



**The Food and Environment  
Research Agency**

**The development and validation of DNA marker methods for the  
verification of meat from wild boar.**

**FINAL REPORT**

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## EXECUTIVE SUMMARY

The objective of this study was to develop a method for the verification of meat from wild boar, by assessing the suitability of two DNA-based methodologies: microsatellite analysis and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. The project approach included identifying a minimum set of microsatellite or PCR-RFLP markers that can differentiate between meat from wild boar and pig breeds, and using the markers to develop a method using an appropriate analysis platform. Ideally, the method would be suitable for routine use by UK public analysts. The outputs of the project were to include a comprehensive standard operating procedure (SOP) outlining the method, as well as data demonstrating the validity of the method.

Of the two methodologies assessed during this project, it was found that microsatellite analysis, using an optimised, informative set of markers, was able to distinguish wild boar meat from pig meat. In comparison, PCR-RFLP analysis was found to be confounded by the modern practice of routinely using cross breeds of common domestic pigs for pork production. The microsatellite-based method was further investigated and was found to be able to correctly identify meat from pure breed wild boar, domestic pig, regardless of the cross breeding, and mixtures containing wild boar and pig DNA.

The microsatellite-based method is not suitable for use by public analysts, since the analysis of the microsatellite data requires the use of capillary electrophoresis equipment capable of differentiating small differences (two base pairs) in DNA fragment size. Nevertheless, the final outputs of the project were a comprehensive SOP, to facilitate transfer to other laboratories, data demonstrating the validity of the developed microsatellite method, and a manuscript to be submitted to a peer-reviewed journal.

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## ABBREVIATIONS

Ct	Threshold cycle
CTAB	Cetytrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphates
EDTA	Ethylenediaminetetraacetic acid
mCyt b	Mitochondrial cytochrome b gene
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
w/w	Weight for weight

## 1.0 INTRODUCTION

The current food labelling regulations stipulate that the identity of meat in a meat product is accurately labelled. This has led to the development of assays that can distinguish one species from another, as there are sufficient differences in the DNA sequence of the target species that can be exploited to design species-specific assays (Brodmann & Moor, 2003; Dooley, *et al.*, 2004; Mendoza-Romero *et al.*, 2004; Laube *et al.*, 2003; Hird *et al.*, 2005, Chisholm, *et al.*, 2008). This is not the case, however, for breeds, where the differences in the DNA sequence are too few to design species-specific PCR primers. Indeed, work at this laboratory in developing real-time PCR methods for the detection of species in meat samples (FSA-funded project Q01083, 'Development of methods for the identification of duck, pheasant, venison, horse, donkey and wild boar in meat products'), confirmed that the *cytochrome b* gene showed too few differences between wild boar and domestic pig for specific primers to be developed. Pigs and wild boar are subspecies and are so closely related that they are able to interbreed to produce fully fertile offspring. Indeed, it is believed that pigs were domesticated from wild boar a couple of times through antiquity, with records of pigs dating back 7000 - 9000 years.

Alternative strategies for breed identification, for example, microsatellite fingerprint pattern analysis using panels of markers or PCR followed by restriction fragment length polymorphism (RFLP) analysis have become popular for the differentiation of pig breeds, including wild boar (Fajardo *et al.*, 2008; Marklund *et al.*, 1998; Alderson & Plastow, 2004). There are over 1200 microsatellites available in databases and described in the literature for distinguishing traditional pig breeds such as Landrace, Pietrain, Large White, Duroc, Hampshire, as well as wild boar (Thuy *et al.*, 2006; SanCristobel *et al.*, 2006; Vernesi *et al.*, 2003). Additionally, PCR-RFLP has been shown to discriminate between wild boar and domestic pig breeds (Fajardo *et al.*, 2008), as well as identifying domestic pig-wild boar hybrids.

This report outlines the work performed to assess panels of microsatellite markers and PCR-RFLPs, available in databases and the literature, for their power to discriminate between meat from wild boar and breeds of pig. It outlines the method developed using a selected panel of microsatellite markers, which has been written up into a comprehensive standard operating procedure (available separately), and describes the investigation into the effect of mixtures on the discrimination of meat from wild boar and pig breeds. Details of the design, optimisation and validation of the microsatellite method are included in the manuscript, which will be submitted to a scientific journal, the title of which is included in the appendix.

## **2.0 MATERIALS AND METHODS**

Materials and methods not included in the manuscript are outlined below.

### **PCR-RFLP**

The PCR-RFLP method of Fajardo *et al.* (2008) was used as a starting point for discriminating the pure wild boar ear tip and hair DNA extracts, from pig hair and buccal swab DNA extracts from known breeds, and the 14 pork samples from supermarkets, of unknown breed. In addition, meat samples from two known breeds (Ayreshire and Large White) and three cross-breeds (Duroc-Gloucestershire Old Spot, Saddleback-Duroc and Hampshire cross breed) were also analysed. The method was carried out exactly as described in the publication. PCR reactions were carried out in a 50 µl final volume comprising 25µl JumpStart™ REDTaq® ReadyMix™ reaction mix (Sigma, Poole, Dorset, UK), with the remaining volume made up of 300 ng template DNA and water. The restriction enzymes *Bst*UI and *Bsp*HI were purchased from New England Biolabs (Hitchin, UK) for digest analysis.

### **Method validation**

The method was validated using DNA extracted from three different pig breeds, and DNA extracted from five different pure wild boar samples. Five separate replicate dilutions of the DNA were prepared for each sample, resulting in a total of 15 pig DNA dilution replicates and 25 wild boar DNA dilution replicates for analysis. Each dilution replicate was then amplified with the full suite of 21 markers required for determination of possible pig-wild boar mixtures. Separate PCR master mixes were made up for each primer pair and for each DNA dilution replicate. Each DNA dilution replicate was analysed on a different 96-well plate, and on a different PCR thermocycler. Finally, each replicate was electrophoresed through a different capillary on the Genetic Analyzer. All PCR products were analysed over three separate runs.

## **3.0 RESULTS AND DISCUSSION**

The work involved in this project to develop a microsatellite-based method to distinguish wild boar from breeds of pig has been written up into a manuscript which has been submitted for publication in a peer-reviewed journal. The title of this manuscript has been included in the appendix. Project work which lead to this manuscript or which did not form part of the publication has been outlined below.

### 3.1 Sample collection

A considerable amount of time and effort was invested in sourcing appropriate and adequate numbers of samples of wild boar and pigs of different breed. It proved extremely difficult to source single, unrelated samples of wild boar or pig from a wide variety of geographical origins. The samples of wild boar were collected, split almost 50:50 between only two geographical areas, the Forest of Dean in the UK (due to annual culling to control the population) and France. The samples from France proved to be particularly time consuming, since they required an import licence. In addition, only hairs could be supplied since they did not require preservation in alcohol, a confounding factor for importation. DNA extracted from hair roots tends to be of lower quantity and quality than from muscle (meat), leading to further difficulties in obtaining good DNA amplification during PCR.

Commercial cuts of wild boar were relatively easy to source online, but were of unknown breeding and were not necessarily 'pure' wild boar. Visually, it is almost impossible to differentiate a pure wild boar from a cross breed, since even with a large dilution of wild boar genes, through generations of breeding with domestic pig, the wild boar phenotypic features of dark coat, coarse hair, and straight tail still prevail. Indeed, during the course of the project it became clear that many commercial producers of wild boar meat cross wild boar with pig, thereby producing a more docile, biddable animal.

Rare and traditional pig breeds were included in the study as previous work, for example the PCR-RFLP method of Fajardo *et al.*, (2008), did not investigate rare breeds. In addition, it was likely that traditional breeds with more defined lineages than common crossed breeds would present unique alleles that would be useful in discrimination studies. However, sourcing rare or traditional breed pig samples in large numbers was challenging. It was found that some of the named traditional breeds were not readily available. To compound this problem it was usual for breeders to have only one boar and perhaps one or two sows. Hence piglet production and slaughter was infrequent, with typically only a single pig butchered at one time. In addition, purchase of a bulk amount of pork was often required (e.g. half a pig) which was not a viable proposition for this project. Small cuts such as a single chop were not readily available. On occasion, small samples were promised, but never arrived.

A large number of Saddleback samples were acquired by visiting farms to obtain hair or buccal samples. Samples of some of the other breeds were also obtained in this manner, but obviously required the cooperation of breeders although, as already mentioned, this type of sample is not ideal due to the poor quality and quantity of DNA.

### 3.2 PCR-RFLP Analysis

The PCR-RFLP method described in Fajardo *et al.*, (2008) targets the MC1R gene, the melanocortin receptor, and uses two restriction enzymes, *Bsp*HI and *Bst*UI. The method was assessed against 21 wild boar DNA samples from the Forest of Dean (17 ear samples, 4 head samples), and 23 wild boar DNA samples (hair) from France. The results are shown in Table 1. All ear and head samples provided enough high quality DNA for PCR-RFLP analysis and all these samples produced a wild boar profile (795 bp fragment with *Bsp*HI, 345 bp, 222 bp, 177 bp fragments with *Bst*UI). Only six hair samples generated enough high quality DNA for the PCR. However, they also showed a pure wild boar profile.

The PCR-RFLP method was also assessed using hair samples taken from 11 British Saddlebacks, buccal swabs from 3 British Saddlebacks, and hair and buccal swabs taken from single Middle White, Large Black, Tamworth, Welsh, Duroc, Hampshire, and Berkshire pigs. Samples of meat were also analysed from a Duroc-Gloucestershire Old Spot cross, a Hampshire cross, a Saddleback-Duroc cross, Ayreshire, Large White, and 14 pork samples from 10 major supermarkets or butchers. These comprised mainly samples from the UK, but also single samples from Denmark, Germany, Ireland, France, and Spain. Of the 11 Saddleback hair samples, only 4 generated DNA of sufficient quality to generate a good PCR product profile. Likewise, the hair samples from the other named breeds failed to yield amplifiable DNA, apart from the Tamworth sample. In addition, the Welsh buccal sample failed to generate good quality DNA.

The results are also shown in Table 1, and it can be seen that the Middlewhite, Tamworth, Hampshire and Berkshire pig breeds all generated a domestic swine Type A profile (539 bp, 256 bp fragments with *Bsp*HI, 345 bp, 222 bp, 177 bp fragments with *Bst*UI). In addition, DNA extracted from hair and a buccal swab from the same individual Tamworth pig generated identical profiles, indicating that the DNA from both sources yielded a true and unambiguous result. In contrast, where numerous individuals were sampled from the same breed (Saddleback), the results generated were mixed. The profiles obtained for the different pig samples can be summarised as: domestic swine Type A, domestic swine Type B (795 bp fragment with *Bsp*HI, 399 bp, 345 bp fragments with *Bst*UI), domestic swine Type A and B cross, or wild boar crossed with domestic swine A or B. For the three DNA samples extracted from buccal swabs, each gave a different profile. Likewise, the DNA extracted from Saddleback hair samples generated profiles ranging from domestic swine B (hair sample 3) to domestic swine B possibly crossed with wild boar (hair sample 1), or a mixture of domestic swine A and B (hair samples 2 and 4). These results indicate that the PCR-RFLP method may not give reproducible results, particularly if DNA quantities are limiting (e.g.

DNA extracts from hair and buccal swabs) or the DNA quality is poor (i.e. from hair). The profiles generated with DNA extracted from the three known cross-breed pig samples, (Duroc-Gloucestershire Old Spot, Saddleback-Duroc and Hampshire cross breed) comprised a combination of all fragment sizes possible from domestic swine type A, B and wild boar. This indicates that the PCR-RFLP method will not give a definitive result with

**Table 1.** Results of PCR-RFLP analysis of pig and wild boar samples

Sample	Enzyme and fragment size (bp)		Assignment according to digest profile (wild boar or domestic swine type)
	<i>Bsp</i> HI	<i>Bst</i> UI	
<i>Wild Boar Samples</i>			
Wild boar tissue UK (x21)	795	345, 222, 177	Wild Boar
Wild boar hair France (x6)	795	345, 222, 177	Wild Boar
<i>Pig samples</i>			
Middlewhite, buccal	539, 256	345, 222, 177	A
Large Black, buccal	795, 539, 256	399, 345, 222, 177	A + B
Tamworth - buccal	539, 256	345, 222, 177	A
Tamworth - hair	539, 256	345, 222, 177	A
Hampshire - buccal	539, 256	345, 222, 177	A
Berkshire - buccal	539, 256	345, 222, 177	A
Saddleback – buccal 1	539, 256	345, 222, 177	A
Saddleback – buccal 2	795	399, 345	B
Saddleback – buccal 3	795, 539, 256	399, 345	A + B
Saddleback – hair 1	795	399, 345, 222, 177	B + Wild Boar?
Saddleback – hair 2	795, 539, 256	399, 345, 222, 177	A + B
Saddleback – hair 3	795	399, 345	B
Saddleback – hair 4	795, 539, 256	399, 345	A + B?
Duroc-Gloucestershire Old Spot - meat	795, 539, 256	399, 345, 222, 177	A + B
Hampshire cross breed	795, 539, 256	399, 345, 222, 177	A + B
Saddleback-Duroc - meat	795, 539, 256	399, 345, 222, 177	A + B
Ayresshire - meat	539, 256	345, 222, 177	A
Large White - meat	539, 256	345, 222, 177	A
<i>Supermarket Pork Samples</i>			
U.K. 1	539, 256	345, 222, 177	A
U.K. 2	539, 256	345, 222, 177	A
U.K. 3	539, 256	345, 222, 177	A
U.K. 4	539, 256	345, 222, 177	A
U.K. 5	539, 256	345, 222, 177	A
U.K. 6	539, 256	345, 222, 177	A
U.K. 7	795, 539, 256	399, 345, 222, 177	A + B
U.K. 8	795, 539, 256	399, 345, 222, 177	A + B
U.K. 9	795, 539, 256	399, 345, 222, 177	A + B
Spain	795, 539, 256	399, 345, 222, 177	A + B
Denmark	795, 539, 256	399, 345, 222, 177	A + B
Germany	539, 256	345, 222, 177	A
Ireland	795, 539, 256	399, 345, 222, 177	A + B
France	539, 256	345, 222, 177	A

cross-breed pigs or meat mixtures. DNA extracted from meat samples from two known breeds (Ayreshire and Large White) generated a clear domestic swine A profile. The DNA extracted from the fourteen supermarket pork samples of unknown breed roughly divided into half generating a pure domestic swine type A profile, and half a mixed A and B profile. As already mentioned, this profile would also mask the presence of wild boar. Following these results, it became clear that, although the PCR-RFLP method could differentiate wild boar, domestic pig type A, and domestic pig type B, it would not differentiate cross-bred wild boar from cross-bred domestic pig and would therefore not be suitable for the identification of the components of possible pork-wild boar mixtures in food products. The remaining samples were, therefore, only subjected to microsatellite analysis, which had the advantage of greater sensitivity if DNA quantity was low, due to the generation of fluorescently-labelled PCR products and their associated increased level of detection.

### **3.3 Microsatellite analysis**

A large number (over 1200) of microsatellites have been identified in pigs (Rohrer *et al.*, 1994; Alexander *et al.*, 1996; Archibald *et al.*, 1995; Ellegren *et al.*, 1994a, 1994b; Ellegren and Basu, 1995; Robic *et al.*, 1994). Thirty microsatellite primer pairs were chosen from the 50 listed in the Pig Panel on the Roslin Institute and INRA ISAG-FAO advisory group websites (<http://www-lgc.toulouse.inra.fr/pig/panel/panel2004.htm>). The rationale was that these markers should be the most useful for the discrimination of pig from wild boar, since they are used to distinguish pig breeds that are closely related. Additionally, full information on these markers (for example expected PCR product size, sequence information and PCR conditions) were readily available from these sources. Finally, markers were chosen with at least one primer pair from each of the 18 chromosome pairs present in pigs, and with one from the X chromosome.

Of the initial 30 primer pairs selected for assessment, 2 produced profiles with too many peaks (IGF1 and S0002), despite attempts at optimisation, and were therefore discounted from the remainder of the project. Of the remaining 28, 9 required optimisation by adjusting the magnesium chloride concentration (reduction from 1.5 mM to 0.9 mM) or raising the annealing temperature from 55°C to 60°C. DNA extracted from hair tended to be of poor quality and quantity, so in these cases the number of PCR cycles was raised to 55 but this resulted in a large number of extraneous peaks. The number of cycles was lowered to 40, but with 100 ng of template DNA in the reaction rather than 20 ng.

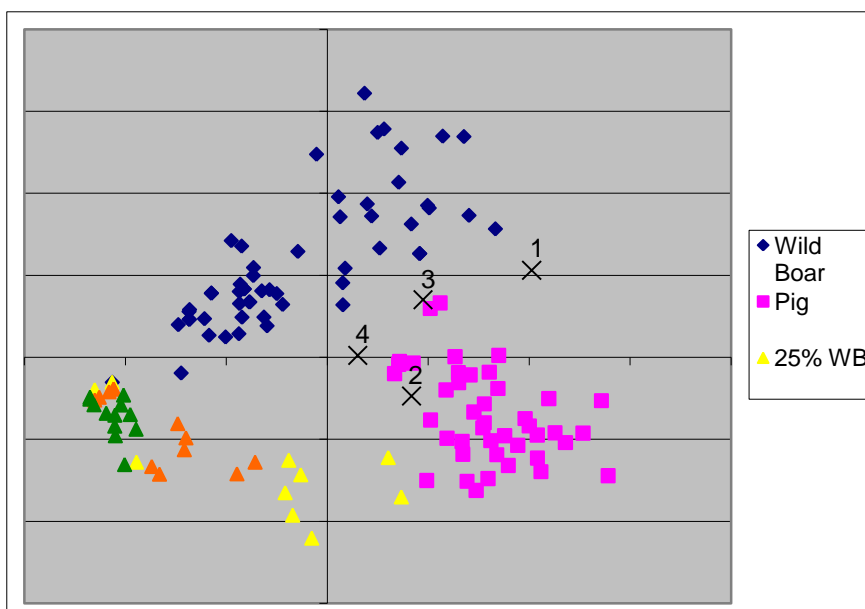
Analysis of the microsatellite data indicated that samples of pure, unprocessed wild boar could be easily discriminated from pure unprocessed pig samples as well as cross-bred pig

samples. Additionally, this could be achieved using 21 primer pairs rather than the total set of 28. Furthermore, for pure meat samples, a core subset of 8 out of the 21 microsatellites primer pairs would suffice to discriminate between wild boar and pig.

### 3.4 Microsatellite analysis of DNA mixes

To simulate commercial samples composed of pig and wild boar in varying amounts, DNA from wild boar was mixed with 12 different pig breeds (Ayreshire, Large White, Saddleback-Duroc, Hampshire, Berkshire, Duroc-Gloucester Old Spot, Hampshire cross, Large Black, Tamworth, Saddleback, Welsh, and Duroc) in ratios of 25:75, 50:50 and 75:25. The mixtures were subjected to microsatellite analysis using the full set of 28 optimised primer pairs from above. Analysis of the data was complicated by the possibility of four alleles being present at each locus due to mixing of the DNA, and not two, as would result from a true pig – wild boar mating. The results are represented graphically in Figure 1 and were not clear cut. Although it was possible to say with certainty that there was a mixture of pig and wild boar present, it was not possible to determine the ratio of each. However, analysis of the DNA mixtures confirmed that only 21 out of the 28 markers were required for the discrimination of pig-wild boar mixtures.

**Figure 1.** Analysis of all samples – graphical representation of assignments based on 21 microsatellite markers



Points of interest on graph –

x 1 = Australian wild boar sample

x 2, 3, 4 = Iron Age sample

## 4.0 CONCLUSION

Two DNA-based methodologies for the verification of meat from wild boar and pig have been assessed and the most appropriate, microsatellite analysis, has been developed, internally validated and written up as an SOP. It was found that the PCR-RFLP method was suitable for the discrimination of pure wild boar from pure pig, but was unable to differentiate mixtures, particularly if common pig breeds were crossed, which would mask the wild boar profile. Likewise, incomplete digestion with either of the enzymes could give a confusing profile. The method was found to not be robust with limiting quantities of DNA from hair and buccal swabs.

In contrast, microsatellite analysis only required small amounts of DNA and produced good results from buccal swabs and even some hair samples. The microsatellite method was capable of distinguishing pure wild boar meat from pure pig breed, or cross-breed, pig meat, using a core set of 8 microsatellite markers with at least 98% likelihood or greater of assignment to the correct breed (pig or wild boar). Mixtures containing 50% or greater wild boar DNA mixed with pig DNA could be determined as mixtures, using a full suite of 21 microsatellite markers. However, the absolute relative quantities of wild boar and pig in such mixtures could not be determined. The developed method is suitable for the analysis of cuts of meat, but is unsuitable for the discrimination of wild boar and pig in commercial processed products, such as sausages, salamis and pates. In order to address this problem, future work could include analysis of DNA that has been extracted from mixtures of wild boar and pig meat in known ratios, or ultimately, meat from known pure wild boar and pig crosses. This could enable development of calibration curves for determining the proportions of wild boar and pig meat in mixtures. As only partial sequence data is available for the wild boar genome (as opposed to the whole pig genome sequence being available), it is difficult to pinpoint differences between the two for discriminatory DNA-based tools. Future work could also focus on highly polymorphic regions such as the ribosomal intergenic transcribed spacer (ITS). These show a high degree of variation between closely-related species, and are commonly used in taxonomic and molecular phylogenetic studies.

In conclusion, in this report a method is outlined that is capable of verifying that a cut of meat is wild boar. This method has been validated internally and is now available for use by other laboratories to identify wild boar meat.

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**Appendix: Draft manuscript to follow, entitled**

Microsatellite analysis for the differentiation of European wild boar (*Sus scrofa scrofa*) from domestic pig breeds (*Sus scrofa domestica*) in food.

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