Int J Legal Med (2012) 126:815–823
DOI 10.1007/s00414-012-0733-3

SHORT COMMUNICATION

Comparison of bacterial DNA profiles of footwear insoles and soles of feet for the forensic discrimination of footwear owners

Haruhisa Goga

Received: 14 February 2012 / Accepted: 13 June 2012 / Published online: 23 June 2012

Abstract It is crucial to identify the owner of unattended footwear left at a crime scene. However, retrieving enough DNA for DNA profiling from the owner's foot skin (plantar skin) cells from inside the footwear is often unsuccessful. This is sometimes because footwear that is used on a daily basis contains an abundance of bacteria that degrade DNA. Further, numerous other factors related to the inside of the shoe, such as high humidity and temperature, can encourage bacterial growth inside the footwear and enhance DNA degradation. This project sought to determine if bacteria from inside footwear could be used for footwear trace evidence. The plantar skins and insoles of shoes of volunteers were swabbed for bacteria, and their bacterial community profiles were compared using bacterial 16S rRNA terminal restriction fragment length polymorphism analysis. Sufficient bacteria were recovered from both footwear insoles and the plantar skins of the volunteers. The profiling identified that each volunteer's plantar skins harbored unique bacterial communities, as did the individuals' footwear insoles. In most cases, a significant similarity in the bacterial community was identified for the matched foot/insole swabs from each volunteer, as compared with those profiles from different volunteers. These observations indicate the probability to discriminate the owner of footwear by comparing the microbial DNA fingerprint from inside footwear with that of the skin from the soles of the feet of the suspected owner. This novel strategy will offer auxiliary forensic footwear evidence for human DNA identification, although further investigations into this technique are required.

H. Goga (✉)
Forensic Science Division, Department of Criminal Investigation,
Okinawa Prefectural Police HQ,
1-2-2 Izumizaki,
Naha, Okinawa 900-0021, Japan
e-mail: kasouken@police.pref.okinawa.jp

Keywords Bacterial DNA profiling · Footwear insole · Skin bacterial community · Forensic · Terminal restriction fragment length polymorphism

Introduction

In criminal investigations, it is essential to link unattended footwear left at a crime scene with the owner of the footwear. In recent years, DNA profiling has provided us with key forensic evidence to identify the owner of the shoes through the use of human skin cells (epithelial cells) sloughed off the sole of the owner's foot that are transferred onto the shoe insole by sweat or abrasion [1]. However, there is often an insufficient quantity of human DNA, also referred to as low copy number (LCN) DNA, retrieved from inside the shoe, which limits the success of attaining a DNA profile [2]. The previous case studies have identified a failure to obtain human DNA profiles from skin samples from the inside of footwear in 51 % of cases (23 of 45), and only partial profiles in 38 % of cases (17 of 45) using commercial short tandem repeat (STR) multiplex typing kits. Aside from the variation in the amount of individual donor DNA transferred from skin surface [3], DNA from forensic samples is also susceptible to degradation through exposure to various environmental factors, such as heat, water, bacteria, and ultraviolet rays, to name a few [4]. Indeed, numerous bacteria reside within regularly worn footwear [5–7], with the inside of the shoe providing a warm and moist location for bacterial inhabitance and growth [8]. Consequently, these bacteria often contaminate forensic skin samples; this represents one of the major causes for failure to determine a DNA profile from skin cell samples from inside of footwear. Furthermore, the elevated temperature and humidity within the footwear likely further increases chemical-induced DNA degradation of forensic samples

within the footwear. Together, these factors severely hamper the successful retrieval and amplification of human DNA from inside the shoe for forensic investigations.

Forensic strategies to target the problem of DNA degradation have been assessed, using a reduced size of PCR amplicons [9] and employing LCN analyses for the limited quantity of DNA [10]. However, problems still persist. Prior studies have already used molecular analysis of bacterial communities to discriminate soil samples for forensic purposes [11]. As such, it would be valuable for forensics investigations to take advantage of the bacteria inside the footwear for the purposes of obtaining forensically relevant information about a wearer, and therefore provide an alternative to human DNA evidence to link the owner with the unidentified footwear.

Human skin can harbor a wide diversity of bacterial communities at any location [12–14], with studies showing that the human foot sole is host to a personalized and relatively stable microbial community [12, 13]. For instance, Fiere et al. recently demonstrated the utility of the bacterial community from the skin on the fingertips in forensic identification, exploiting the bacteria that are transferred to the surfaces of objects that people regularly touch, such as computer keyboard and mouse [15]. However, the sampling of human fingertip microflora as a reference for these bacterial communities is the question at issue from the study of Tims et al. [16]. Given this, it is hypothesized that bacteria inside regularly worn footwear would be transferred from the owner's plantar skin to the insole of the footwear, and that the bacterial community profile between the footwear insole and the owner's plantar skin would be similar, as compared with the plantar skin of another person who has never worn the footwear. As such, this may offer a useful tool for owner–footwear discrimination.

Terminal restriction fragment length polymorphism, T-RFLP, is a simple and reproducible method that is widely used for the analysis of microbial diversity [17, 18]. Furthermore, it can also be easily introduced into forensic laboratories that are already performing human DNA typing on a routine basis. If bacterial profiling and comparison of the owner–shoe bacterial community is to be considered as a forensic tool, it is surmised that three hurdles must be overcome. First, a sufficient amount of bacterial DNA must be successfully recovered from both the footwear insole and the plantar skin surface. Second, the bacterial community profiles on each footwear insole must be unique. This is likely to be the case, as previous studies examining the skin bacterial community profiles from the soles of feet showed unique profiles [12, 13]. Third, the bacteria on the insole needed to significantly match with the bacteria on the plantar skin of the owner, but not with that of another individual, as determined by bacterial community profile comparison.

To address these three questions, volunteers were swabbed for bacteria from their footwear insoles and the soles of their feet, and the similarities between these two microbial community profiles were compared. The goal of this pilot study was to discriminate the owner of the footwear by the bacteria present on the insoles of the footwear and the soles of the feet using bacterial 16S ribosomal RNA (rRNA) genes with T-RFLP analysis to determine its usefulness as a forensic application. Here, a series of studies that demonstrate and discuss the validity and utility of this approach is presented.

Materials and methods

Human subjects and footwear specimens

Fourteen unrelated Japanese people (13 males and 1 female) who work at the same office volunteered for this study. All volunteers were healthy at the time of sampling. A total of 14 pairs of regularly used footwear (all different types of sneakers) were provided by the volunteers. Information about volunteers and their footwear is described in Table 1. All of the volunteers usually wore socks when wearing their sneakers. The footwear from each volunteer had never been worn by any of the other volunteers in this study. Informed consent was obtained from all volunteers.

Sample collection

Swabbing has been shown to be an ideal method for sample collection for microbial community analysis [12, 15]. Therefore, to collect bacterial samples, the bare feet of each volunteer and the insoles from their pair of footwear were swabbed for 3 min with sterilized cotton swabs (#104; Kawamoto Corporation, Osaka, Japan) premoistened with 400 μL of sterilized distilled water. Sample collection from the plantar skins of each volunteer was conducted before washing during the day time. The samples were collected between February 18, 2011 and October 19, 2011 (Table 1). Moistened swabs that were not used to collect a sample were used as a negative (extraction) control to check for sample contamination. All sample swabs were stored at −20 °C until DNA extraction for less than 1 week. A previous study demonstrated that storage conditions (−20 or 20 °C) had little influence on temporal stability of swabbed bacterial community composition, even after 2 weeks [15].

Second sample collection for a parallel investigation

A second, smaller investigation was carried out in parallel to examine the effects of footwear insole material and the duration of wearing the footwear on the bacterial diversity

Table 1 Sample information

Footwear	Footwear type	Owner's sex	Owner's age	Sample collection date
1	Sneaker	Male	28	February 18, 2011
2	Sneaker	Male	53	March 11, 2011
3	Sneaker	Male	57	March 11, 2011
4	Sneaker	Male	51	March 30, 2011
5	Sneaker	Male	47	April 1, 2011
6	Sneaker	Male	37	May 1, 2011
7	Sneaker	Male	29	May 4, 2011
8	Sneaker	Female	31	May 20, 2011
9	Sneaker	Male	27	May 20, 2011
10	Sneaker	Male	55	July 4, 2011
11	Sneaker	Male	33	July 7, 2011
12	Sneaker	Male	31	October 19, 2011
13	Sneaker	Male	27	October 19, 2011
14	Sneaker	Male	47	October 19, 2011

of the footwear insole. Four male individuals from the original cohort of volunteers (owners of the footwear #1, 5, 9, and 11; see Table 1), all of whom had already been swabbed for the main study, were randomly selected and issued with an identical new pair of sneakers that were purchased at the same shoe store at the same time. These four subjects were requested to wear the sneakers for approximately 1 month (July 25, 2011 to September 2, 2011). Following this, the same sampling technique was used to collect samples from these four subjects on September 2, 2011. The sneakers were not worn by any of the other volunteers.

DNA extraction

Sample DNA was extracted from the cotton swabs using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) as modified by Fierer et al. [15]. Cotton tips of stored swabs were cut with sterilized scissors and directly immersed into bead tubes containing 60 μL of Solution C1. Bead tubes were incubated at 65 °C for 10 min and then vortexed for 2 min. The remaining steps were performed in accordance with the manufacturer's protocol. Extraction controls were included for every extraction procedure. Extracted DNA samples were stored at −20 °C until used.

Amplification and digestion

The bacterial 16S rRNA gene from each DNA sample was amplified in duplicate simultaneously by polymerase chain reaction (PCR) using 5′-6-FAM oligonucleotide primers: 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) [11, 18]. The amplified product was approximately 1,300 bp. The PCR reaction composition included 2.5 μL of 10× Ex Taq® buffer (Takara Bio Inc., Shiga, Japan), 2 μL of dNTP Mixture (Takara Bio), 0.5 μM of each primer, 0.6 U of TaKaRa Ex Taq® Hot Start Version (Takara Bio), 2.5 μL of template DNA and sterilized distilled water in a final volume of 25 μL. PCR reactions were denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step of 72 °C for 10 min [18] using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Negative controls using sterilized distilled water were included to check for sample DNA contamination. All PCR products (7.5 μL) were digested at 37 °C for 3 h with restriction enzyme *Hha*I [11, 18] in a reaction containing 5 U of *Hha*I (Takara Bio) and 1 μL of 10× buffer M (Takara Bio) in a final volume of 10 μL. The digestion was terminated by deactivating the restriction enzyme at 95 °C for 10 min.

Electrophoresis

Digested PCR products (1 μL) were electrophoresed using Genetic Analyzer 3130xl (Applied Biosystems), 3130 POP-4™ polymer (Applied Biosystems) and a 36-cm Capillary Array (Applied Biosystems). Hi-Di™ Formamide (15 μL; Applied Biosystems) and GeneScan™ -500 LIZ® Size Standard (0.1 μL; Applied Biosystems) as an internal size standard were used per sample, and denatured at 95 °C for 3 min, followed by chilling on ice for 3 min. Electrokinetic injection was performed at 1.2 kV for 18 s. Fragments were separated at 15 kV for 1,500 s at 60 °C.

Analysis of T-RFLP profiles and community comparison

Microbial T-RFLP data were analyzed using GeneMapper® ID software (ver.3.2.1; Applied Biosystems). DNA

fragments in the range of 90 to 500 bp with a detection limit of 50 relative fluorescent units (RFUs) were included. GeneMapper ID sizing tables containing peak positions and peak heights (same as RFU) per samples were exported to Microsoft Excel. Initially, consensus bacterial T-RFLP profiles for each sample were constructed [19], where reproducible peaks that appeared in every replicate profile between the duplicate samples were identified, and non-producible peaks were eliminated as background noise. The average size and height of each reproducible peak were calculated, and the sets of newly calculated averaged size and height of reproducible peaks were assigned as the bacterial T-RFLP profiles of the each sample for subsequent use. Bacterial T-RFLP profiles were then normalized to allow comparisons to be made between samples of equal size (i.e., equal amounts of DNA) [20, 21]. Briefly, the peak heights of comparative average profiles of each sample were summed, and the larger total peak height was divided with the smaller one to determine the correction factor. The height of each peak in a larger total height profile was divided by the correction factor to standardize to the smaller one. Any peak with a new height below 50 RFU was excluded from the analysis. The similarity index was calculated by multiplying the number of terminal restriction fragments (T-RFs) shared between comparable profiles (±1 base confidence interval) by 2, and dividing by the total number of peaks present in both profiles [11, 20].

$$S_{AB} = \frac{2N_{AB}}{N_A + N_B}$$

where

S_{AB} Similarity index, an indicator of similarity between sample A and B, ranges from 0–1
N_{AB} Number of T-RFs shared by sample A and B (±1 bp)
N_A Total number of T-RFs in sample A
N_B Total number of T-RFs in sample B

Statistics

PASW statistics 18 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. Student's one-tailed t test was carried out for the comparison of the average number of T-RFs and similarity indices of bacterial profiles. Independent two-sample t test was performed to test the difference between the means of two sample sets in the microbial collection study and the microbial variability study. One-sample t test was performed for the bacterial profile comparison study between footwear insole with owner's plantar skin and footwear insole with other individual's plantar skin. Results were considered significant at $P<0.05$.

Results and discussion

Recovery of bacterial DNA profiling

For the initial collection study, the bacteria from both the footwear insoles and the soles of the feet of the volunteers (herein referred to as plantar skin) were successfully sampled by swabbing, with sufficient DNA attained for bacterial 16S rRNA gene amplification in all samples. It is noteworthy that every bacterial T-RFLP profile had a significant peak height and adequate number of T-RFs for the following comparison study. Bacterial community profiles from the insoles included a total number of T-RFs ranging between 5 and 30 (an average of 15T-RFs) per sample. The plantar skin bacterial profiles included a total number of T-RFs ranging between 6 and 37 (an average of 12T-RFs) per sample. Notably, the average number of T-RFs from the shoe insoles was significantly higher than that from plantar skins ($P=0.019<0.05$). These results demonstrate that sufficient quantities of bacterial DNA can be simply recovered from both insoles and plantar skin. This retrieval of ample amounts of trace DNA evidence from a forensic sample, with minimal difficulties, offers the potential of this technique to be put into practice for criminal investigations. In the study of Hillier et al., only 23 % of samples (10 of 43) taken from the footwear insole gave human DNA profiles using commercial STR typing kits [2]. As such, microbial DNA profiling may offer an easy solution in cases where it is difficult to obtain human DNA profiling from the surface of a skin-contacted object, as noted by Fiere et al. [15]. It would be interesting to compare bacterial DNA profiling and human DNA profiling directly to ascertain which is easier to obtain from the shoe insole. Thus, this initial collection study indicates that, in cases where valuable human DNA profiles cannot be obtained from LCN and degraded samples from inside of the previously worn shoes, bacterial DNA profiles from the insoles of shoes and the suspect's soles might offer an alternative genetic method for identifying the owner of the shoes in criminal investigations.

Bacterial variability in footwear insole and plantar skin

For the microbial diversity study, the bacterial community profiles of the insoles and the plantar skins were compared between the left and right sides for each individual and between individuals. The results showed a significantly higher average similarity of bacterial profiles from the plantar skin within an individual, as compared with the profiles of plantar skin between different individuals (Fig. 1a). This observation demonstrates that bacterial variability on the plantar skin surface is higher between individuals than within the same individual, as noted by previous studies [12–14]. With the exception of the index finger, human skin-associated bacterial communities are similar in paired symmetric sites (right and

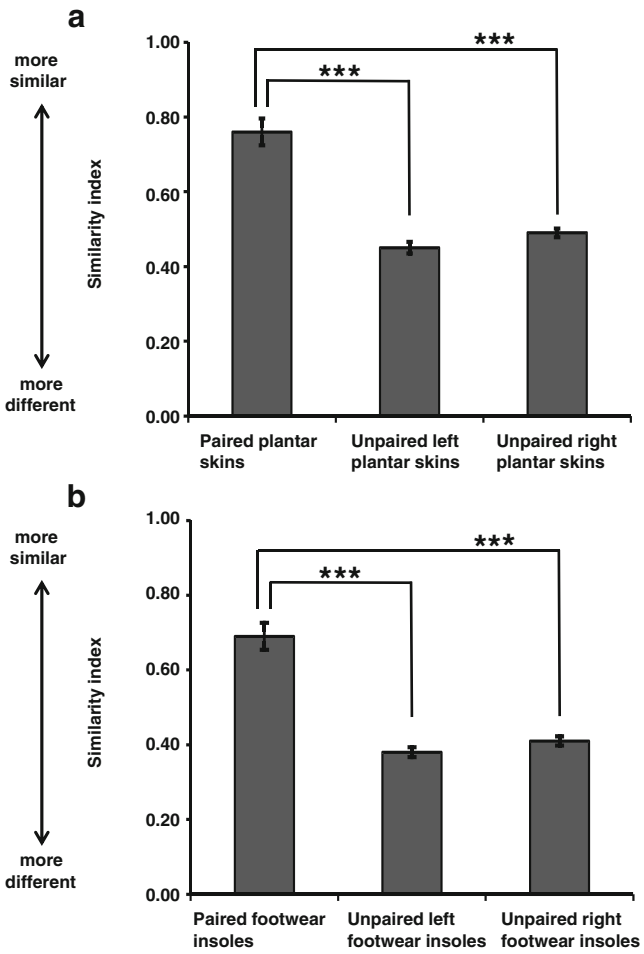

Fig. 1 Interpersonal and intrapersonal similarity of bacterial communities in soles of foot and footwear insoles: **a** similarities between left and right plantar skin of the same individual (n=14) versus similarities between left or right plantar skins of different individuals (n=91 each), **b** similarities between left and right insoles within the pair of footwear (n=14) versus similarities between left or right insoles from the footwear of different individuals (n=91 each). Higher value of similarity index indicates an increased average similarity in the bacterial communities, as compared with other bacterial communities. ***P<0.001, significant differences. *Bars* show the mean of similarity index calculated for each samples. *Error bars* show the standard error (±SEM) of the mean

left sides of the body), although they do not completely match [12–14]. Similarly, the average similarity of bacterial profiles from a pair of left and right footwear insoles was significantly higher than the similarity between the paired insoles of different individuals (Fig. 1b), also indicating that interpersonal bacterial variability is higher than intrapersonal bacterial variability for footwear insoles.

Eliminating the effects of shoe insole and duration of wearing

Because the footwear (sneaker) provided by each volunteer was different, and the duration for which the shoes had previously been worn varied among the volunteers, this small study next sought to eliminate the effects of the material of shoe insole and the period of time the shoes have been worn in inducing bacterial variability between individuals. Interestingly, the results showed a very similar pattern as observed in the original cohort (Fig. 1b), with a significantly higher average similarity of bacterial profiles from the footwear insoles within the individual, as compared with the profiles of insoles between different individuals (Fig. 2). This result, though from a small sample size, indicates that the previous results of interpersonal bacterial variability from Fig. 1 are not caused by differences in their insole materials or the period of time wearing the shoes.

The results from Figs. 1 and 2 indicate that each human plantar skin and their respective pair of insoles harbor a microbial fingerprint that is unique to a specific individual. This is because the bacteria present inside regularly worn footwear are likely to be derived from the owner's plantar skin. These results are as expected. Previous reports show that infectious microorganisms that often lead to skin disorders on the soles of human feet can shift to the footwear insole, even if a sock is worn [22–25]. The dominant bacterial strains inside footwear, *Staphylococcus* and *Corynebacterium* [6], are also the dominant bacterial strains on human plantar skin [12, 13]. These data strongly support the hypothesis in this study that the origin of bacteria inside the footwear comes from the owner.

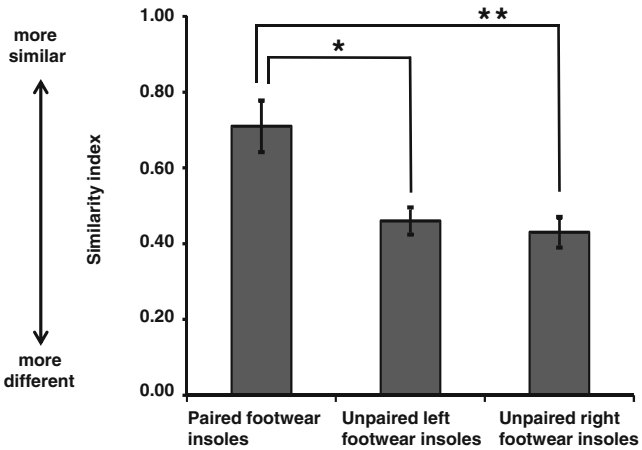

Fig. 2 Bacterial diversity from the footwear insoles of four male volunteers with identical footwear worn for the same duration (1 month). Bacterial community similarities between the left and right insole within a pair of footwear (n=4) versus similarities between left or right insoles from the same type of footwear between different volunteers (n=6 each). Higher value of similarity index indicates an increased average similarity in the bacterial communities as compared with other bacterial communities. *P<0.05, **P<0.01, significant differences. *Bars* show the mean of similarity index calculated for each samples. *Error bars* show the standard error (±SEM) of the mean

Comparison of bacterial profiles between footwear insole and plantar skin

Thus far, the results demonstrate that people leave different and distinguishable "bacterial footprints" on their footwear insoles that are unique to each person. Next, this study sought to determine whether microbial community profiling of footwear insoles was different enough to distinguish the owner of the footwear from a range of individuals.

The bacterial profiles from insoles were compared the plantar skin of the owner as well as the plantar skins of other individuals who had never worn the footwear. In 11 out of 14 cases, the right footwear insole could be correctly linked with the right foot of the owner of the shoe, as compared with profiles from the non-owner's plantar skins (Fig. 3a). For left insoles, 10 of 14 cases showed significant similarity between the owner's plantar skin and insoles, as compared to the non-owner's plantar skins (Fig. 3b). In most of the cases, it is noteworthy to point out that the actual values of the similarity indices of the bacterial profiles between owners and their shoes were lower than the expected values for bacterial variability (Figs. 1 and 2), although the similarity indices were significantly higher than that between unrelated individuals and the insole (Fig. 3a, b). The number of individuals/shoes examined is relatively small, and two types of errors (type I, false positive and type II, false negative) can be made with hypothesis testing according as the level of significance of $P<0.05$. However, the majority of this comparison study supports the hypothesis that a significant similarity exists between the bacteria on the

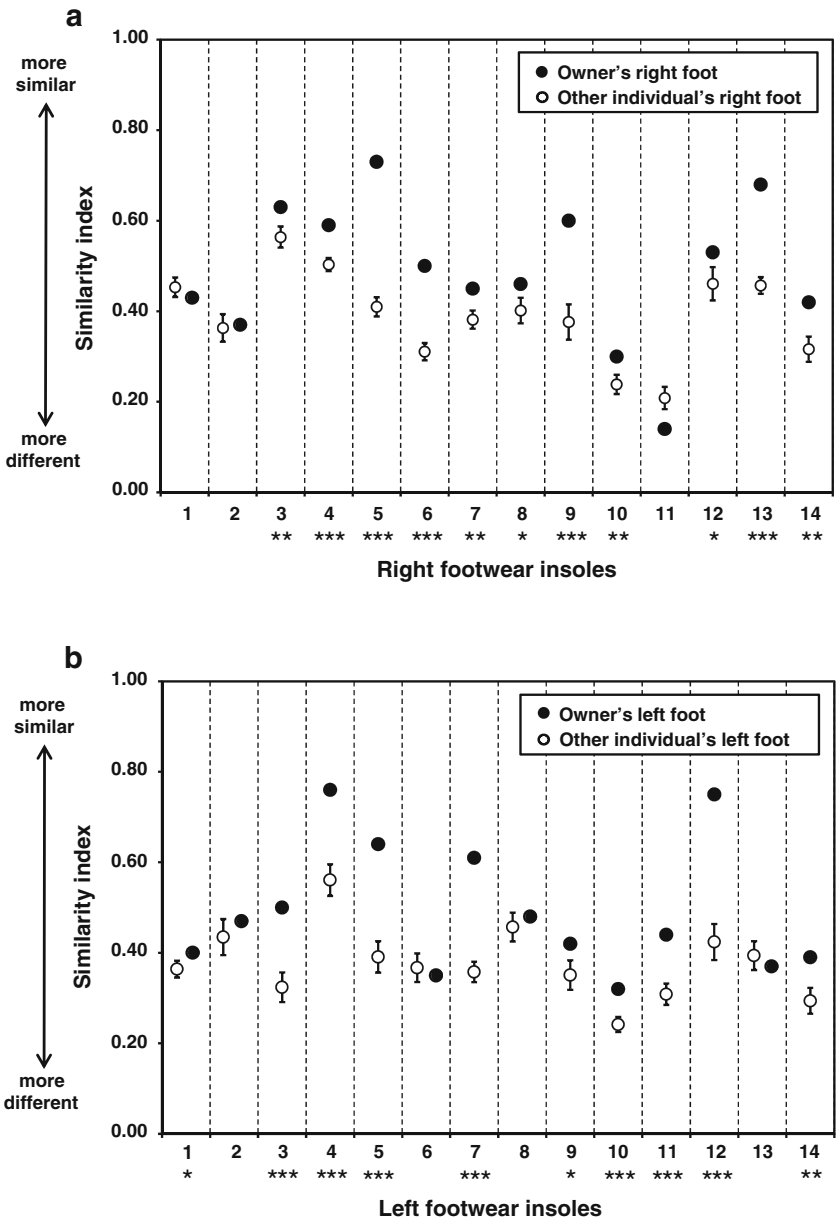

Fig. 3 Comparing similarity of bacterial profiles between footwear insole and plantar skin from the owner ($n=1$) versus the average similarity of bacterial profiles between the footwear insole and plantar skins from other individuals who have never worn the footwear ($n=13$): **a** right footwear insole versus sole of right foot, **b** left footwear insole versus sole of left foot. A relatively high similarity index implies that two bacterial communities are more similar than the combination of the two communities. Footwear *number with an asterisk* represents that the bacterial community of the owner's plantar skin is significantly similar to that of footwear insole as compared with the averaged similarity of other individual's plantar skin to footwear insole (*$P<0.05$, **$P<0.01$, ***$P<0.001$). *Error bars* show the standard error (±SEM) of the mean. See Table 1 for footwear number displayed on the *horizontal axis*

owner's plantar skin and the bacteria on the right and left insoles, as compared with other individuals.

The potential use of bacterial DNA profiling from shoe insoles in forensic settings

Overall, the results indicate that plantar skin-associated bacteria are retained on the insoles of footwear, even if the person is wearing socks, and that unattended footwear found at a crime scene can be matched to their owner by comparing microbial DNA profiling. However, considerable fluctuation in the degree of resemblance of the bacterial profiles between the insole and the owner's plantar skin can be seen, depending on the samples. In addition, a few cases showed a lack of significance between the insole and the owner or another individual (Fig. 3a, b). A similar degree of fluctuation was also observed in the hand swab study by Fiere et al [15], although this point was not addressed. Several reasons could explain these problems. The first is due to additional bacterial contamination. Tims et al. compared physical fingerprint microflora before or after hand washing and cautioned that human fingertip skin-associated microflora was too dynamic to be used as a forensic marker due to the constant bacterial contamination by touching [16]. This is inconsistent with the argument of Fiere et al. [15], and may be due to differences in the method by which hand skin bacteria were sampled. Fiere et al. collected reference bacterial samples from the volunteers' hand without washing or after their own arbitrary treatment [15]; in contrast, Tims et al. collected reference sample from all the volunteers after standardized hand washing [16]. Washing significantly altered the composition of the hand skin bacterial community [14]. Therefore, for human fingertip microflora, a more crude bacterial profile from hands would serve as a better reference when making comparisons between subject's hands and transferred bacterial profiles on touched objects. Additional studies are needed to confirm this.

It is unlikely that bacterial contamination is the reason for problems in this study, as insoles and the plantar skin remain wholly covered by the footwear, and the soles of the feet were therefore less exposed to exogenous microbial contamination than other areas of the skin, such as the fingertips. However, contamination to footwear insoles may arise from people taking off their shoes in indoors, especially at their home where microorganisms are generally ubiquitous [26–29]. It is possible that the individual's socks or feet pick up nonendogenous bacterial species from the indoor floor when walking. These transient microbial contaminants adhered to the sole of a sock or the sole of a bare foot are then transferred to their shoe insoles each time they put their shoes back on. This, in turn, creates a more complicated bacterial community on the shoe insole that would be sufficient to explain the fluctuation and lack of significance in the bacterial profiles between the insole and owner's plantar skin; for example, footwear #2 (Fig. 3 a, b). This hypothesis is supported by the findings in the collection study where the numbers of T-RFs from shoe insoles were higher than that from plantar skins. Also, results from others are in support of this hypothesis [24, 30]: infectious microbes that cause foot skin disorders frequently adhere to the soles of feet when people walk without shoes on indoor floors, and patients with skin infections on their feet can disseminate their virulent microbes to others by walking without shoes in common areas.

These points clearly outline how the adhesion of exogenous microbes on the soles of feet may help to describe the significantly lower similarity index for the right insole of footwear #11. Hillier et al. showed that the inside of footwear can sometimes generate mixed human DNA profiles attributed to "secondary transfer" [2]. Therefore, #11 must be regarded as an outlier in this study, possibly due to high bacterial contamination from others (Fig. 3a). Surprisingly, though, the left insole from footwear #11 was significantly matched to the bacteria from the plantar skin from the owner, as compared with other individuals (Fig. 3b). This discrepancy between the right and left insole within a pair of shoes was also seen in four of the other cases (footwear #1, 6, 8, 13 in Fig. 3a, b). As shown by the transplant experiment of skin bacteria in another study [13], the ecological conditions inside worn shoes can be an advantage for some but a disadvantage for other types of transferred bacterial species or strains, which shifts the bacterial DNA profiles of shoe insoles, thus creating issues with identification attempts via this approach. However, this factor may be less influential on bacterial profiles of footwear insoles than on bacterial contamination to insoles, at least in sneaker-type shoes. This is because the result of the bacterial profile resemblance between owner plantar skin and the insoles was inconsistent between the right or left insoles in one pair of shoes; paired insoles would be expected to have the same ecological conditions. In addition to bacterial contamination, dominant and nondominant foot discrepancies within individuals may also have an impact on the results in this study because the dominant foot would frequently come into contact with various types of environmental surfaces than the nondominant foot causing more bacterial contamination, as observed in a previous handedness study [14].

Another reason could be due to the analysis method used in this research, which may have caused fluctuations in similarity values depending on the footwear specimen. As observed in Fig. 1a, b, the T-RFLP analysis had enough power to detect a difference in the bacterial profiles from different insoles and plantar skins. However, other studies indicate that bacterial 16S rRNA T-RFLP analyses cause too much noise to compare the similarities between bacterial communities because bacterial 16S rRNA gene is common

to a very large number of species [20, 31]. Moreover, more consideration is required as to whether the simple similarity index with standardization of DNA quantities among samples for bacterial profile comparisons used in this paper and by others [11, 20, 21] is an optimum indicator for sample discrimination. For this reason, it will be important to compare the microbial community between insoles and human plantar skins using an alternative method in future studies. A simplified T-RFLP analysis [31], which targets a specific group of microorganisms, may offer a solution to the problems in this comparison, both the bacterial contamination and the noise of analysis. In addition, improvements in this study, such as the use of a larger sample size with a better ratio of age and gender, and more variation in the type of footwear (e.g., leather shoes, sandals, and boots), are necessary in future investigations before this strategy can be applied to forensic casework. Furthermore, it is unknown how long bacteria persist on footwear insoles after they are last handled, and the influence of artificial environmental factors for footwear, such as storage conditions, washing, and antibiotic treatments, should be investigated to test the changes in bacterial DNA profile of the footwear insole.

From a literature search, this study appears to be the first to demonstrate the utilization of bacteria from the inside of footwear to connect the footwear with the owner. The utilization of bacterial evidence persisting on insoles offers forensic teams the chance to identify the owner of the footwear amongst the pool of suspects or victims of crime. In this age of rapid progress, there has been an advancement of forensic microbiological investigations using molecular analyses. More refined technology for comparing microbial community will sharpen the ability of forensic investigators to discriminate the owner between potential suspects. As proposed by Fierer et al. [15], there is an expectation that this approach could discriminate between identical twins as studies have shown that identical twins with the same human STR profiles actually harbor notable variations in their gut microbial community [32, 33]. In addition, women are known to have a higher level of variability in their hand skin-associated microbiome than men [14], suggesting that shoes owned by women may also be easier to distinguish than shoes owned by men. This potential was not observed in this study; the only female volunteer (the owner of footwear #8) showed only a slight difference in the similarity index between the owner and other individuals (Fig. 3a, b). This sample size of one, however, deems this observation irrelevant. The genetic approach in this study is not perfect as of now; however, it will offer a novel strategy to discover forensic footwear evidence as an auxiliary investigative tool for current human DNA profiling.

Acknowledgments The author thanks all of the volunteers who provided bacterial samples for this study. Special thanks to Dr. Minoru Nakazato (Lab Director of Forensic Science Division, Department of Criminal Investigation, Okinawa Prefectural Police HQ) for his encouragement during the early days of the study and to Dr. Takeshi Arakawa (Molecular Microbiology Group, Department of Tropical Infectious Diseases, COMB, Tropical Biosphere Research Center, University of the Ryukyus) for his helpful discussions on the work in this paper.

References

1. Bright J, Petricevic SF (2004) Recovery of trace DNA and its application to DNA profiling of shoe insoles. Forensic Sci Int 145:7–12
2. Hillier E, Dixon P, Stewart P, Yamashita B, Lama D (2005) Recovery of DNA from shoes. Can Soc Forensic Sci J 38:143–150
3. Daly DJ, Murphy C, McDermott SD (2012) The transfer of touch DNA from hands to glass, fabric and wood. Forensic Sci Int Genet 6:41–46
4. Poinar HN (2003) The top 10 list: criteria of authenticity for DNA from ancient and forensic samples. Int Congr Ser 1239:575–579
5. Iwasaki F (2002) The research on the bacterial filth of the inside shoes (in Japanese with English title). J Jpn Soc Med Study Footwear 16:51–54
6. Matsuura Y (1998) The research on the effectiveness of antibacterial shoes (in Japanese). J Jpn Soc Med Study Footwear 12:27–30
7. Li H, Zhao C, Zhou J, Shao H, Chen W (2011) Isolation, purification and identification of bacteria from the shoes worn by children. Afr J Biotechnol 10:4133–4137
8. Mitsui M (2009) Effects of shoe microclimate on wear comfort (in Japanese with English title). Sen'i Gakkaishi 65:166–170
9. Butler JM, Shen Y, McCord BR (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 48:1054–1064
10. Smith PJ, Ballantyne J (2007) Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci 52:820–829
11. Horswell J, Cordiner SJ, Maas EW, Martin TM, Sutherland KBW, Speir TW, Nogales B, Osborn AM (2002) Forensic comparison of soils by bacterial community DNA profiling. J Forensic Sci 47:350–353
12. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC et al (2009) Topographical and temporal diversity of the human skin microbiome. Science 324:1190–1192
13. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R (2009) Bacterial community variation in human body habitats across space and time. Science 326:1694–1697
14. Fierer N, Hamady M, Lauber CL, Knight R (2008) The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proc Natl Acad Sci U S A 105:17994–17999
15. Fierer N, Lauber CL, Zhou N, McDonald D, Costello EK, Knight R (2010) Forensic identification using skin bacterial communities. Proc Natl Acad Sci U S A 107:6477–6481
16. Tims S, van Wamel W, Endtz HP, van Belkum A, Kayser M (2010) Microbial DNA fingerprinting of human fingerprints: dynamic colonization of fingertip microflora challenges human host inferences for forensic purposes. Int J Leg Med 124:477–481
17. Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522
18. Osborn AM, Moore ERB, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol 2:39–50
19. Dunbar J, Ticknor LO, Kuske CR (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA

gene terminal restriction fragment analysis. Appl Environ Microbiol 66:2943–2950
20. Meyers MS, Foran DR (2008) Spatial and temporal influences on bacterial profiling of forensic soil samples. J Forensic Sci 53:652–660
21. Heath LE, Saunders VA (2006) Assessing the potential of bacterial DNA profiling for forensic soil comparisons. J Forensic Sci 51:1062–1068
22. Knudsen EA (1986) Isolation of dermatophytes from footwear with adhesive tape strips. J Med Vet Mycol 25:59–61
23. Watanabe K, Taniguchi H, Nishioka K, Maruyama R, Katoh T (2000) Preventive effects of various socks against adhesion of dermatophytes to healthy feet (in Japanese with English abstract). Nihon Ishinkin Gakkai Zasshi 41:183–186
24. Maruyama R, Fukuyama K, Katoh T, Sugimoto R, Taniguchi H, Watanabe K, Nishioka K (2003) Prevention of dermatophytoses (in Japanese with English abstract). Nihon Ishinkin Gakkai Zasshi 44:265–268
25. Tanaka K, Katoh T, Irimajiri J, Taniguchi H, Yokozeki H (2006) Preventive effects of various types of footwear and cleaning methods on dermatophyte adhesion. J Dermatol 33:528–536
26. Abraham JH, Gold DR, Dockery DW, Ryan L, Park J, Milton DK (2005) Within-home versus between-home variability of house dust endotoxin in a birth cohort. Environ Health Perspect 113:1516–1521
27. Tringe SG, Zhang T, Liu X, Yu Y, Lee WH, Yap J et al (2008) The airborne metagenome in an indoor urban environment. PLoS ONE. doi:10.1371/journal.pone.0001862
28. Rintala H, Pitkäranta M, Toivola M, Paulin L, Nevalainen A (2008) Diversity and seasonal dynamics of bacterial community in indoor environment. BMC Microbiol. doi:10.1186/1471-2180-8-56
29. Flores GE, Bates ST, Knights D, Lauber CL, Stombaugh J, Knight R, Fierer N (2011) Microbial biogeography of public restroom surfaces. PLoS ONE. doi:10.1371/journal.pone.0028132
30. Katoh T (2006) Dermatomycosis and environment (in Japanese with English abstract). Nihon Ishinkin Gakkai Zasshi 47:63–67
31. Lenz EJ, Foran DR (2010) Bacterial profiling of soil using genus-specific markers and multidimensional scaling. J Forensic Sci 55:1437–1442
32. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE et al (2009) A core gut microbiome in obese and lean twins. Nature 457:480–484
33. Turnbaugh PJ, Quince C, Faith JJ, McHardy AC, Yatsunenko T, Niazi F et al (2010) Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. Proc Natl Acad Sci USA 107:7503–7508