



Short communication

Rapid characterisation of cell cultures by matrix-assisted laser desorption/ionisation mass spectrometric typing

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Misidentification or cross-contamination of cultured cell lines used for scientific or diagnostic purposes are a continuing challenge for laboratories and tissue culture repositories. Institutions dedicated to veterinary virology are particularly affected since the variety of viruses under investigation often requires the parallel maintenance of numerous different cell lines from different host species. To provide rapid but yet exact characterisation of cell cultures, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometric typing was applied to 66 cell culture samples representing 34 species from insects to primates. A reference spectra library was generated that allows unambiguously the identification of all 66 cell lines. Spectrum-based phylogenetic analysis showed that clustering was mainly driven by taxonomy and allows the species determination of unknown samples.

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In his recent white paper titled 'Eradication of cross-contaminated cell lines: A call for action' (Nardone, 2007), Roland Nardone has listed numerous reported cases of misidentification or cross-contamination of cell lines, a problem obviously persisting for decades. Additionally, large numbers of unreported or unrecognised cases may be assumed (Masters et al., 2001). Procedures for the authentication or species determination of cell lines such as karyotyping, 2D-electrophoretic profiling, as well as restriction length polymorphism, short tandem repeat or isoenzyme analysis are available but do not seem to be part of the daily routine in most laboratories, for reasons of cost, time or equipment. Availability of a linear MALDI mass spectrometer assumed, MALDI typing could therefore serve as a rapid and economic alternative for the confirmation of the identity of cell cultures.

The Collection of Cell Lines in Veterinary Medicine within the Friedrich-Loeffler-Institut is an animal tissue culture repository dedicated to the collection and characterisation of cell cultures used in veterinary virology. As propagation and characterisation of animal viruses depends generally on the choice of a suitable cell line, most likely derived from the host species preferred by particular viruses, numerous cell lines from different species are conserved at the Collection of Cell Lines in Veterinary Medicine for "in-house" and public distribution. Authenticity of deposited cell lines is assured routinely by karyotyping and sequence analysis of

part of the cytochrome b gene (Wolf et al., 1999). As an additional quality control, a rapid examination method of all incoming and outgoing cell samples is desirable, which is sufficiently powerful to confirm unambiguously the identity of outgoing cell cultures and sort out misidentified incoming cell samples before they undergo any further labour-intensive or costly genetic or biochemical characterisation.

For this purpose, a rapid procedure for the identification and taxonomic classification of microorganisms (Heller et al., 1987; Platt et al., 1988; Sauer et al., 2008) based on MALDI-TOF mass spectroscopic analysis of whole cell extracts was expanded to cultured animal cells. Sixty-four well characterised stable cell lines (Table 1) covering a wide range of species and two batches of primary chicken cell cultures prepared independently were obtained from the Collection of Cell Lines in Veterinary Medicine. Biotyper software (Bruker Daltonics, Bremen, Germany), designed originally for the identification of bacteria, was used for the generation of a reference spectra collection, the identification procedure and phylogenetic calculations.

For the construction of a custom-made reference spectra database, the guidelines of the manufacturer were followed (Sauer et al., 2008). Briefly, extracts of authentic samples were prepared and spotted on six sample spots of a steel MALDI target. From every spot four single spectra with 500 shots each were acquired, and a reference spectrum was calculated from the 24 single spectra. Reference spectra contain the usual parameters of mass spectra (peak mass and intensity) and additional information on the reproducibility of the mass peaks in form of the frequency of occurrence

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Table 1

List of cell lines used for MALDI typing.

Code/medium	Symbol	Species	Common name	Organ/tissue
0755/IF	WN-R	Bison bonasus	European bison	Kidney
0030/MH	KM-R	Bos taurus	Cattle	Milt
0050/IF	KNS-R	Bos taurus	Cattle	Nasal mucosa
0261/MHE	MDBK	Bos taurus	Cattle	Kidney
0970/MH	FKD-R1	Bos taurus	Cattle	Intestine
0972/IF	FP-R	Bos taurus	Cattle	Rumen
0977/IF	KSE-R	Bos taurus	Cattle	Coronary band
0978/IF	FE-R	Bos taurus	Cattle	Udder
0083/MHE	MDCK	Canis lupus familiaris	Dog	Kidney
1061/MHE	MDCK-2	Canis lupus familiaris	Dog	Kidney
0122/MH	ZN-R1	Capra hircus	Goat	Kidney
0127/IF	ZZ-R	Capra hircus	Goat	Tongue
0142/MHE	MA-104	Chlorocebus sabaues	African green monkey	Kidney
0078/MHE	Vero	Chlorocebus sabaues	African green monkey	Kidney
0228/MHE	Vero 76	Chlorocebus sabaues	African green monkey	Kidney
0256/IF	TGE	Columba livia	Domestic pigeon	Embryo
0999/IF	QM-9	Coturnix coturnix japonica	Japanese quail	Muscle
0816/ME	CCB	Cyprinus carpio	Common carp	Brain
0843/ME	KF-1	Cyprinus carpio	Common carp	Fin
1112/ME	KFC	Cyprinus carpio	Common carp	Fin
1091/I	FLN-R	Eptesicus serotinus	Common serotine	Kidney
0351/G	EaA	Estigmene acrea	Saltmarsh caterpillar	Larva
0138/MH	KE-R	Felis catus	Domestic cat	Embryo
^a NA/MH	^a NA	Gallus gallus domesticus	Chicken	Liver (primary)
^a NA/MEH	^a NA	Gallus gallus domesticus	Chicken	Fibroblasts (primary)
0417/MEH	LMH	Gallus gallus domesticus	Chicken	Liver
1029/IF	DF-1	Gallus gallus domesticus	Chicken	Fibroblasts
0082/MEH	HeLa	Homo sapiens	Human	Cervical carcinoma
0141/MH	Hep-2	Homo sapiens	Human	Cervical carcinoma
0960/IF	HT-29	Homo sapiens	Human	Intestinal carcinoma
0962/IF	NCI-H460	Homo sapiens	Human	Lung carcinoma
1151/R	LoVo	Homo sapiens	Human	Colorectal carcinoma
1001/IF	HN-R	Lepus europaeus	European hare	Kidney
0200/MH	PF	Meleagris gallopavo	Turkey	Embryo
0179/MEH	BHK-21	Mesocricetus auratus	Golden hamster	Kidney
1102/MH	FMN-R	Microtus arvalis	Common vole	Kidney
1129/IF	FMG-R	Microtus arvalis	Common vole	Brain
0281/IF	MTH-R	Mus musculus	House mouse	Thymus
0686/IF	RTG-2/f	Oncorhynchus mykiss	Rainbow trout	Ovary
1104/ME	CHSE-214	Oncorhynchus tshawytscha	Chinook salmon	Embryo
0109/MH	RK13	Oryctolagus cuniculus	Rabbit	Kidney
0901/MEH	KMK-R	Oryctolagus cuniculus	Rabbit	Bone marrow
1014/IF	KFT-R	Oryctolagus cuniculus	Rabbit	Thymus
0011/MEH	PtK2	Ovis aries	Sheep	Kidney
0017/MEH	PO	Ovis aries	Sheep	Kidney
0043/MH	SFT-R	Ovis aries	Sheep	Thymus
0175/MH	ESH-L	Ovis aries	Sheep	Skin
0201/MH	ESP	Ovis aries	Sheep	Plexus
0271/MH	EGE	Pica pica	Magpie	Embryo
0057/MH	FHM	Pimephales promelas	Fathead minnow	Caudal fin
0183/MH	LTA	Rattus norvegicus	Norway rat	Fibroblasts
0926/L	ASK	Salmo salar	Atlantic salmon	Kidney
0352/G	SF9	Spodoptera frugiperda	Fall armyworm	Larva
0379/MEH	WSL-R	Sus scrofa	Pig	Liver
0008/MEH	SPEV	Sus scrofa domestica	Domestic pig	Kidney
0086/MEH	EFN	Sus scrofa domestica	Domestic pig	Kidney
0086-R/MEH	EFN-R	Sus scrofa domestica	Domestic pig	Kidney
0170/IF	EFH-12	Sus scrofa domestica	Domestic pig	Testicle
0262/MEH	SK-6	Sus scrofa domestica	Domestic pig	Kidney
0606/IF	ST	Sus scrofa domestica	Domestic pig	Testicle
0005-1/MEH	PK15	Sus scrofa domestica	Domestic pig	Kidney
0072/MH	Tb1Lu	Tadarida brasiliensis	Brazilian free-tailed bat	Lung
1131/MH	TH-1	Terrapene carolina	Common box turtle	Heart
0483/MH	SKH	Trachemys scripta elegans	Red-eared slider	Heart
0350/G	High5	Trichoplusia ni	Cabbage looper	Larva
0322/MEH	Fufe	Vulpes vulpes	Redfox	Embryo

For construction of the reference database, cells were grown in their preferred cell culture medium: IF: Iscove's DMEM/F12, 1:1; ME: MEM with Earle's salts; MH: MEM with Hanks' salts; MEH: ME/MH, 1:1; R: RPMI 1640; G: Grace's insect medium; L: Leibovitz 15; D: DMEM with Earle's salts, low glucose; IE: InsectExpress SF9-S2 (PAA, Pasching, Austria).

^a NA denominates two chicken primary cell batches which do not carry codes.

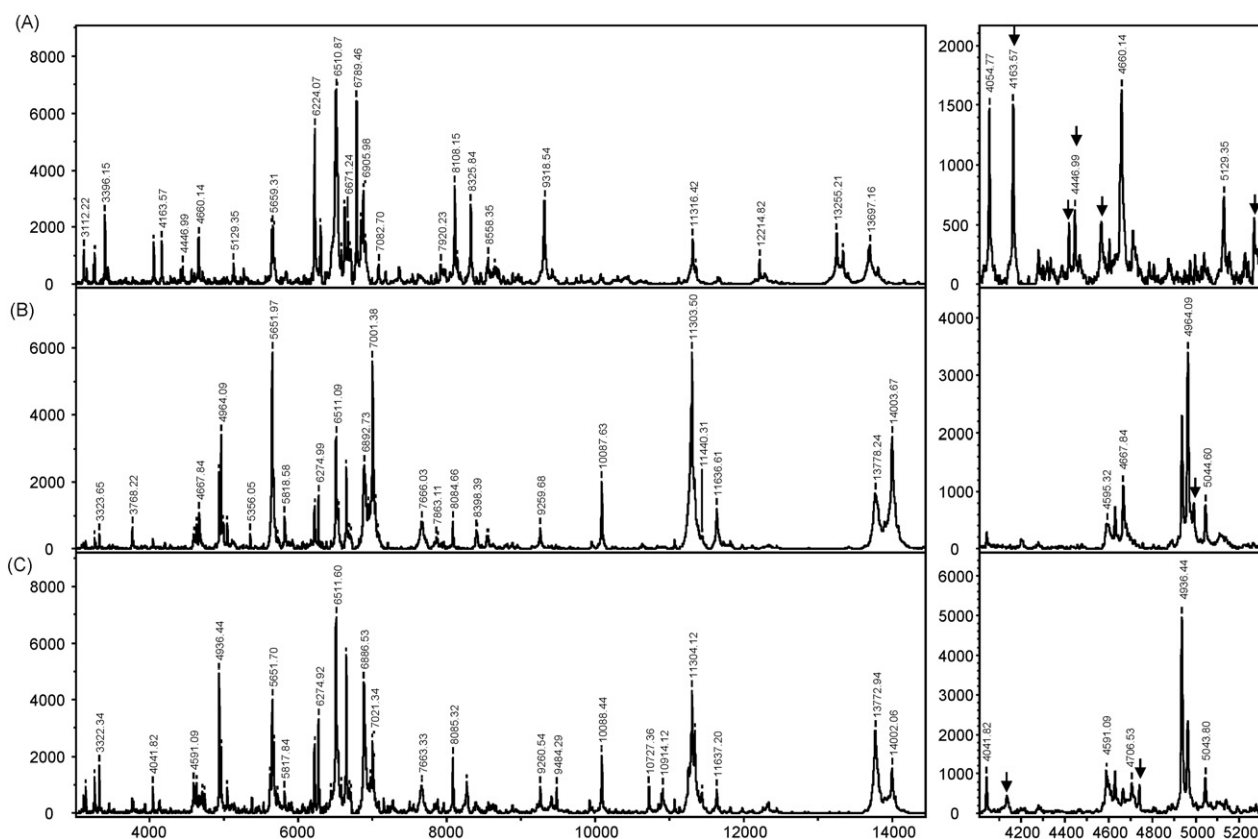


Fig. 1. Sample spectra from cell lines 0352 (panels A, SF9, *Spodoptera frugiperda*), 0962 (panels B, NCI-H460, *Homo sapiens*) and 0078 (panels C, Vero, *Chlorocebus sabaeus*). In the mass range from 4000 Da to 5300 Da of the magnified spectrum (on the right) major masses unique for the insect cell and masses differentiating the two primate cells are highlighted by arrows. Masses are given in Da on the x-axis, intensities in arbitrary units on the y-axis.

of every peak in the underlying 24 single spectra. Reference spectra were generated within the mass range of 3500–15,000 Da with the following default parameters: compression of the spectrum data by a factor of 10, baseline smoothing by the Savitsky–Golay algorithm (25 Da frame size), baseline correction by 2 runs of the multipolygon algorithm, and peak search by spectra differentiation. The number of peaks was limited to 100 per reference spectrum and all peaks of a reference spectrum were normalized to the most intense peak with an intensity of 1.0. Reference spectra of all samples were joined in a database which was the basis for all further calculations. Biotyper software estimates the similarity between spectra by a scoring algorithm on the basis of mass matches within 2 weighted tolerance frames of 200 ppm and 600 ppm and the frequency of a matched mass in the 24 single spectra underlying the reference spectrum. Scores range between 3 (identical) and 0 (completely unrelated). In the ‘identification’ mode the software compares a sample spectrum with the spectra of the database on the basis of similarity scores and returns ranked hit lists of the most similar spectra. Scores are also the basis for the calculation of dendrograms for which ‘correlation’ and ‘average’ were selected as the distance measure and the linkage algorithm in the respective menu. Cell culture conditions were chosen for the requirements of the respective cell line. Usually, cells were grown to confluent monolayers on plastic cell culture flasks with 25 cm² growth area (Corning, Wiesbaden, Germany) in suitable cell culture media containing 10% fetal calf serum, at 37 °C in a humidified 5% CO₂ atmosphere. Whole cell extracts were prepared as described for bacteria (Sauer et al., 2008) with slight modifications. Adherent cell monolayers were washed with sorbitol buffer (150 mM sorbitol supplemented with Complete™ protease inhibitors, Roche Diagnostics, Mannheim, Germany) three times, scraped into 1 mL

sorbitol buffer and transferred to a microcentrifuge tube. Cells were thoroughly resuspended and the optical density at 600 nm was adjusted to 0.2 with sorbitol buffer. 300 µL of the suspension was transferred to a fresh tube, cells were precipitated by addition of 900 µL ethanol (98%) and sedimented for 5 min at 10,000 × g. The supernatant was carefully removed and the sediment resuspended in 50 µL of 70% formic acid. After mixing with 50 µL acetonitrile, the suspension was centrifuged as above and the supernatant was transferred to a fresh tube. 1.5 µL of the extract was spotted to a steel MALDI target and allowed to dry at ambient temperature. Finally, the dried extract was overlaid with 1.5 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid as matrix and was again allowed to dry.

Spectra were acquired with an Ultraflex I instrument (Bruker) in linear positive mode in the range from 2000 to 15,000 Da. Acceleration Voltage was 25 kV and the instrument was calibrated in the range from 4364 to 10,299 Da with reference masses of an extract of *E. coli* DH5-α strain prepared according to Sauer et al. (2008). Example spectra of an insect cell line and two cell lines derived from primates are shown in Fig. 1. All peaks highlighted there were present in 18 or more of the 24 reference spectra used for the calculation of the reference spectrum of the respective species.

When reference spectra from cell lines represented in the spectra collection were examined with the identification procedure of Biotyper software against the database with 66 entries, the respective cell line was in all cases identified as the top-ranking entry of the hit list. Without exception, the highest-ranking entries of the hit list were the cell cultures of the reference database which originated from the same species (Fig. 2) if spectra from other cell lines of the same species were available. Scores of cell lines from

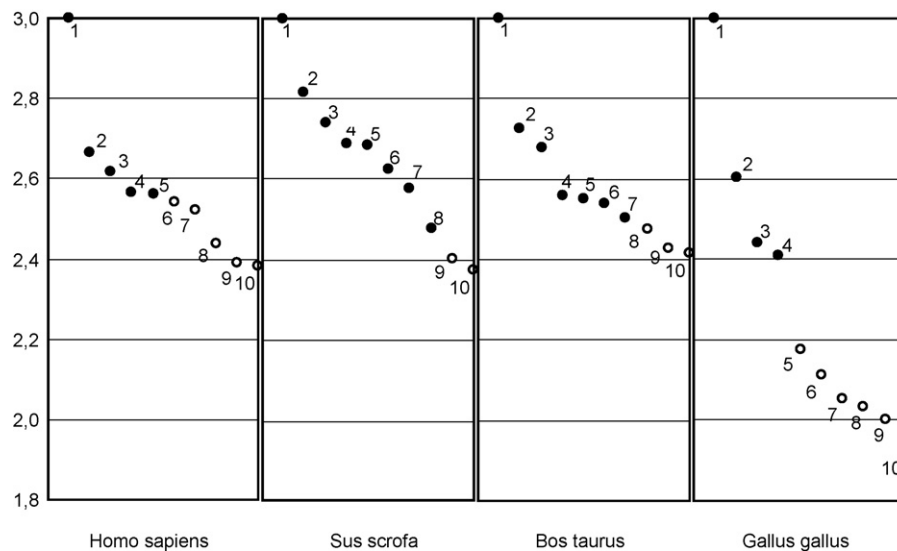


Fig. 2. Score distribution of database queries on cell lines from *Homo sapiens*, *Sus scrofa*, *Bos taurus*, and *Gallus gallus*. Reference spectra representing the 4 species were examined with the identification procedure of Biotyper software against a database of 66 cell lines. Similarity scores are labelled with the rank numbers of the resulting hit lists. Filled symbols indicate hits representing the same species as the examined cell line, open symbols mark cell cultures from species differing from the examined cell line. Note that highest scores (y-axis) were always obtained for the examined cell line itself (rank 1, score 3.0), followed by all other cell lines from the same species that were available in the database. Logarithmic scores vary from 3 (identical) to 0 (unrelated).

the same as the examined species ranged between 3.0 and a lower limit between 2.6 and 2.4 in all cases (Fig. 2). In no case where a species was represented in the spectra collection by a single cell line only, the score of the second-ranking entry, a cell line from a different species, exceeded 2.6, indicating that the score range from 2.6 to 3.0 was reserved for database entries representing the species of the examined cell line and thus identified samples originating from the same species as the highest-ranking entry of the hit list.

To test the procedure under realistic conditions, the identification procedure was performed with single spectra with 500 shots of all cell cultures present in the database and samples of human, bovine, swine and murine origin that were not represented in the database. In all cases the top-scoring database entry represented the species of the sample under examination indicating that species of unknown samples can be identified by MALDI typing. The courses of the scores over the top-ranking database entries differed from the results obtained with bacterial samples. Whereas in the latter case a more or less sharp decrease of the scores was observed for species differing from the examined sample (data not shown), the decline of the scores was flatter and without distinct steps at species borders for the samples from tissue cultures, indicating that spectra from cell cultures showed a higher degree of similarity than those from bacteria. Indeed, comparison of the mass lists from cell culture samples revealed that four masses (5660, 6529, 6657, and 11,318 Da) were found in all, 8 more in 65 of the 66 reference spectra, suggesting that a more critical selection of masses may further improve the performance of the procedure. None of the four masses present in all 66 reference spectra was found in mass spectra of fetal calf serum or cell culture medium samples suggesting that they had not been carried over from the medium but rather represented conserved proteins.

The dendrogram constructed from the 66 reference spectra reflects phylogenetic relations of most of the species well (Fig. 3). Clustering was dominated by taxonomy, whereas other characteristics like the tissue type, organ, or passage number had no noticeable influence. In this regard, it is interesting to note that of the four closely clustering cell samples from chicken, two were primary cells (highlighted by an asterisk in Fig. 3) and two were stable cell lines, indicating that MALDI-TOF spectra remain stable

as species signatures in the course of the development of a stable cell line.

The robustness of the procedure to variation of culture conditions and to contamination was tested with a selection of cell lines used widely. Species identification was not hampered by variation of the culture medium or cell density. Cells were grown in different but suitable media (BHK: IF, MEH, D; Vero: D, IF, ME, MH, MEH, R; High5: IE, G, M; MDCK: IF, MEH, D; abbreviations as in Table 1) or to confluence and to 5% cell density in their respective standard medium indicated in Table 1 (BHK, MDBK, RK13). Reference spectra were generated and examined with the identification procedure of Biotyper software against the database of confluent cells grown in their preferred medium (Table 1). In all cases the species of the cells were identified with scores exceeding 2.8 for cells grown in different media and scores over 2.6 for the cells grown to different cell densities. Fresh contamination of cell cultures was simulated by analysis of mixtures from extracts of Hela and BHK cells at ratios of 1:4, 1:1, and 4:1. In the identification procedure the resulting spectra produced hit lists with cells from both species ranking highest but with insignificant scores for the 1:4 and 4:1 mixtures so that the procedure is not recommended for screening for fresh contamination. Viral contamination was simulated by infection with Pseudorabies virus (PrV) (Kaplan and Vatter, 1959), an alphaherpesvirus with broad host range. BHK (gold hamster), Hela (man), MDBK (cattle), and SPEV (pig) cells were infected with PrV at a multiplicity of infection of 5.0, prepared for mass spectrometry 12 h post-infection and the resulting spectra were tested against the database of non-infected cells. Irrespective of the infection, all host-cell species could be identified with scores over 2.6. Spectra of PrV-infected cells were also examined against a database in which reference spectra from non-infected and the four reference spectra from infected cells had been joined. Under these conditions, the infected cell culture under examination always ranked highest followed by non-infected representatives of the species of the respective host-cell, indicating that the procedure may be expanded to identify viral contamination in cell cultures if appropriate references are available. In this respect it was interesting to note that similarity scores between PrV-infected cells from different species were considerably higher than between their non-infected counterparts.

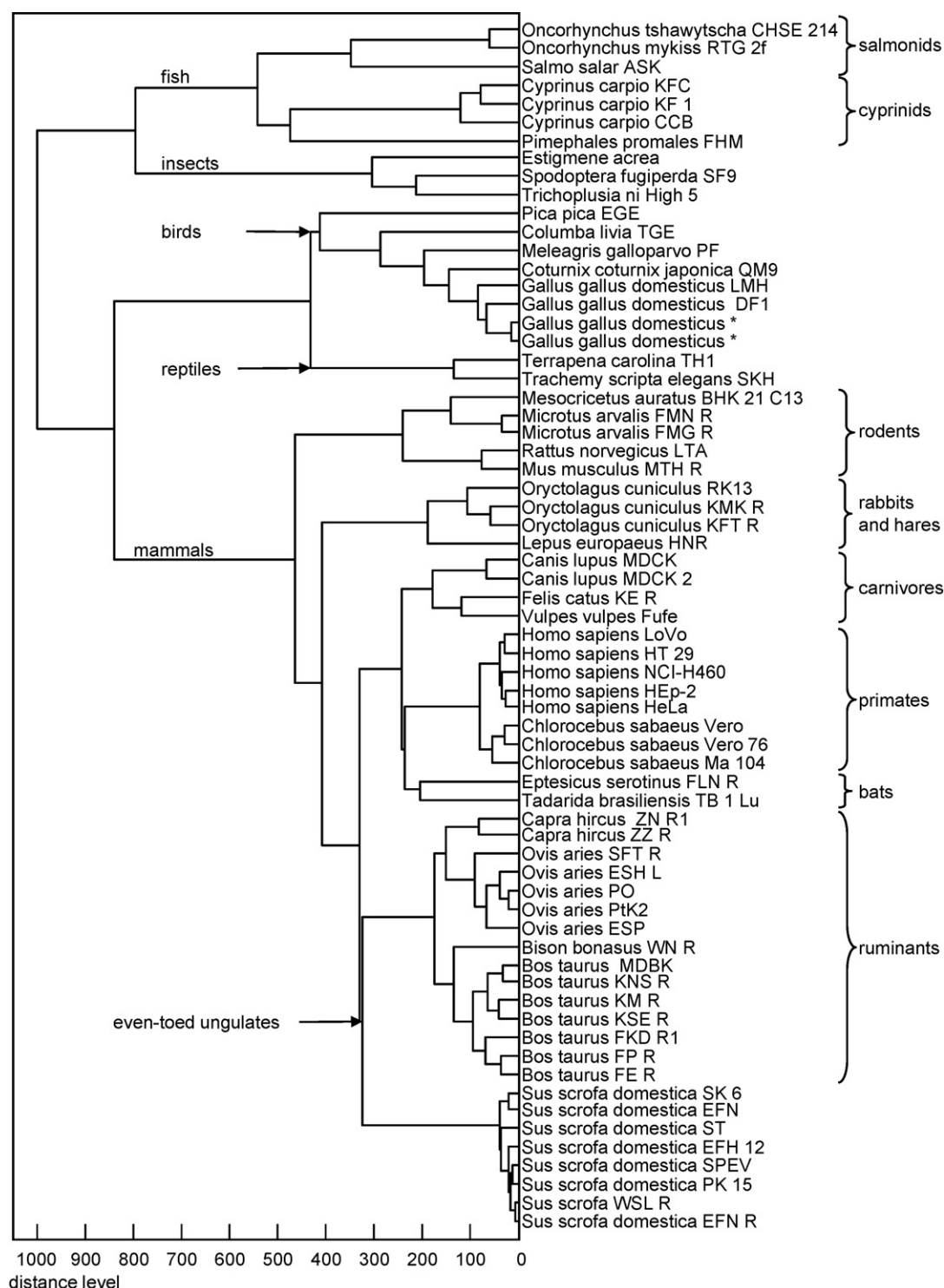


Fig. 3. Phylogenetic tree constructed from reference spectra of cell lines from a wide range of animal species. Systematic species designations and relevant taxonomic groups (in English) are given. Two independent chicken primary cell preparations are indicated with an asterisk. Phylogenetic distances are based on similarity scores calculated from the reference spectra as described in the text.

The procedure described above identified unambiguously cell lines for which reference spectra were available in the spectra library. Depending on the availability of reference spectra from the same or related species, unknown samples could be classified taxonomically to the level of species identification. The procedure is rapid, the cost per sample negligible, construction of laboratory-specific spectrum libraries is feasible and scheduled MALDI-TOF based assessment of the authenticity of the cell lines in use is

straightforward. Results for single samples can be expected within an hour, so that ongoing experiments do not suffer serious delays. For tissue culture repositories with access to a linear MALDI mass spectrometer, MALDI typing may be of use as a rapid and inexpensive additional quality control for incoming and outgoing cell culture samples. Cell-specific mass lists may serve as a characteristic bar-code complementing cytological, biochemical, genetical or virological data collected for its characterisation. Further refine-

ment of the statistical analysis e.g. by creation of laboratory-specific background mass lists or by identification of species-specific or cell line-specific mass signatures may allow a more detailed characterisation of cell lines which could be required e.g. for intra-species differentiation or in-process applications in the course of protein (e.g. vaccine) production.

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