems for agriculture and forestry, including damage to farms and crops in mountainous villages. It is thought that one of the reasons for the increasing numbers of wild boar is that Inobuta (a cross between wild boars and domestic pigs) or domestic pigs have accidentally escaped from breeding farms and interbred with feral populations. Breeding farms for wild boar and Inobuta are located in many prefectures in Japan (Kodera and Kanzaki 2001). However, little is known about the frequency of escapes of Inobuta from these farms. Wild boar and Inobuta that have escaped from farms may affect the fertility of wild boar and the genetic structure of wild boar populations. Hunters sometimes find white spots on the ears and/or feet of Japanese wild boar, and this suggests that there is gene flow from Inobuta into wild boar populations. However, little is known about the genetic effects of escaped Inobuta on feral populations.

Recently, Ishiguro et al. (2002) developed a genetic profiling method for Japanese wild boar, using mitochondrial DNA (mtDNA) and the nuclear glucosephosphate isomerase-processed pseudogene (*GPIP*) to distinguish Inobuta from Japanse wild boars. In Wakayama prefecture, there have been rumors that white spots on wild boars are the result of interbreeding with released or escaped Inobuta.

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In the present study, we examined the genetic profile of mtDNA haplotypes and *GPIP* genotypes of 176 wild boars in Wakayama prefecture and in a neighboring region of Osaka prefecture using tissue samples obtained over a 3-year period to estimate gene flow in wild boar populations. Based on analysis of mtDNA and *GPIP*, all 176 samples were classified as belonging to the Japanese wild boar lineage, without the typical genetic markers of Inobuta. The genetic profile of wild boar is a useful tool for estimation of the size and genetic variability of the feral population.

Materials and methods

Samples

A total of 176 wild boar tissue samples (155 muscle samples and 21 liver samples) were collected from animals killed by hunting in Wakayama prefecture (159 samples) and Osaka prefecture (17 samples) (Table 1). Among the 176 samples, 27 samples were obtained from 3 locations from November 2004 to February 2005; another 118 samples were obtained from 10 locations from November 2005 to February 2006; and 31 samples were obtained from 3 locations from November 2006 to February 2007 (Table 1). The sampling sites were plotted on a 5-km mesh map, and their positions are shown in Fig. 1 and Fig. 2.

DNA extraction and mtDNA analysis

Total DNA was extracted from the tissue samples using the DNeasy Tissue Kit (Qiagen Science, MD). The mtDNA D-loop region (574-bp) was amplified using 2 primers (mit112 and mit106) and was purified using a QIAquick PCR Purification Kit (Qiagen) (Ishiguro and Nishimura 2005). The DNA sequence of the 574-bp mtDNA was determined by performing direct DNA sequencing using 2 primers (mit11 and mit12), as described elsewhere (Ishiguro et al. 2002; Ishiguro and Nishimura 2005). Haplotypes of the 574-bp mtDNA were determined by comparison with the DNA sequences in a pig mtDNA database (J1–J20, Japanese wild boar; M17–M20, Ryukyu wild boar; M21–M39, East Asian domestic pig; M40–M55, European domestic pig and wild boar; M56–M60, East Asian wild boar; Ishiguro and Nishimura, 2005). Novel haplotypes of the 574-bp mtDNA, which were not previously present in pig mtDNA databases, were submitted to the nucleotide databases of GenBank, EMBL, and DDBJ. Parsimonious network analysis of mtDNA haplotypes was performed using the split decomposition method (Dopazo et al. 1993).

GPIP haplotype analysis

The 507-bp *GPIP* sequence was amplified using 2 primers (GPIP1 and GPIP6) and was purified using the QIAquick PCR Purification Kit. The 507-bp DNA sequence was determined by performing direct DNA sequencing, and the *GPIP* alleles (*GPIP1*, *GPIP3*, *GPIP3a*, *GPIP4* and *GPIP4a*) were determined by comparison with previously reported *GPIP* sequences (Ishiguro et al. 2002; Ishiguro and Nishimura 2005).

Results

Distribution of mtDNA haplotypes

The distribution of mtDNA haplotyes of the 176 wild boars is summarized in Table 1. Five mtDNA haplotypes were detected: J10 (123 samples), J15 (1 sample), J21 (32 samples), J22 (5 samples) and J23 (15 samples). No mtDNA haplotypes characteristic of Asian domestic pigs (M21–M39) or European domestic pigs (M40–M55) were detected. Three novel mtDNA haplotypes (J21, J22 and J23) were detected, and their DNA sequences have been submitted to the nucleotide databases of GenBank, EMBL, and DDBJ with the following accession numbers: AB302179 (J21), AB302180 (J22), and AB302181 (J23). To assess genetic relationships among the present 5 mtDNA haplotypes, a parsimonious network was constructed using the 20 mtDNA haplotypes of Japanese wild boars that we previously reported (Ishiguro and Nishimura 2005). The 4 mtDNA haplotypes differed from the J10 mtDNA haplotype by only 1 nucleotide substitution and were located adjacent to each other in the parsimonious network (Fig. 3). Haplotype J10 (70%) was the predominant haplotype in Wakayama prefecture. Haplotype J23 was predominant in the neighboring area (Sennan city) of Osaka prefecture.

The geographical distribution of the 5 mtDNA haplotypes is plotted in Fig. 1. Haplotype J10 was predominant in 71 out of 81 samples (87%) from Tanabe city and was predominant in 28 out of 56 samples (50%) from 5 sampling sites (Gobo city, Yura cho, Hidaka cho, Hidakagawa cho, and Inami cho) in western Wakayama prefecture. Although the diversity of mtDNA haplotypes is dependent on the sampling area and size of samples, the diversity of the mtDNA haplotypes from the 5 sites in western Wakayama prefecture was markedly greater than the diversity of the mtDNA haplotypes from Tanabe city.

Table 1. Source and genetic profile of the 176 Japanese wild boars

Year	No. of samples	Location	mtDNA haplotype[a)]	No. of samples	*GPIP* genotype[b)]	No. of samples
2005	27	Tanabe City	J10	9	*GPIP1/GPIP1*	3
					GPIP3a/GPIP3a	6
		Sennan City	J10	5	*GPIP1/GPIP1*	2
					GPIP3a/GPIP3a	3
			J23	5	*GPIP1/GPIP1*	3
					GPIP3a/GPIP3a	2
		Kozagawa Cho	J10	8	*GPIP1/GPIP1*	4
					GPIP3a/GPIP3a	4
2006	118	Tanabe City	J10	40	*GPIP1/GPIP1*	32
					GPIP3a/GPIP3a	7
					GPIP3a/GPIP3b	1
			J21	5	*GPIP1/GPIP1*	4
					GPIP3a/GPIP3a	1
		Gobo City	J10	2	*GPIP1/GPIP1*	1
					GPIP3a/GPIP3a	1
		Sennan City	J10	2	*GPIP1/GPIP1*	2
			J23	2	*GPIP1/GPIP1*	2
		Hashimoto City	J10	4	*GPIP1/GPIP1*	4
		Hidakagawa Cho	J10	9	*GPIP1/GPIP1*	7
					GPIP3a/GPIP3a	2
			J21	19	*GPIP1/GPIP1*	8
					GPIP3a/GPIP3a	7
					GPIP3a/GPIP3b	4
			J22	3	*GPIP1/GPIP1*	1
					GPIP3a/GPIP3a	2
		Hidaka Cho	J10	9	*GPIP1/GPIP3a*	2
					GPIP3a/GPIP3a	2
					GPIP3a/GPIP3b	5
			J21	2	*GPIP1/GPIP1*	2
			J22	1	*GPIP1/GPIP1*	1
		Yura Cho	J10	4	*GPIP1/GPIP1*	4
			J21	1	*GPIP3a/GPIP3a*	1
			J22	1	*GPIP3a/GPIP3b*	1
			J15	1	*GPIP3a/GPIP3b*	1
		Inami Cho	J21	4	*GPIP1/GPIP1*	3
					GPIP3a/GPIP3a	1
		Kozagawa Cho	J10	7	*GPIP1/GPIP1*	5
					GPIP3a/GPIP3a	1
					GPIP3a/GPIP3b	1
			J21	1	*GPIP3a/GPIP3a*	1
		Susami Cho	J10	1	*GPIP3a/GPIP3a*	1
2007	31	Tanabe City	J10	22	*GPIP1/GPIP1*	18
					GPIP3a/GPIP3a	4
			J23	5	*GPIP1/GPIP1*	5
		Sennan City	J23	3	*GPIP1/GPIP1*	3
		Susami Cho	J10	1	*GPIP3a/GPIP3a*	1
Total	176			176		176

[a)] mtDNA haplotype previously defined by Ishiguro and Nishimura (2005).
[b)] *GPIP* genotype is designated previously (Ishiguro et al. 2002).

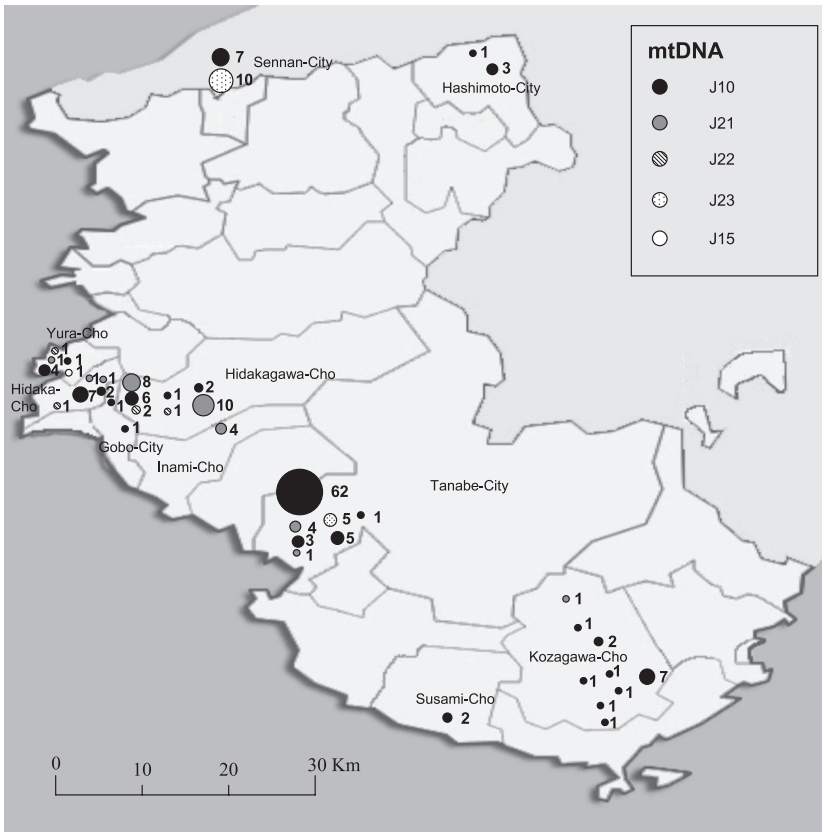

Fig. 1. Distribution of mtDNA haplotypes detected in 176 Japanese wild boars. The circles with numbers indicate the relative predominance of the mtDNA haplotypes.

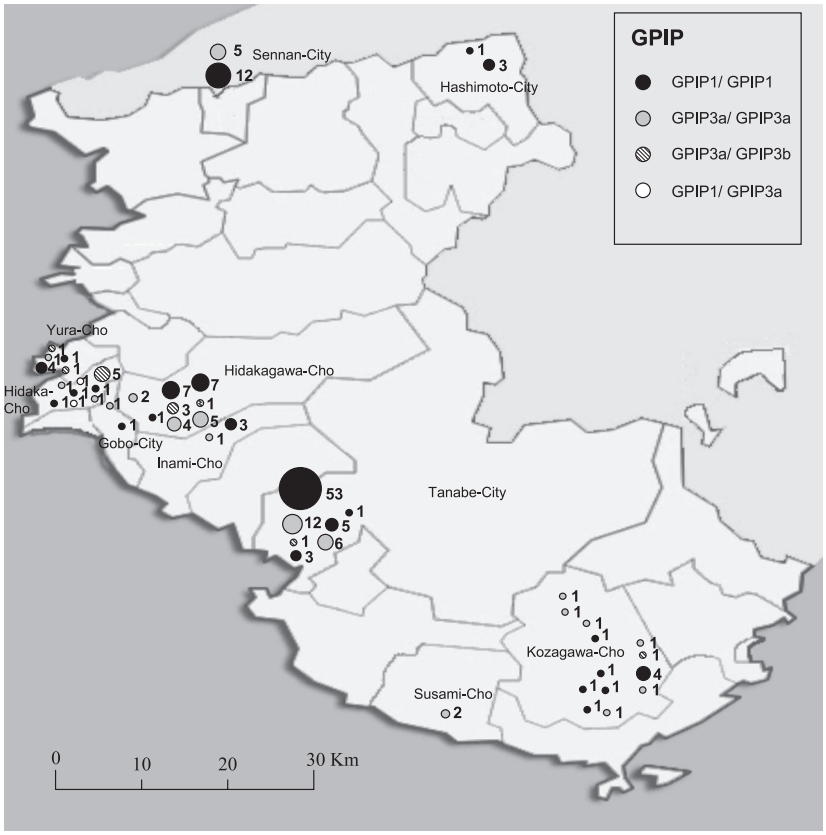

Fig. 2. Distribution of the *GPIP* genotypes detected in 176 Japanese wild boars. The circles with numbers indicate the relative predominance of the *GPIP* genotypes.

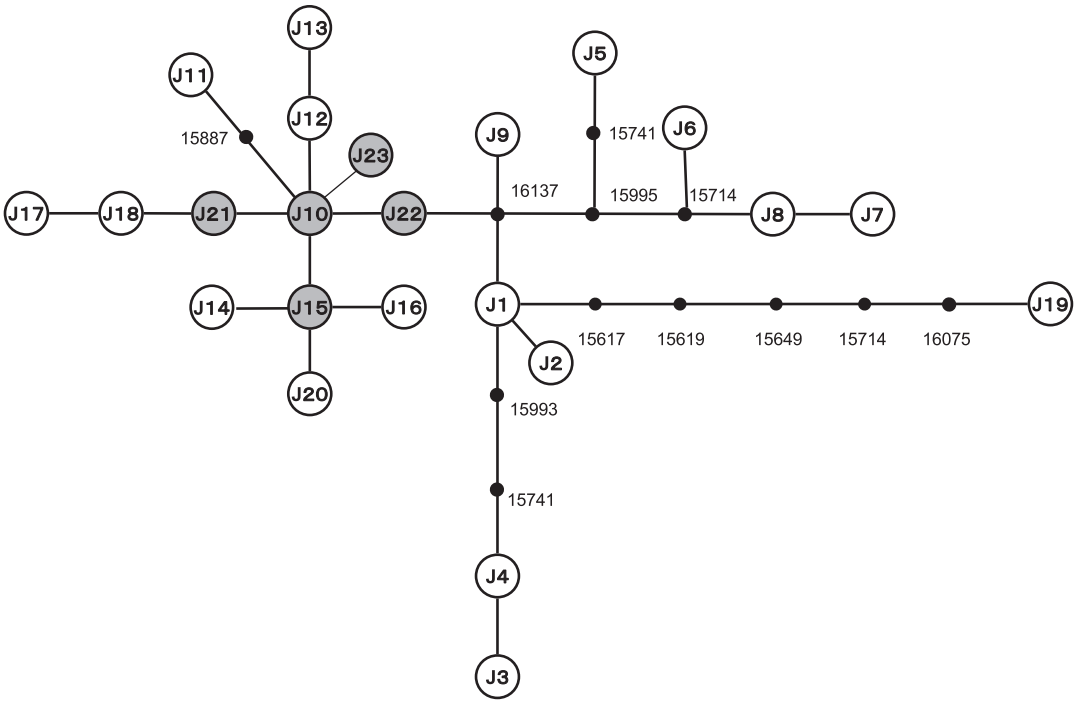

Fig. 3. Parsimonious network constructed using 23 mtDNA haplotypes from Japanese wild boars. These 23 mtDNA haplotypes comprise 3 novel mtDNA haplotypes (J21, J22 and J23) found in the present study and 20 mtDNA haplotypes reported previously (Ishiguro and Nishimura 2005). Five mtDNA haplotypes (J10, J15, J21, J22 and J23) were detected in the present study. The nucleotide position numbers indicate nucleotide substitutions. Nucleotide positions are numbered according to the complete pig mtDNA reference (Ursing and Arnason 1998).

Table 2. Nucleotide substitutions of the *GPIP* locus in domestic pig and wild boar

Type	Allele	Sequence at nucleotide position								Domestic pig and wild boar
		181	188	225	233	316	388	389	415	
Asian type	*GPIP1*	G	G	G	C	A	G	A	C	Japanese wild boar, Asian wild boar
	GPIP3	A	•	•	•	•	•	T	T	Japanese wild boar, Asian wild boar, Domestic pigs
	GPIP3a	A	A	•	•	•	•	T	T	Japanese wild boar, Ryukyu wild boar
	GPIP3b	A	•	•	•	•	•	•	T	Japanese wild boar
European type	*GPIP4*	A	•	A	•	G	C	T	T	Domestic pigs
	GPIP4a	A	•	A	T	G	C	T	T	Domestic pigs

Distribution of GPIP genotypes

To estimate the genetic influence of Inobuta on wild boars, we examined nucleotide substitutions at 8 variable sites in the 507-bp fragment of the *GPIP* gene, and used the resulting data to determine *GPIP* alleles (Tables 1 and 2). Three *GPIP* alleles (*GPIP1*, *GPIP3a* and *GPIP3b*) were identified; the nucleotide substitutions of the novel allele *GPIP3b* are shown in Table 2. No European alleles (*GPIP4* and *GPIP4a*) were detected. Table 1 shows the *GPIP* genotypes of wild boars at each sampling site. Four *GPIP* genotypes (*GPIP1/GPIP1*, *GPIP3a/GPIP3a*, *GPIP3a/GPIP3b,* and *GPIP1/GPIP3a*) were detected and plotted on the map (Fig. 2). The *GPIP1/GPIP1* genotype (65%) was predominant, especially in 62 of the 81 samples (77%) from Tanabe city. Four genotypes were found in the 56 samples from western Wakayama prefecture. The diversity of the genotypes from western Wakayama prefecture was greater than the diversity of the genotypes from Tanabe city, which is similar to the findings of the mtDNA haplotype analysis.

Discussion

In the present study, we examined the genetic influence of domestic pigs and Inobuta on Japanese wild

boars in Wakayama prefecture using mtDNA haplotypes (maternal inheritance) and *GPIP* genotypes (biparental inheritance). The combination of mtDNA and *GPIP* analyses is suitable for detection of crossbred lineages in wild boar populations (Ishiguro et al. 2002). We did not detect mtDNA haplotypes characteristic of domestic pigs or European *GPIP* alleles. Thus, the present findings do not constitute evidence of gene flow from domestic pigs or Inobuta into Japanese wild boar populations. In previous studies (Ishiguro et al. 2002; Ishiguro and Nishimura 2005), the results of both mtDNA and *GPIP* analyses provided evidence of gene flow from domestic pigs to Japanese wild boars in limited areas in other prefectures. Inobuta is generally produced on breeding farms by crossing a male Japanese wild boar with a female domestic pig, usually belonging to a European lineage. The Inobuta piglets produced by this crossbreeding have the same mtDNA haplotypes as their domestic pig mother. Consequently, mtDNA haplotype analysis easily identifies F_1 Inobuta piglets as belonging to a European lineage of domestic pig. *GPIP* allele analysis detects biparental genetic characters from both the father and mother. *GPIP* alleles are specific for Asian or European lineages of wild boars, but not for subspecies of wild boar or breeds of domestic pigs (Table 2). The *GPIP* genotypes of F_1 Inobuta piglets are likely to be identified as heterogeneous *GPIP* alleles composed of Japanese wild boar lineage and European pig lineage. However, when multiple crossbreeding between escaped Inobuta and native wild boars occurs among feral populations, the European lineage of the Inobuta is rapidly diluted and the European *GPIP* allele disappears within several generations. Thus, even if domestic pig lineage of the mtDNA haplotype and/or *GPIP* genotype is not detected in wild boars, such a finding is not conclusive evidence of the absence of Inobuta lineage. In the present study, in which we examined the genetic profile of 176 wild boars using tissue samples obtained over a 3-year period, we did not detect genetic evidence of Inobuta lineage. The mtDNA haplotypes characteristic of East Asian and European domestic pigs have been detected in the Japanese wild boar population in Miyazaki prefecture, indicating that there has been frequent gene flow from domestic pigs into Japanese wild boar populations in Miyazaki prefecture (Okumura et al. 2001). Thus, reliable assessment of gene flow from domestic pigs into wild boar populations requires repeated examination of the genetic profile of wild boars.

The white spots on hair or a white wild boar "Albino" are sometimes seen by hunters. Their appearance on wild boars would be suspected to be influenced by escaped domestic pigs or the crossbred Inobuta without detailed genetic examinations. The present analyses help to elucidate these possibilities. The female domestic pigs used to produce Inobuta are generally of breeds such as Berkshire and Duroc (Wakayama livestock center: http://www.pref.wakayama.lg.jp/prefg/070109/gaiyou/004/004.html). The coat color of F_1 Inobuta is usually black, brown, or a mix of black and brown; F_1 Inobuta do not have white areas on their coats. Coat color variation in pigs is controlled by somatic mutations of several genes, including melanocortin receptor 1 and c-Kit (Marklund et al. 1998; Kijas et al. 1998). It is unclear whether white spots on Japanese wild boars are the result of mutations in these genes. In fact, 2 of the present samples obtained in Tanabe city in 2007 were from animals with white spots, but mtDNA and *GPIP* analysis of those samples did not indicate an Inobuta genetic profile. Those results indicate that some white spots on wild boars are not due to gene flow from Inobuta into the wild boar population.

In the present study, we detected 3 novel mtDNA haplotypes (J21, J22 and J23) in wild boars in Wakayama prefecture. The 3 novel mtDNA haplotypes differ from the J10 mtDNA haplotype by only 1 nucleotide substitution; therefore, they are closely related to each other (Fig. 3). The distribution pattern of mtDNA haplotypes is extremely similar to that of the *GPIP* genotypes (Figs. 1 and 2). The diversity of mtDNA haplotypes and *GPIP* genotypes in Tanabe city is different from their diversity in Gobo city, Yura cho, Hidaka cho, Hidakagawa cho, and Inami cho. It is unclear why the sampling sites in Wakayama prefecture had such great profile diversity; this diversity may be due to artificial release or migration of wild boars from other areas or prefectures. It is difficult to assess the genetic diversity of wild boars based on documents. Genetic profile analysis is a useful method for studying the genetic structure of wild boar populations in a limited area and can provide data that is useful for management of wild animals.

Acknowledgments: This study was supported in part by Grants-in-Aid (18405034) from the Ministry of Education, Science, Sports, and Culture of Japan, and by the Research Project for Utilizing Advanced Technologies from the Ministry of Agriculture, Forestry and Fisheries. We thank many hunters who assisted our collecting samples.

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Received 10 June 2007. Accepted 22 September 2007.