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Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years

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Abstract

In the early 1900s, the wolf (*Canis lupus*) was extirpated from France and Switzerland. There is growing evidence that the species is presently recolonizing these countries in the western Alps. By sequencing the mitochondrial DNA (mtDNA) control region of various samples mainly collected in the field (scats, hairs, regurgitates, blood or tissue; $n = 292$), we could (1) develop a non-invasive method enabling the unambiguous attribution of these samples to wolf, fox (*Vulpes vulpes*) or dog (*Canis familiaris*), among others; (2) demonstrate that Italian, French and Swiss wolves share the same mtDNA haplotype, a haplotype that has never been found in any other wolf population world-wide. Combined together, field and genetic data collected over 10 years corroborate the scenario of a natural expansion of wolves from the Italian source population. Furthermore, such a genetic approach is of conservation significance, since it has important consequences for management decisions. This first long-term report using non-invasive sampling demonstrates that long-distance dispersers are common, supporting the hypothesis that individuals may often attempt to colonize far from their native pack, even in the absence of suitable corridors across habitats characterized by intense human activities.

INTRODUCTION

Several hundred years ago, wolf (*Canis lupus*) distribution covered the entire Northern Hemisphere, including all biomes (Mech, 1970). During the past 250 years, wolf populations in Europe underwent a continuous decline because of human persecution and degradation of ecological conditions (Breitenmoser, 1998), leading to the fragmentation of their geographic distribution. Eradication was complete in western Europe by the end of the nineteenth century, except for a few isolated populations in Italy (Ciucci & Boitani, 1991; Boitani, 1992) and in the Iberian Peninsula (Blanco, Reig & de la Cuesta, 1992). The last populations in the Alps disappeared before the end of the nineteenth century (Breitenmoser, 1998), and in France the last wolf was killed in 1939 (de Beaufort, 1987). In contrast, large populations have survived up to now in central and eastern Europe.

In the early 1970s, the lowest population size of the Italian isolate in the Apennines was estimated at approximately 100 wolves (Cagnolaro *et al.*, 1974; Zimen & Boitani, 1975). Since the end of the 1970s, the wolf has been legally protected in Italy (poison prohibition in 1976) and in Europe (Bern Convention in 1979). As a consequence, Italian populations increased and recolonized northwards through the entire Apennine range (Boitani, 1992; Randi *et al.*, 2000). Wolves reached the northwestern Apennines in 1985 (Meriggi, 1995) and southern French territories in Mercantour National Park some time before 1992 (two individuals observed; Houard & Lequette, 1993). From the early 1990s substantial field data, collected through a network of field observers, attested to wolf presence in France and Switzerland (Breitenmoser, 1998; Poulle, Lequette & Dahier, 1999). The comeback of a large predator in Alpine areas where it had disappeared several decades ago leads to huge sociological and economical controversies. In addition to legal-protection status, wolf expansion has been promoted by changes in ecological conditions (e.g. the increase of wild ungulate popula-

tions). Also, recent agricultural practices (e.g. extensive grazing, less guarding and large sheep flocks) in France and in Switzerland facilitate wolf attacks on livestock (Meriggi & Lovari, 1996), and generate conflicts of interest between stockbreeders and people in charge of wolf conservation (Breitenmoser, 1998). One of the key difficulties in managing conflicts between agricultural practices and wolf protection lies in certifying wolf presence and/or predation in a region. The wolf is a very elusive carnivore and direct observations are scarce. Furthermore, conventional analysis of field signs (tracks, scats, hairs, dead prey) do not allow the distinction between wolf and dog (*Canis familiaris*) with a high confidence level (e.g. T. Dahier, pers. comm.). Yet, reliable species identification can be achieved via non-invasive sampling and mitochondrial DNA (mtDNA) amplification and sequencing. Indeed, it has been shown that hair and faeces collected in the field can be used for this purpose (e.g. Höss *et al.*, 1992; Taberlet & Bouvet, 1992; see reviews in Morin & Woodruff, 1996; Kohn & Wayne, 1997; Taberlet, Waits & Luikart, 1999).

Recent studies have demonstrated that wolves from Italy share a single mtDNA control region haplotype. Such a haplotype has never been found in other genetic surveys of wolf populations or in domestic dogs worldwide (Vilà *et al.*, 1997; Randi *et al.*, 2000). As a consequence, mtDNA control region sequencing can be used to distinguish between wolf and dog, and to identify unambiguously the Italian among all the other wolf lineages. This approach represents a valuable tool to certify the presence of wolves in a region where field observations suggest a recent colonization process.

The purpose of this paper is (1) to demonstrate the usefulness of non-invasive genetic sampling as a routine management tool for lineage or species identification, and as a general approach to long-term studies of range expansions; (2) to identify the wolf lineage, allowing the distinction between wolves stemming from the Italian-expanding population and other populations; (3) to present a review of all wolf genetic identifications in France and in Switzerland for a better assessment of the recent colonization process.

MATERIALS AND METHODS

Sampling

A total of 256 unidentified samples (Table 1), including 190 faeces (74.2%), 40 hairs (15.6%), 22 tissues from dead animals (8.6%) and four regurgitates (1.6%), was collected in France and Switzerland between 1992 and 2001 by field correspondents of the French Wolf-Network (coordinated by the Office National de la Chasse) and the Swiss Wolf Project (coordinated by KORA, coordinated research projects for the conservation and management of carnivores in Switzerland). The geographic origins and sample types are given in Appendix 1. Before DNA extraction, tissue samples were stored in 80% ethanol, faeces and regurgitate samples were stored for several weeks or months at -20°C , whereas hairs were preserved dry in paper envelopes. In addition, we analyzed for reference purposes 21 wolf samples from different European populations and 15 dog samples from different breeds (Appendix 2).

DNA extraction

The 292 samples were processed between 1996 and 2001 in two different laboratories (Grenoble, France; Lausanne, Switzerland). DNA from tissue samples was extracted using the QIAamp tissue kit (QIAGEN), following supplier's instructions. DNA from faeces, regurgitate and hair was extracted in a physically separated facility dedicated only to low-DNA-content samples. DNA extractions from 150–200 mg of faeces were carried out using either the QIAamp tissue or the DNA stool kits (QIAGEN), and the regurgitates were processed using the QIAamp tissue kit (QIAGEN). For hair samples, standard Chelex method (Walsh, Metzger & Higuchi, 1991) was used. For each extraction experiment, control assays (blank extractions) were performed for monitoring cross-contamination among samples, possible human contaminations and PCR carry-over.

Table 1. Results of the genetic analyses for all unidentified putative wolf samples collected in the field (I, Italy; F, France; CH, Switzerland)

| | Identified | | Dog | Fox | Other | Total | Non-identified | Total |
|-------------|------------|-------|-----|-----|-------|-------|----------------|-------|
| | Wolf | Other | | | | | | |
| | I/F/CH | | | | | | | |
| Faeces | 66 | | 41 | 70 | 2 | 179 | 11 | 190 |
| | 65 | 1 | | | | | | |
| Hairs | 11 | | 7 | 3 | 1 | 22 | 18 | 40 |
| | 11 | 0 | | | | | | |
| Tissue | 20 | | 2 | 0 | 0 | 22 | 0 | 22 |
| | 16 | 4 | | | | | | |
| Regurgitate | 2 | | 0 | 1 | 0 | 3 | 1 | 4 |
| | 2 | 0 | | | | | | |
| Total | 99 | | 50 | 74 | 3 | 226 | 30 | 256 |

DNA amplification

We amplified each DNA extract using primers L15995 (called 'forward' in Taberlet & Bouvet, 1994) and H16498 (Fumagalli *et al.*, 1996), targeting the left domain of the mtDNA control region. Amplifications were carried out in a 25 µl reaction volume using 1U/tube AmpliTaq® Gold DNA Polymerase (Applied Biosystems), 1x PCR Buffer II (Perkin–Elmer), 2.5 µM MgCl₂ solution (Applied Biosystems), 100 µM each dNTP (PCR Nucleotide Mix, Roche Molecular Biochemicals), 200 µg/ml Bovine Serum Albumin (BSA, Roche Molecular Biochemicals) and 1 µM each primer. Template volumes were 5–10 µl for hair, faeces and regurgitate DNA extracts, and 2 µl for tissue samples. PCR amplifications were carried out on either a GeneAmp PCR System 9600 or 9700 (Applied Biosystems) for 35–55 cycles according to the number of target molecules in DNA extracts and using the following cycling parameters: initial step at 95°C for 10 min, and then 95°C for 30 sec, 50–54°C for 30 sec, 72°C for 60 sec. PCR products were visualized on a 1.6% agarose gel and purified using the QIAquick PCR Purification Kit (QIAGEN) following supplier's instructions.

DNA sequencing

Double-strand cycle sequencing was conducted using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Unincorporated fluorochromes were removed using spin columns purification or ethanol precipitation. Electrophoresis of purified sequencing products was performed in a 5% polyacrylamide gel (Long Ranger™ gel, FMC), loaded on a ABI PRISM™ 377 DNA or 373XL Sequencer (Applied Biosystems). Both strands were sequenced.

Species and lineage identification

For species and/or lineage identifications, we compared the sequences obtained from unidentified samples with our wolf and dog reference sequences, and with the available homologous sequences in DNA databases using BLAST 2.0 at <http://www.ncbi.nlm.nih.gov/BLAST/>.

RESULTS AND DISCUSSION

Results are summarized in Table 1. In total, 226 samples (88.3%) could be successfully sequenced, leading to the identification of 99 wolf, 50 dog and 74 fox (*Vulpes vulpes*) samples, and three non-canid species (one *Cervus elaphus* hair, and two mustelid faeces). Thirty samples did not yield enough DNA during the extraction procedure and were not further analyzed. The success rate was much higher for faeces (94.2%) than for hairs (55.0%).

All but five wolf mtDNA sequences (94.9%) corre-

spond to a single haplotype identical to the Italian haplotype (W4 in Vilà *et al.*, 1997, or W14 in Randi *et al.*, 2000). Four out of the five remaining wolf sequences are identical or nearly identical with other known haplotypes: LS3 and D0500-01 samples correspond to W22 in Vilà *et al.* (1997); T3499-01 and T3499-02 samples (France) correspond to W16 in Randi *et al.* (2000), differing only by a single nucleotide substitution (transition). The last sequence (sample WCH-079) is close to several sequences of European and North American wolves from our reference data set or from GenBank (minimum divergence of three nucleotide substitutions). Accession numbers of all the mtDNA haplotypes produced in this study are given in Appendices 1 and 2.

Efficiency of non-invasive genetic sampling for species identification

Our study clearly demonstrates the usefulness of non-invasive genetic sampling for the identification of free-ranging canids, in particular those causing damage to livestock. Faecal and hair samples are often the only way to prove the presence of an elusive animal like the wolf. However, it appears that some samples cannot be analyzed (11.7%). We did not assess the causes of failure, but DNA degradation and the presence of PCR inhibitors are likely to affect the possibility of amplifying a relatively long mtDNA fragment (e.g. 342 base pairs for the wolf). The success rate we obtained (88.3%) should be considered as a minimum. Further experiments, such as additional DNA extractions from the same material and/or additional PCRs, would probably have increased the success rate, at least for faeces. However, we did not repeat unsuccessful DNA extractions in this study.

The PCR parameters we used for DNA extracts from hair and faeces are extremely sensitive (55 cycles) and could allow the amplification of a single target molecule. In such conditions, however, there is a high risk of contamination either by sample cross-contamination, or by PCR carry-over. These two potential error risks were monitored by appropriate laboratory procedures, including several negative controls (extraction negative controls, PCR negative controls) during the analysis, and by an appropriate laboratory design including a dedicated room only used for DNA extraction and PCR setup of ancient and/or low-DNA-content samples and a physical separation of pre- and post-PCR areas. Furthermore, when the identification of a wolf faeces sample was found far from known packs (e.g. samples FLV98 and F6699 found in Vercors and the eastern Pyrenees, respectively; see Appendix 1), experiments were systematically done independently at least twice from the extraction to the sequencing in order to rule out the possibility of contamination.

Nevertheless, as mtDNA is maternally inherited, and given that our approach is only based on mtDNA sequencing, we can only identify the maternal lineage. Because all members of the genus *Canis* are known to hybridize, a hybrid between a female dog and a male wolf will be identified as dog and not wolf (and vice

versa). However, several studies using both mtDNA (Wayne *et al.*, 1992; Randi, Francisci & Lucchini, 1995; Vilà & Wayne, 1999; Randi *et al.*, 2000) and allozyme markers (Randi, Lucchini & Francisci, 1993; Lorenzini & Fico, 1995) do not demonstrate serious introgression between dogs and wolves in Europe.

Reliability of lineage identification

As indicated in the Introduction, two recent studies involving more than 100 samples demonstrated that wolves from Italy exhibit a unique mtDNA control-region haplotype which has never been found either in domestic dogs or in other wolf populations world-wide

(Vilà *et al.*, 1997; Randi *et al.*, 2000). In such conditions, the identification of the Italian lineage is presumed unambiguous. Wolf samples collected in France or Switzerland and bearing the Italian mtDNA lineage indicate that the maternal ancestors of these individuals originate from Italy. Among the samples identified as wolves, we found five individuals bearing a haplotype different from the Italian lineage. Two that displayed an unusual confidence in humans before their capture near Montpellier (France) in 1999 exhibited a haplotype typical of southeast Europe, and thus probably originated from captivity. A third sample (a skull) was in fact a hoax set up by journalists, and possessed a North American haplotype, probably also coming from a

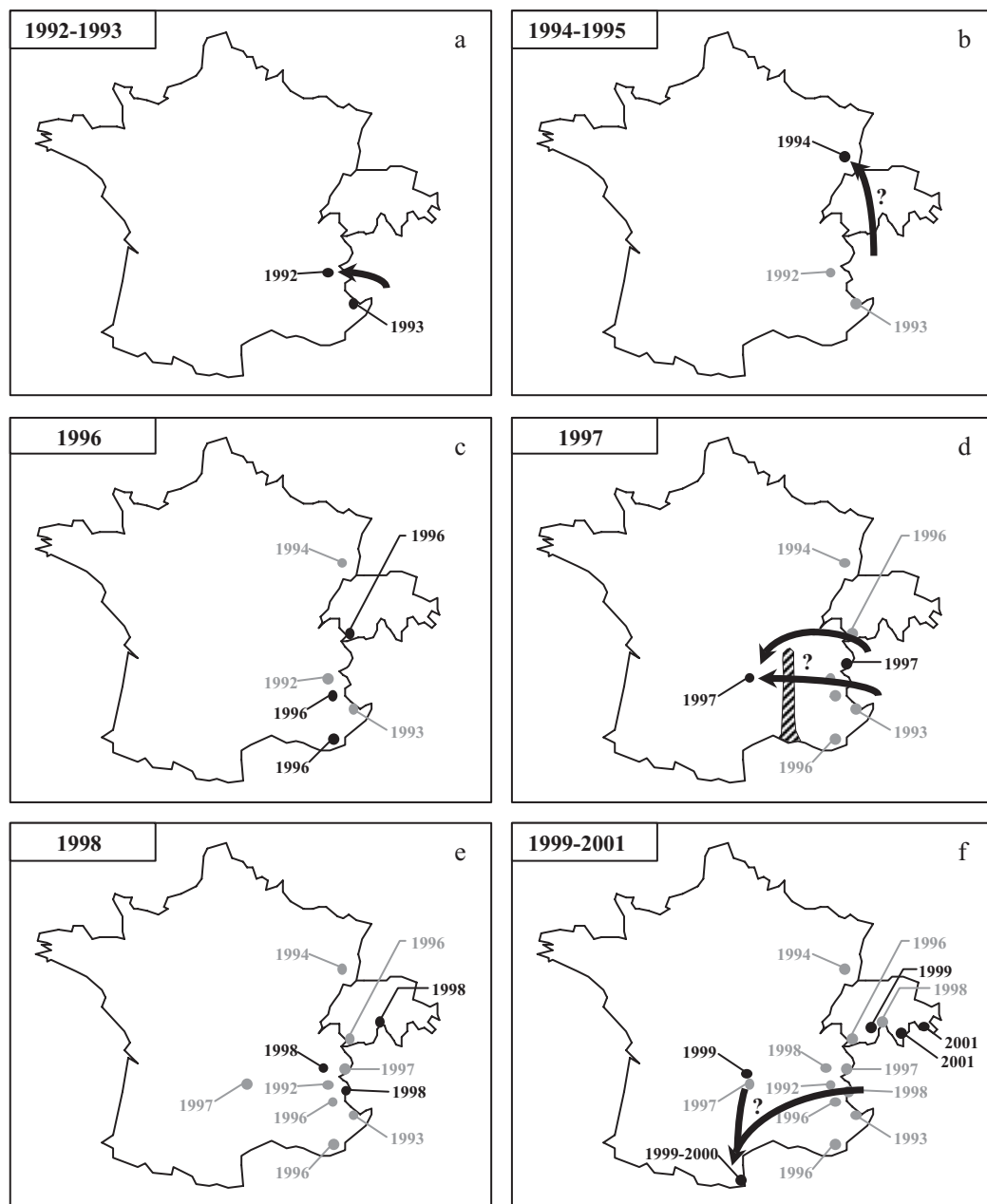


Fig. 1. Time of first genetic detection (black circles) of wolves of the Italian lineage in different regions of France and Switzerland. Arrows correspond to putative colonization routes. Cross-hatched areas indicate regions of high human activity, that might act as a barrier to dispersal.

captive population. A fourth sample (a skull found in the field) also bears the same North American haplotype, suggesting again a captive origin. For the last sample (faeces) collected in southeastern Switzerland, it is difficult to interpret reliably a single piece of data that might suggest either a current colonization from eastern Europe, or a captive source. It is clear that a precise knowledge of the genetic lineage could have a dramatic impact on management decisions, such as the possible removal of individuals of captive origin.

Wolf expansion in France and Switzerland

This study describes the use of long-term non-invasive genetic sampling to examine natural recolonization by a large carnivore. Our results show that the wolf has been present in the French and Swiss Alps since at least 1992 and 1996, respectively. Isolated data concerning wolf arrival in a particular region are often difficult to interpret, and provide relatively little information concerning the colonization process *per se* (see above). However, the unique combination of 10 years of field and genetic data enables the reconstruction of the pattern of expansion of the species in these countries (Fig. 1). The presence of wolves from the Italian lineage in locations far away from where packs have been identified seems to be the rule rather than the exception. In our data set, it is the case for sample LMFG found in 1992 in Aspres-les-Corps (Isère, France; Fig. 1(a)), sample LV3 found in 1994 in Vosges (France, Fig. 1(b)), sample LS1 found in 1996 in Valais (Switzerland, Fig. 1(c)), sample Lf.Cant. found in Massif Central (France) in 1997 (Fig. 1(d)), and finally sample F6699-01 found in 1999 in the Pyrenees (France, Fig. 1(f)). Wolves are characterized by a high dispersal potential, and seem to have a mechanism of expansion divided into two successive colonization steps (Mech, 1970). The first step is the dispersal of single, nomadic individuals, which explore new areas for mates. The second phase corresponds to the installation of packs, providing environmental conditions are appropriate. The establishment of the first pack in Mercantour National Park (France) occurred in 1992–93 (Houard & Lequette, 1993).

As the time between the arrival of the first long-distance dispersers and the presence of a stable pack can take several years (4 to 6 years according to Poulle *et al.*, 1999), it is likely that wolves were already present in the French Alps before 1992, the date of the first genetic identification of an 'Italian' wolf in the area. Given the current presence of wolves of presumed natural origin on both sides of the French Rhône valley, which is characterized by intense road traffic and a high level of urbanization, areas of high human activity do not seem to constitute a major barrier to wolf dispersal. This confirms the strong dispersal capability of this canid species (Mech, 1987), and suggests that natural corridors may not be as important as previously thought for dispersal. Our results are also consistent with the scenario of a natural return of wolves in France and

Switzerland from the expanding Italian population. Although our genetic analyses cannot rule out the possibility of illegal releases of captive 'Italian' wolves, we think that this is unlikely and is certainly the least parsimonious explanation, for a species recolonizing its traditional historical range, where it had been eradicated by man for a period of less than a century. Its present conservation status and the massive reconstitution of its wild ungulate prey populations, as well as its high dispersal potential, may explain its rapid and large-scale expansion since the 1970s. More generally, the results show that non-invasive genetic sampling can be used for long-term analyses of range expansions in wild species.

Perspectives

Since 1993, several hundred faeces samples have been collected in Mercantour National Park for studies on diet and health conditions, as well as for genetic analysis. In parallel with mtDNA sequencing for species and lineage identification, and thanks to improvements in non-invasive genetic techniques (Kohn *et al.*, 1999; Taberlet *et al.*, 1999), this large sample can now be subjected to nuclear DNA analyses (microsatellites). These markers will enable us to identify single wolves and assess their temporal and spatial distribution, to estimate the population size, and to track the evolution of pack composition.

The return of the wolf in portions of its former historical range represents a real challenge to predator and prey management. Molecular tools, together with field data, potentially provide a unique opportunity to understand thoroughly the ongoing colonization processes in a large carnivore species, and to establish a basic framework for a rational management in a controversial and sensitive sociological context.

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Appendix 1. List of samples analyzed for species identification. Sample type: F, faeces; H, hair; R, regurgitate; T, tissue. Country: CH, Switzerland; F, France. Genetic ID: D, dog; F, fox; O, other species; Wit: wolf of the Italian lineage; W: wolf from another lineage. Sequence lengths are 316 bp for dogs, 332 bp for foxes and 342 bp for wolves. Shorter lengths are indicated by * and the specified sequence length.

| Code | Sampling date | Sample type | Country | Geographic origin | Genetic ID | Haplotype | Accession no. |
|----------|---------------|-------------|---------|--------------------------|------------|-------------|---------------|
| F0400-16 | 23/03/00 | F | F | Alpes de Haute Provence | D | D18 *290 bp | AF487730 |
| F0401-01 | 02/02/01 | F | F | Alpes de Haute Provence | D | D11 | AF338782 |
| F0599-12 | 13/07/99 | F | F | Hautes Alpes (05) | D | D1 | AF338772 |
| F0600-01 | 18/11/00 | F | F | Alpes Maritimes | D | D20 *292 bp | AF487733 |
| F0600-02 | 18/11/00 | F | F | Alpes Maritimes | D | D21 *301 bp | AF487734 |
| F2600-10 | 25/08/00 | F | F | Drôme | D | D11 | AF338782 |
| F2600-13 | 05/07/00 | F | F | Drôme | D | D19 | AF487731 |
| F3800-03 | ??/??/00 | F | F | Isère (38) | D | D10* 227 bp | AF338781 |
| F6600-04 | ??/??/00 | F | F | Pyrénées Orientales (66) | D | D3 | AF338774 |
| F6601-01 | 14/04/01 | F | F | Pyrénées Orientales | D | D3 | AF338774 |
| F7499-01 | 23/07/99 | F | F | Haute Savoie (74) | D | D8 | AF338779 |
| FCE98-2 | 25/11/98 | F | F | Hautes Alpes (05) | D | D9 | AF338780 |
| FCI99-1 | 23/01/99 | F | F | Isère (38) | D | D2 | AF338773 |
| FCI99-2 | 23/01/99 | F | F | Isère (38) | D | D2 | AF338773 |

| Code | Sampling date | Sample type | Country | Geographic origin | Genetic ID | Haplotype | Accession no. |
|----------|---------------|-------------|---------|------------------------------|------------|-------------|---------------|
| FCI99-3 | 02/01/99 | F | F | Isère (38) | D | D1 | AF338772 |
| FCP98-5 | 06/01/98 | F | F | Alpes de Haute Provence (04) | D | D4 | AF338775 |
| P3899-15 | 19/04/99 | H | F | Isère (38) | D | D1 | AF338772 |
| P7399-14 | 25/03/99 | H | F | Savoie (73) | D | D7 | AF338778 |
| P8301-01 | 06/05/01 | H | F | Var | D | D22 | AF487735 |
| P8498-02 | ??/??/98 | H | F | Vaucluse (84) | D | D3 | AF338774 |
| P8498-03 | ??/??/98 | H | F | Vaucluse (84) | D | D1 | AF338772 |
| T0699-06 | 27/06/99 | T | F | Alpes Maritimes (06) | D | D2 | AF338773 |
| T0699-07 | 27/06/99 | T | F | Alpes Maritimes (06) | D | D2 | AF338773 |
| WCH-002 | 1998 | F | CH | Graubünden | D | D1 | AF338772 |
| WCH-003 | 1998 | F | CH | Graubünden | D | D1 | AF338772 |
| WCH-017 | 01/04/99 | F | CH | Valais | D | D14 | AF338785 |
| WCH-028 | 28/10/99 | H | CH | Valais | D | D2 | AF338773 |
| WCH-030 | 28/09/99 | F | CH | Valais | D | D3 | AF338774 |
| WCH-037 | 05/03/00 | F | CH | Vaud | D | D7 | AF338778 |
| WCH-042 | 09/07/00 | F | CH | Valais | D | D23 | AF487747 |
| WCH-044 | 28/04/00 | F | CH | Valais | D | D23 | AF487747 |
| WCH-050 | 17/07/00 | F | CH | Valais | D | D8 | AF338779 |
| WCH-056 | 01/09/00 | F | CH | Valais | D | D24 | AF487748 |
| WCH-057 | 01/09/00 | F | CH | Valais | D | D2 | AF338773 |
| WCH-064 | 27/02/01 | F | CH | Valais | D | D8 | AF338779 |
| WCH-065 | 27/02/01 | F | CH | Valais | D | D8 | AF338779 |
| WCH-066 | 27/02/01 | F | CH | Valais | D | D8 | AF338779 |
| WCH-067 | 27/02/01 | F | CH | Valais | D | D23 | AF487747 |
| WCH-068 | 27/02/01 | F | CH | Valais | D | D23 | AF487747 |
| WCH-069 | 27/02/01 | F | CH | Valais | D | D24 | AF487748 |
| WCH-070 | 27/02/01 | F | CH | Valais | D | D8 | AF338779 |
| WCH-071 | 27/02/01 | F | CH | Valais | D | D25 | AF487749 |
| WCH-072 | 27/02/01 | F | CH | Valais | D | D24 | Nuc19 |
| WCH-074 | 27/02/01 | F | CH | Valais | D | D24 | AF487748 |
| WCH-075 | 27/02/01 | F | CH | Valais | D | D25 | AF487749 |
| WCH-076 | 2001 | H | CH | - | D | D26 | AF487750 |
| WCH-078 | 03/03/01 | F | CH | Valais | D | D25 | AF487749 |
| WCH-086 | 26/08/00 | F | CH | Valais | D | D8 | AF338779 |
| WCH-107 | 2001 | F | CH | Valais | D | D27 | AF487751 |
| WCH-108 | 2001 | F | CH | Valais | D | D27 | AF487751 |
| F0400-13 | 01/10/00 | F | F | Alpes de Haute Provence | F | F7 | AF338795 |
| F0400-15 | ??/09/00 | F | F | Alpes de Haute Provence | F | F15 *319 bp | AF487736 |
| F0401-02 | 16/03/01 | F | F | Alpes de Haute Provence | F | F18 *305 bp | AF487739 |
| F0401-06 | 21/06/01 | F | F | Alpes de Haute Provence | F | F19 | AF487740 |
| F0501-27 | 29/05/01 | F | F | Hautes Alpes | F | F3 | AF338791 |
| F0501-32 | 19/05/01 | F | F | Hautes Alpes | F | F20 *319 bp | AF487741 |
| F0599-08 | 25/03/99 | F | F | Hautes Alpes (05) | F | F3 | AF338791 |
| F2600-11 | 25/08/00 | F | F | Drôme | F | F16 | AF487737 |
| F2601-20 | 04/07/01 | F | F | Drôme | F | F22 *315 bp | AF487743 |
| F3800-02 | ??/??/00 | F | F | Isère (38) | F | F8 | AF338796 |
| F3801-03 | 29/01/01 | F | F | Isère | F | F23 *320 bp | AF487744 |
| F3801-16 | 26/03/01 | F | F | Isère | F | F24 *320 bp | AF487745 |
| F3801-17 | 29/01/01 | F | F | Isère | F | F2 | AF338790 |
| F3899-01 | 09/05/99 | F | F | Isère (38) | F | F2 | AF338790 |
| F3899-11 | 06/06/99 | F | F | Isère (38) | F | F7 | AF338795 |
| F6600-05 | 31/08/00 | F | F | Pyrénées Orientales | F | F17 | AF487738 |
| F6699-07 | 15/01/99 | F | F | Pyrénées Orientales (66) | F | F6 | AF338794 |
| F7300-02 | 15/02/01 | F | F | Savoie | F | F2 | AF338790 |
| F7301-03 | 18/05/01 | F | F | Savoie | F | F3 | AF338791 |
| F7399-02 | 11/03/99 | F | F | Savoie (73) | F | F3 | AF338791 |
| F7399-06 | 16/03/99 | F | F | Savoie (73) | F | F3 | AF338791 |
| F7399-16 | 11/03/99 | F | F | Savoie (73) | F | F2 | AF338790 |
| F7399-17 | 11/03/99 | F | F | Savoie (73) | F | F3 | AF338791 |
| F7399-27 | 17/08/99 | F | F | Savoie (73) | F | F4 | AF338792 |
| F7399-28 | 18/08/99 | F | F | Savoie (73) | F | F3 | AF338791 |
| F7399-31 | 10/09/99 | F | F | Savoie (73) | F | F4 | AF338792 |
| F7399-32 | 10/09/99 | F | F | Savoie (73) | F | F3 | AF338791 |
| FCA98-1 | 04/12/98 | F | F | Savoie (73) | F | F2 | AF338790 |
| FCA98-2 | 01/10/98 | F | F | Savoie (73) | F | F2 | AF338790 |
| FCA98-3 | 04/10/98 | F | F | Savoie (73) | F | F3 | AF338791 |
| FCE98-1 | 24/11/98 | F | F | Hautes Alpes (05) | F | F2 | AF338790 |
| FCH98-2 | 13/12/98 | F | F | Hautes Alpes (05) | F | F10 | AF338798 |
| FCH99-1 | 06/01/99 | F | F | Hautes Alpes (05) | F | F2 | AF338790 |
| FCI99-6 | 03/03/99 | F | F | Isère (38) | F | F2 | AF338790 |
| FCP98-1 | 01/12/98 | F | F | Alpes de Haute Provence (04) | F | F9* 300 bp | AF338797 |
| FCP98-2 | 04/12/98 | F | F | Alpes de Haute Provence (04) | F | F5* 308 bp | AF338793 |
| FCV98-1 | 02/12/98 | F | F | Drôme (26) | F | F1 | AF338789 |

| Code | Sampling date | Sample type | Country | Geographic origin | Genetic ID | Haplotype | Accession no. |
|-----------|---------------|-------------|---------|------------------------------|------------|-------------|---------------|
| P0501-02 | 11/02/01 | H | F | Hautes Alpes | F | F21 *320 bp | AF487742 |
| P0600-02 | 23/01/00 | H | F | Alpes Maritimes (06) | F | F13 | AF338801 |
| P7301-01 | 10/03/01 | H | F | Savoie | F | F25 *308 bp | AF487746 |
| WCH-013 | 17/01/99 | F | CH | Valais | F | F2 | AF338790 |
| WCH-021 | 07/06/99 | F | CH | Valais | F | F2 | AF338790 |
| WCH-023 | 14/07/99 | F | CH | Valais | F | F2 | AF338790 |
| WCH-027 | 20/09/99 | F | CH | Graubünden | F | F14* 181 bp | AF338802 |
| WCH-031 | 06/11/99 | F | CH | Valais | F | F2 | AF338790 |
| WCH-032 | 08/08/99 | F | CH | Valais | F | F2 | AF338790 |
| WCH-033 | 18/10/99 | F | CH | Valais | F | F11 | AF338799 |
| WCH-035 | 18/10/99 | F | CH | Valais | F | F12 | AF338800 |
| WCH-038 | 17/05/00 | F | CH | Valais | F | F2 | AF338790 |
| WCH-046 | 17/07/00 | F | CH | Valais | F | F2 | AF338790 |
| WCH-047 | 14/07/00 | R | CH | Valais | F | F2 | AF338790 |
| WCH-048 | 14/07/00 | F | CH | Valais | F | F4 | AF338792 |
| WCH-049 | 24/04/00 | F | CH | Valais | F | F2 | AF338790 |
| WCH-052 | 01/06/00 | F | CH | Valais | F | F26 | AF487752 |
| WCH-053 | 01/06/00 | F | CH | Valais | F | F27 | AF487753 |
| WCH-054 | 28/08/00 | F | CH | Fribourg | F | F2 | AF338790 |
| WCH-063 | 27/02/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-073 | 27/02/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-077 | 08/03/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-082 | 01/04/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-083 | 19/04/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-085 | 15/08/00 | F | CH | Valais | F | F4 | AF338792 |
| WCH-087 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-088 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-089 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-090 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-091 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-092 | 04/05/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-095 | 05/06/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-101 | 20/09/01 | F | CH | Ticino | F | F2 | AF338790 |
| WCH-103 | 21/09/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-104 | 19/10/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-105 | 19/10/01 | F | CH | Valais | F | F2 | AF338790 |
| WCH-106 | 19/10/01 | F | CH | Valais | F | F2 | AF338790 |
| Allevard | 27/07/98 | F | F | Isère (38) | Wit | W1 | AF338803 |
| Allevard2 | 07/09/98 | F | F | Isère (38) | Wit | W1 | AF338803 |
| Besse | 09/08/98 | H | F | Isère (38) | Wit | W1 | AF338803 |
| Bramans | 08/08/98 | H | F | Savoie (73) | Wit | W1 | AF338803 |
| Clavans1 | 14/08/98 | H | F | Isère (38) | Wit | W1 | AF338803 |
| Clavans2 | 14/08/98 | H | F | Isère (38) | Wit | W1 | AF338803 |
| D0501-01 | 24/08/01 | T | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| F0401-04 | 17/05/01 | F | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| F0401-05 | 18/05/01 | F | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| F0500-03 | 10/08/00 | F | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| F0500-04 | 04/10/00 | F | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| F0501-31 | 19/06/01 | F | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| F2600-12 | 25/08/00 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| F2699-04 | 02/04/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| F2699-08 | 05/08/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| F2699-12 | 10/09/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| F2699-13 | 14/09/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| F3800-05 | 08/02/00 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F3800-15 | 16/07/00 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F3899-08 | 11/05/99 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F3899-18 | 03/09/99 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F3899-19 | 12/09/99 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F3899-21 | 19/09/99 | F | F | Isère (38) | Wit | W1 | AF338803 |
| F6600-02 | ??/??/00 | F | F | Pyrénées Orientales (66) | Wit | W1 | AF338803 |
| F6699-01 | 15/01/99 | F | F | Pyrénées Orientales (66) | Wit | W1 | AF338803 |
| F6699-02 | 15/01/99 | F | F | Pyrénées Orientales (66) | Wit | W1 | AF338803 |
| F6699-03 | 27/02/99 | F | F | Pyrénées Orientales (66) | Wit | W1 | AF338803 |
| F6699-05 | 05/03/99 | F | F | Pyrénées Orientales (66) | Wit | W1 | AF338803 |
| F7301-05 | 22/05/01 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-04 | 12/03/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-05 | 14/03/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-07 | 16/03/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-10 | 18/03/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-11 | 20/03/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-15 | 11/04/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-19 | 23/04/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |

| Code | Sampling date | Sample type | Country | Geographic origin | Genetic ID | Haplotype | Accession no. |
|----------|---------------|-------------|---------|------------------------------|------------|-----------|---------------|
| F7399-21 | 05/04/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-23 | 12/06/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| F7399-29 | 30/08/99 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| FCI99-4 | 14/02/99 | F | F | Isère (38) | Wit | W1 | AF338803 |
| FCP98-3 | 04/12/98 | F | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| FCP98-4 | 21/12/98 | F | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| FCP98-6 | 18/12/98 | F | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| FCV99-1 | 25/01/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| FCV99-2 | 24/01/99 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| FLI98-1 | 27/07/98 | F | F | Isère (38) | Wit | W1 | AF338803 |
| FLI98-2 | 07/09/98 | F | F | Isère (38) | Wit | W1 | AF338803 |
| FLV98-1 | 22/11/98 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| FLV98-2 | 22/11/98 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| LC.Canj | 22/09/96 | F | F | Var | Wit | W1 | AF338803 |
| LDAV1 | 01/11/97 | F | F | Savoie (73) | Wit | W1 | AF338803 |
| Lf.Cant. | 10/10/97 | T | F | Cantal | Wit | W1 | AF338803 |
| LM1 | 08/06/95 | T | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| LM3 | 01/07/93 | T | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| LM4 | 02/09/95 | T | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| LM6 | 17/09/96 | T | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| LM7 | 17/09/96 | T | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| LMFG | 14/06/92 | T | F | Isère (38) | Wit | W1 | AF338803 |
| LV3 | 16/06/94 | T | F | Vosges | Wit | W1 | AF338803 |
| P0499-01 | 01/03/99 | H | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| P0601-01 | 19/05/01 | H | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| P0699-05 | 05/12/99 | H | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| P6399-01 | 17/07/99 | H | F | Puy de Dôme (63) | Wit | W1 | AF338803 |
| P6399-02 | 17/07/99 | H | F | Puy de Dôme (63) | Wit | W1 | AF338803 |
| P6399-03 | 17/07/99 | H | F | Puy de Dôme (63) | Wit | W1 | AF338803 |
| P7399-18 | 20/04/99 | H | F | Savoie (73) | Wit | W1 | AF338803 |
| Queyras1 | 15/06/98 | F | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| Queyras2 | 18/06/98 | F | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| T0501-01 | 29/01/01 | T | F | Hautes Alpes (05) | Wit | W1 | AF338803 |
| T0699-02 | 16/04/99 | T | F | Alpes de Haute Provence (04) | Wit | W1 | AF338803 |
| T3800-11 | 20/11/00 | T | F | Isère (38) | Wit | W1 | AF338803 |
| Tiolache | 22/11/98 | F | F | Drôme (26) | Wit | W1 | AF338803 |
| Vomi1 | 01/01/97 | R | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| Vomi2 | 01/04/97 | R | F | Alpes Maritimes (06) | Wit | W1 | AF338803 |
| LS1 | 18/06/96 | F | CH | Valais | Wit | W1 | AF338803 |
| LS2 | 18/06/96 | F | CH | Valais | Wit | W1 | AF338803 |
| TLS98-1 | 27/11/98 | T | CH | Valais | Wit | W1 | AF338803 |
| WCH-001 | 27/11/98 | T | CH | Valais | Wit | W1 | AF338803 |
| WCH-011 | 18/12/98 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-012 | 19/12/98 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-014 | 15/01/99 | T | CH | Valais | Wit | W1 | AF338803 |
| WCH-018 | 03/06/99 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-019 | 03/06/99 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-024 | 14/07/99 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-029 | 07/03/00 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-036 | 16/10/99 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-041 | 30/06/00 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-043 | 30/06/00 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-045 | 03/07/00 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-055 | 25/08/00 | T | CH | Valais | Wit | W1 | AF338803 |
| WCH-062 | 10/01/01 | F | CH | Ticino | Wit | W1 | AF338803 |
| WCH-081 | 11/04/01 | F | CH | Graubünden | Wit | W1 | AF338803 |
| WCH-084 | 16/04/00 | F | CH | Valais | Wit | W1 | AF338803 |
| WCH-094 | 20/04/01 | F | CH | Graubünden | Wit | W1 | AF338803 |
| D0500-01 | 11/05/00 | T | F | Hautes Alpes (05) | W | W3 | AF338805 |
| LS3 | ??/??/?? | T | ? | ? | W | W3 | AF338805 |
| T3499-01 | 05/06/99 | T | F | Hérault (34) | W | W2 | AF338804 |
| T3499-02 | 05/06/99 | T | F | Hérault (34) | W | W2 | AF338804 |
| WCH-079 | 20/04/00 | F | CH | Graubünden | W | W11 | AF487754 |
| FCA98-4 | 19/09/98 | F | F | Savoie (73) | O | O1 | |
| FCA98-5 | 19/09/98 | F | F | Savoie (73) | O | O2 | |
| WCH-022 | 01/01/99 | H | CH | Valais | O | O3 | |
| F0599-15 | 10/08/99 | F | F | Hautes Alpes (05) | - | | |
| F2699-05 | 02/04/99 | F | F | Drôme (26) | - | | |
| F6600-01 | ??/??/00 | F | F | Pyrénées Orientales (66) | - | | |
| F6698-01 | 01/11/98 | F | F | Pyrénées Orientales (66) | - | | |
| F6699-04 | 01/03/99 | F | F | Pyrénées Orientales (66) | - | | |
| F7399-08 | 17/03/99 | F | F | Savoie (73) | - | | |
| F7399-09 | 17/03/99 | F | F | Savoie (73) | - | | |

| Code | Sampling date | Sample type | Country | Geographic origin | Genetic ID | Haplotype | Accession no. |
|----------|---------------|-------------|---------|------------------------------|------------|-----------|---------------|
| F7399-12 | 23/03/99 | F | F | Savoie (73) | - | | |
| FCH98-1 | 14/06/98 | F | F | Hautes Alpes (05) | - | | |
| FCI99-5 | 14/02/99 | F | F | Isère (38) | - | | |
| P0499-02 | ??/??/99 | H | F | Alpes de Haute Provence (04) | - | | |
| P0600-01 | 23/01/00 | H | F | Alpes Maritimes (06) | - | | |
| P3800-01 | ??/??/00 | H | F | Isère (38) | - | | |
| P6699-06 | 05/03/99 | H | F | Pyrénées Orientales (66) | - | | |
| P7399-03 | 11/03/99 | H | F | Savoie (73) | - | | |
| P7399-13 | 23/03/99 | H | F | Savoie (73) | - | | |
| P8499-01 | ??/??/99 | H | F | Vaucluse (84) | - | | |
| PCA98-01 | 04/12/98 | H | F | Savoie (73) | - | | |
| PCA98-02 | 04/12/98 | H | F | Savoie (73) | - | | |
| PCH99-01 | 15/01/99 | H | F | Hautes Alpes (05) | - | | |
| PCI98-01 | 06/10/98 | H | F | Isère (38) | - | | |
| PLA98-01 | 17/11/98 | H | F | Alpes de Haute Provence (04) | - | | |
| PLI99-01 | 09/02/99 | H | F | Isère (38) | - | | |
| WCH-004 | 30/11/98 | H | CH | Valais | - | | |
| WCH-020 | 05/06/99 | R | CH | Valais | - | | |
| WCH-039 | 09/07/00 | H | CH | Valais | - | | |
| WCH-040 | 09/07/00 | H | CH | Valais | - | | |
| WCH-051 | 18/07/00 | H | CH | Valais | - | | |
| WCH-080 | 10/04/01 | F | CH | Graubünden | - | | |
| WCH-102 | ??/??/?? | H | CH | ? | - | | |

Appendix 2. List of samples used as reference. Species: D, dog; W, wolf. Sample type: F, feces; H, hair; T, tissue. Sequence lengths are 316 bp for dogs, 332 bp for foxes and 342 bp for wolves.

| Code | Sampling date | Species | Sample type | Country | Haplotype | Accession no. |
|--------------------------------------|---------------|---------|-------------|----------|-----------|---------------|
| CB1 (beagle) | 1996 | D | H | France | D4 | AF338775 |
| CB2 (beagle) | 1996 | D | H | France | D4 | AF338775 |
| CB3 (beagle) | 1996 | D | H | France | D11 | AF338782 |
| CBA1 (German shepherd) | 1996 | D | H | France | D5 | AF338776 |
| CBA2 (German shepherd) | 1996 | D | H | France | D16 | AF338787 |
| CBAxG (German shepherd × groendhal) | 1996 | D | H | France | D17 | AF338788 |
| CC1 (cocker) | 1996 | D | H | France | D5 | AF338776 |
| CC2 (cocker) | 1996 | D | H | France | D5 | AF338776 |
| CH (Alaskan husky) | 1996 | D | H | France | D3 | AF338774 |
| CL1 (golden retriever) | 1996 | D | H | France | D13 | AF338784 |
| CL2 (golden retriever) | 1996 | D | H | France | D1 | AF338772 |
| CSHxL (samoyede-husky × g.retriever) | 1996 | D | H | France | D15 | AF338786 |
| LPort2 | 1997 | D | F | Portugal | D6 | AF338777 |
| LPort3 | 1997 | D | F | Portugal | D6 | AF338777 |
| Nick (samoyede) | 1996 | D | H | France | D12 | AF338783 |
| FESP9901 | 1999 | W | F | Spain | W10 | AF338812 |
| FESP9902 | 1999 | W | F | Spain | W7 | AF338809 |
| FESP9903 | 1999 | W | F | Spain | W7 | AF338809 |
| LI1 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI10 | 1996 | W | H/T | Italy | W1 | AF338803 |
| LI2 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI3 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI4 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI5 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI6 | 1995 | W | H/T | Italy | W1 | AF338803 |
| LI7 | 1996 | W | H/T | Italy | W1 | AF338803 |
| LI8 | 1996 | W | H/T | Italy | W1 | AF338803 |
| LI9 | 1996 | W | H/T | Italy | W1 | AF338803 |
| LPol | 1994 | W | H | Poland | W9 | AF338811 |
| LPort | 1997 | W | F | Portugal | W6 | AF338808 |
| LRoum | 1996 | W | T | Romania | W8 | AF338810 |
| LWCRO10 | 1997 | W | T | Croatia | W4 | AF338806 |
| LWCRO11 | 1997 | W | T | Croatia | W5 | AF338807 |
| LWCRO12 | 1997 | W | T | Croatia | W4 | AF338806 |
| LWCRO4 | 1997 | W | T | Croatia | W4 | AF338806 |
| LWCRO6 | 1997 | W | T | Croatia | W4 | AF338806 |