Methods for Species Detection on Leather and Collagen

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Abstract

Animal species identification on leather and collagen is a matter of high interest because of reasons concerning consumer protection, religion, recourse claims, product counterfeiting, medical approval and scientific matters e.g. restoration of ancient items. Besides other established methods for species discrimination, methods for leather relevant species detection are classified in three categories: histological examination and molecular analysis based on either DNA or proteins. Here, we reviewed all three methods with regard to the usage for animal species identification in leather and collagen products.

Keywords: animal species detection, leather, collagen, microscopy, PCR-RFLP, LC-MS

Introduction

Animal species detection on leather and collagen has different motivations. Especially in high price segments of leather (e.g. kid, lamb), an unambiguous assignment is of high relevance. The global fragmentation of production chains in the leather industry impedes the information transfer related to a leathers animal origin. Today, consumers must mostly trust in the declarations of the leather supplier, because often leathers are not adequately labelled related to the animal species. In recourse claims, the information about the animal species origin is useful as well. Also during restoration of ancient or medieval leather, the knowledge about a leathers animal species origin is of crucial importance. With this knowledge, conclusions can be drawn to used fats or finishes for e.g. leather wallpapers or leather book covers. Furthermore, species discrimination is of prime importance also for the leather component collagen, which is used in medical devices, for example.

Methods for species detection on leather are classified in three categories: histological examination and molecular analysis based on either DNA or proteins. Here, we review three methods with regard to the usage for animal species identification in leather and collagen products.

Species identification based microscopy

Because of the unique leather structure, the species origin of unknown leather can be distinguished by microscopical investigation of the leather's surface. In cases regarding the species origin of full grain leather, microscopy is still the method of choice. Microscopy is a

low-cost method with easy and fast handling. In histological analysis, the surface of unknown leather is examined. Nevertheless much experience with the interpretation of the pictures is required.

The leather origin strongly corresponds with the sequence of the hair channels on the leather surface and therefore the grain pattern (figure 1). For example, the grain pattern of cattle is more uniform, whereas the grain pattern from sheep leather is mainly arranged in rows. Characteristic for pig leather are wrinkles and defined lines in the grain pattern (Fuchs et al., 2008).

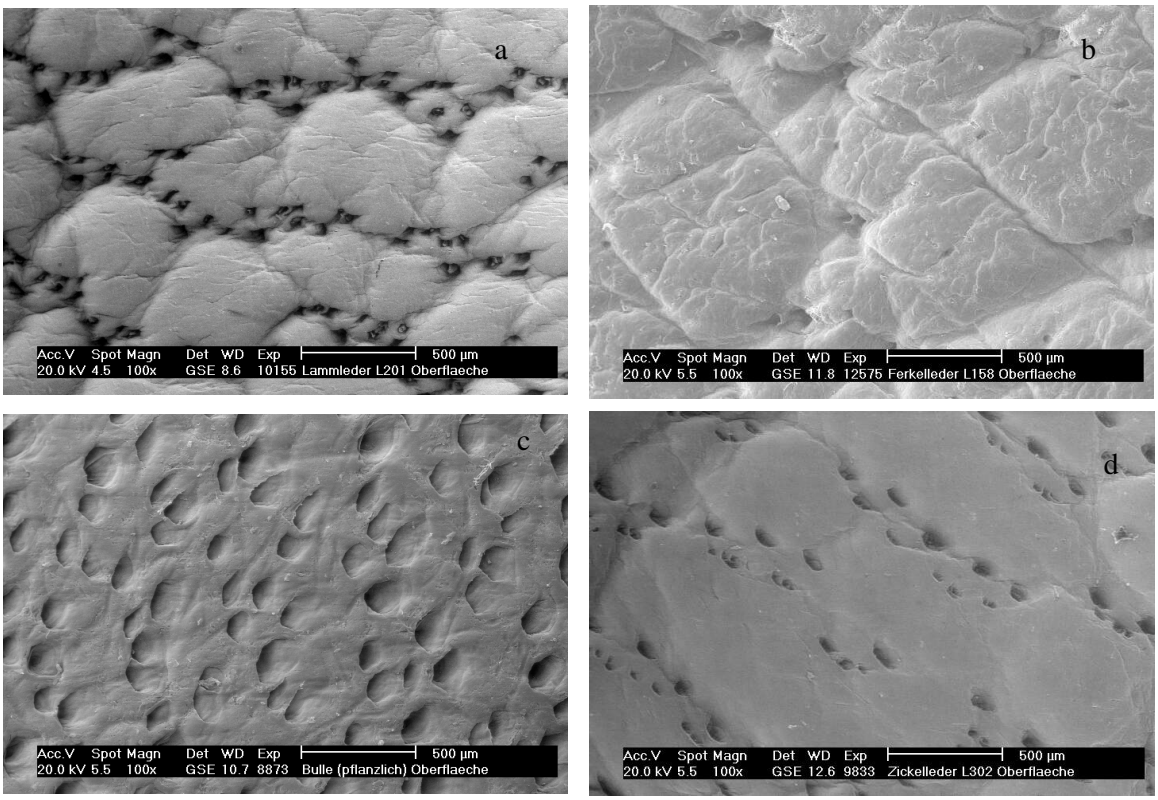

Figure 1: Microscopy pictures of the leathers' grain side of different animal species: a) lamp (sheep), b) pig, c) cattle, d) kid (goat).

During histological analysis, the leather sample is compared to pictures of leathers of known species origin. Many leathers are well distinguishable, e.g. that of cattle, pig, sheep and exotic species. However, the differentiation of sheep, hair sheep and goat may be not easy.

The microscopic evaluation of colored or coated leathers which are possibly embossed is challenging because most finishes cover the hair pores and the embossed structure superposes the original grain. From such leathers the coat must be removed before microscopy, which is a challenge in some cases. It is also hard to distinguish splitleather and other leathers without grain like Nubuk or Velours and of course, leathers from closely related species.

Species identification based on DNA: restriction fragment length polymorphism (RFLP)

Species determination of leather is also possible with DNA-based methods. Besides other DNA-based methods, the PCR/ RFLP is the method of choice for animal species detection of leather. The method is based on an amplification of animal DNA from all different kinds of leather in a Polymerase Chain Reaction (PCR) and on the proximate identification of the species by restriction fragment length polymorphism (RFLP) (Meyer et al., 1995). However, isolating amplifiable DNA from leather is a hard challenge. During tanning, the naturally occurring DNA in the skin is damaged by the extreme acidic pH and by oxidising and reducing agents; and during further progress the DNA is exposed to elevated temperatures and humid environments. Furthermore, the composition of additives is unknown, such as vegetable tannins, fats, colours and further finishes (Vuisoz et al., 2007) and therefore no knowledge is available about the amplifiability (Reynolds et al., 1992) of the purified DNA. Such leather additives can either inhibit, hinder or of course enhance the PCR, but the detailed interaction between eluted additives from the leather with the enzyme in PCR is not investigated up to now.

An isolation of animal species specific DNA from leather is possible by an enzymatic or chemical digestion of a small leather piece, normally 50 mg. The isolated DNA is a mixture of genomic and mitochondrial DNA (mtDNA) fragments. In PCR/ RFLP, only the mtDNA is of interest, because mtDNA is available in high copy numbers in an animal cell and therefore more mtDNA than genomic DNA is extractable from leather. Regardless of which DNA isolation kit is used, the isolated mtDNA is only available in combination with contaminations such as PCR inhibitors, which were extracted along with the DNA from the leather. Thus, the obtained DNA solution must be diluted before an amplification of the mtDNA in PCR to avoid false negative results. Furthermore, the criteria for amplifying ancient DNA must be considered during a PCR with DNA extracted from leather: (1) generation of a PCR product of less than 200 base pairs, (2) exclusion of contaminating sequences (Burger et al., 2002) (3) a second optional amplification by nested PCR in case of small amounts of isolated mtDNA. For amplification, universal primers are used, which amplify a DNA fragment from the highly conserved cytochrome-b-gene of the vertebrates. Accordingly, mtDNA from e.g. cattle, sheep, goat and pig are amplified using the same PCR protocol. The obtained PCR product is afterwards analyzed by means of species-discriminating RFLP. A schematic overview of the functionality of the RFLP is shown in figure 2. The amplified DNA fragments were cleaved in species-specific DNA fragments with the help of a restriction enzyme. By an electrophoretic separation of the DNA fragments, a species-specific banding pattern is generated (figure 3).

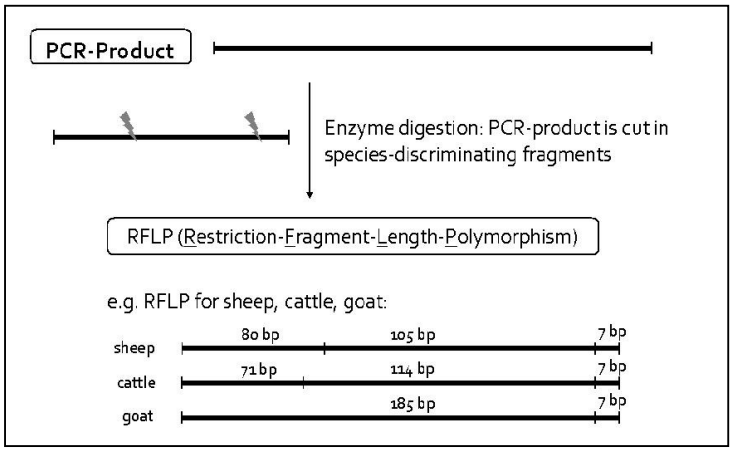

Figure 2: Schematic overview of the used RFLP method for species identification on leather.

Possible drawbacks of the animal species detection on leather with PCR/ RFLP are the small amounts of extracted DNA and the extracted inhibitors along with the DNA. Both factors can lead to insufficient amplification of specific DNA in a first PCR. Thus, after a first negative PCR, the used DNA has to be checked by nested PCR and dilution of the DNA samples. Up to now, the main animal species in leather production are distinguishable with PCR/ RFLP, which are cattle, sheep, goat and pig. Nevertheless, this method is not promising for ancient leather samples. To date the general recall ratio in our lab is 89% for the species determination of leather samples. 11% of analyzed leather samples do not show an interpretable result because of either coextraction of polymerase inhibitors during DNA isolation or extremely damaged DNA usually due to postmortem DNA damage in ancient and medieval leathers.

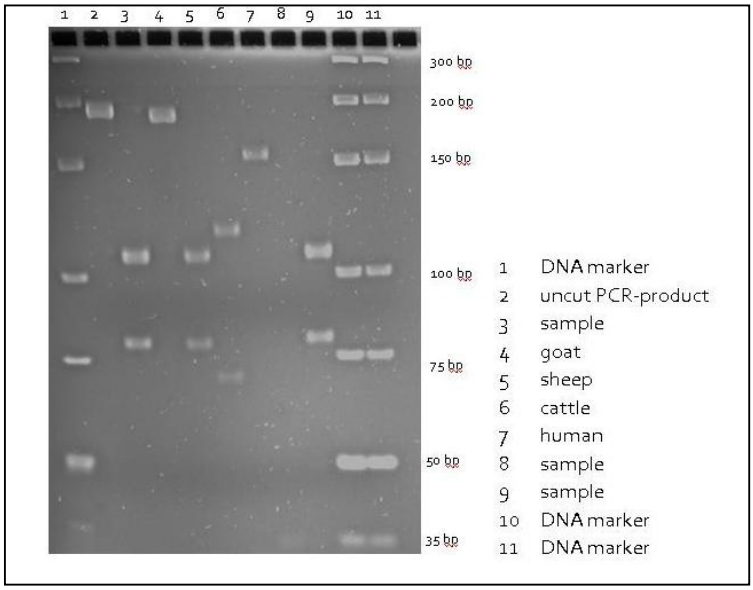

Figure 3: Separated species discriminating DNA-fragments. The PCR-product was cleaved with a restriction enzyme. Species specific DNA pattern are generated by separating the DNA fragments.

Species identification based upon mass spectrometry

Species detection is also possible on protein basis by matrix assisted laser desorption/ ionisation mass spectrometry (MALDI-MS) or liquid chromatography coupled with mass spectroscopy (LC-MS). If the DNA remainder of certain samples for species determination is not high enough, proteomic workflows for protein determination are a third method of choice. To date, a couple of mass spectrometric methodologies exist for the elucidation of the animal species in several end products. Hollemeyer et al. (2002) reported a method using MALDI-ToF (Matrix-assisted laser desorption/ionization coupled with time-of-flight analysis of ions) for the classification of feathers, down and hair which is constantly improved (Hollemeyer et al. 2008; Hollemeyer et al. 2012). A similar method was described for the identification of animal species from bone collagen using MALDI-ToF (Buckley et al. 2009).

The identification method described more deeply in this work, is based on LC-MS. The newly developed approach was inspired by the work of Zhang et al. (2006). This group found a selection of marker peptides that are unique to a given species of either bovine or porcine origin in gelatin samples (Zhang 2006; Zhang et al. 2009) and applied LC-MS for species identification. In the so called ”bottom-up” or ”shotgun” approach for MS based proteomics, the correlation between the peptide and the related protein is lost, because the first step is a tryptic digestion and, as in case of leather solvation, of all protein contained in a sample. In order to get rid of salts and contaminants that accompany the targeted peptides, the samples undergo solid phase extraction using either C8 or C18 phases. The tryptic peptides are eluted with adjustable concentrations of acetonitrile and are concentrated in a vacuum centrifuge. The analytic separation of the peptides is done by reversed-phase HPLC and samples are directly infused into a mass spectrometer. Here we used a Shimadzu Prominence HPLC coupled to a QTRAP 4000 linear ion trap (AB Sciex). Chrome tanned leather samples (Crust) had to be de-tanned prior to enzymatic digestion as described by Datta (1973) and as well as by a modified protocol. Database searches with the obtained mass spectra can be done either using MASCOT MS/MS Ions Search (www.matrixscience.com) or for the distinct species determination with the software ProteinPilot (ABSciex) applying user specified search parameters.

The identification of the correct species is a matter of computer aided data processing. The procedure takes advantage of the data processing algorithm of the program ”Protein Pilot” (AB Sciex). The program assigns an ”unused” score to every detected protein. This score reflects the possibility of a mass spectrum to be explained by differing sequences, and for a peptide to belong to more than one unique protein due to sequence homology. Peptides adding their unused score and hence their degree of detection evidence to a certain protein, do not contribute to the detection and hence the unused score of any another protein.

The species determination algorithm developed by us is additionally based on the sequence comparison of cattle, pig and horse collagen. For the species determination, the collagen types I, II and III are considered. Only unique tryptic peptides that occur once in a certain species were chosen for species determination. After recording the spectra for a sample in an LC-MS run and data processing by Protein Pilot, the contribution scores of every of the unique peptides are summed up in a table, e.g. table 1. The species, for which the unused score is the highest, represents the true origin of the sample.

The limitations of the method are determined by the strength and type of leather processing (esp. tanning, retanning, fatting, hydrophobing). Generally every sample that is susceptible for trypsin hydrolysis, if necessary after proper pretreatments like de-fatting and de-tanning, can be measured and evaluated as well.

Table 1: Example of an analysis result of LC-MS based species determination. The table contains the sum of the peptide contribution scores for unique peptides belonging to a certain species or collagen type

Contrib. scores	Cr cattle	Cr pig	Cr cattle	Cr pig	Salmon
bovine					
COL1A1	7.0	0.0	2.3	0.0	0.0
COL1A2	16.1	0.0	16.0	0.0	0.0
COL2A1	0.0	0.0	0.0	0.0	0.0
COL3A1	6.6	0.0	4.0	0.0	0.0
Sum	**29.7**	**0.0**	**22.3**	**0.0**	**0.0**
porcine					
COL1A1	0.0	4.0	0.0	4.2	0.0
COL1A2	0.0	14.2	0.0	15.9	0.0
COL2A1	0.0	0.0	0.0	0.0	0.0
COL3A1	0.0	4.4	0.0	11.6	0.0
Sum	**0.0**	**22.7**	**0.0**	**31.7**	**0.0**

Conclusions

A leathers animal origin is determined with either microscopy, with analysis of isolated leather DNA, or with an analysis of extracted proteins. Which method is used, depends on the leather sample to be analysed. Microscopy of the leather is suitable for normal leathers with no or hardly any finish. For collagen products, microscopy is not applicable. Species detection based on PCR-RFLP is proven on a broad range of leathers. A successful species determination based on extracted DNA is possible on automotive chrome-tanned leather, untreated glace leather, leather wall papers, but also on collagen fleeces, gelatine solutions, and also parchment. The success rate for species determination based on DNA is 89% for leather products. But because the used additives for leather products are mostly unknown, the success in species determination depends on the particular case. The LC-MS method is of great value in cases, where the RFLP fails due to absence or too small amounts of DNA. The limitations of the method are samples that represent mixtures of different species origin as might occur in food or medical devices.

The best result for species detection on leather and collagen products is gained by combining different methods. This approach is advisable for ancient or challenging samples. In the near future, the DNA and protein based species detection on leather and collagen products will be the methods of choice and will be extended to distinguish between a larger number of species. They provide, in contrast to microscopy, secure conclusions related to the animal origin of a particular sample.

References

Buckley, M., Collins, M., Thomas-Oates, J., and Wilson, J. C., 2009, Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, Rapid communications in mass spectrometry, 23, 3843–54.

Burger, J., Schoon, R., Zeike, B., Hummel, S., Herrmann, B., 2002, Species Determination using Species-discriminating PCR-RFLP of Ancient DNA from Prehistoric Skeletal Remains, Ancient Biomolecules, 4, 19-23.

Datta, SS., 1973, An Introduction to the Principles of Leather Manufacture, in: Indian Leather Technologist Association, Lal Bazar, Calcutta.

Fuchs, K., Fuchs, M., Derichs, L. 2008, Faszination Leder. Alltägliches und Exotisches unter der Lupe. Edition Chimaira, Frankfurt am Main.

Hollemeyer, K., Altmeyer, W., and Heinzle, E., 2002, Identification and Quantification of Feathers, Down, and Hair of Avian and Mammalian Origin Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, Analytical Chemistry, 74, 5960–5968.

Hollemeyer, K., Altmeyer, W., Heinzle, E., and Pitra, C., 2008, Species identification of Oetzi 's clothing with matrix-assisted laser desorption / ionization time-of-flight mass spectrometry based on peptide pattern similarities of hair digests, Rapid communications in mass spectrometry, 22, 2751–2767.

Hollemeyer, K., Altmeyer, W., Heinzle, E., and Pitra, C., 2012, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry combined with multidimensional scaling, binary hierarchical cluster tree and selected diagnostic masses improves species identification of neolithic keratin sequences from furs of the Tyrolean Iceman Oetzi, Rapid communications in mass spectrometry, 26, 1735–45.

Meyer, R., Höfelein, C., Lüthy, J. Candrian, U., 1995, Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food, J.A.O.A.C. Int., 78, 1542-1551.

Reynolds, R., Saiki, R., Grow, M. et al., 1992, The development and evaluation of new genetic markers for the application of PCR to forensic casework, in: C. Rittner, P.M. Schneider (Eds.), Advantages in Forensic Haemogenetics, Springer, Heidelberg, pp 29-31.

Vuissoz, A., Worobey, M., Odegaard, N. et al., 2007, The survival of PCR-amplifiable DNA in cow leather. Journal of Archaeological Science, 34, 823-829.

Zhang, G., Sun, A., Li, W., Liu, T., and Su, Z., 2006, Mass spectrometric analysis of enzymatic digestion of denatured collagen for identification of collagen type, Journal of chromatography A, 1114, 274–7.

Zhang, G., Liu, T., Wang, Q., Chen, L., Lei, J., Luo, J., Ma, G., and Su, Z., 2009, Mass spectrometric detection of marker peptides in tryptic digests of gelatin: A new method to differentiate between bovine and porcine gelatin, Food Hydrocolloids, 23, 2001–2007.