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Abstract

In this study, we investigated and compared characteristics of the bacterial community structures on hair (scalp hair) and scalp in 18 individuals. Significant differences were found between the sites, in terms of cell density, alpha and beta diversity, and relative abundance of the phyla, Firmicutes and Proteobacteria, whereas no difference was found in relative abundance of the phylum Actinobacteria. Bacteria of the genus *Cutibacterium* showed similar relative abundance at both sites, whereas those of genus *Pseudomonas* were highly abundant on hair, and those of genus *Staphylococcus* were significantly lesser in abundance on hair than on scalp. Statistical correlations between the sites were high for the individual relative abundance of five major operational taxonomic units (OTUs). This suggests that the bacterial community structure on hair is composed of hair-specific genus, *Pseudomonas*, and skin-derived genera, *Cutibacterium* and *Staphylococcus*, and is distinguishable from other human skin microbiomes.

Key words: human scalp hair; bacterial community structure; quantitative PCR; NGS

Introduction

The entire human body surface, including hair and skin, is colonized by a wide variety of microorganisms, including bacteria, fungi, and viruses (1-3). Human skin, the largest organ of the human body, is colonized by 10^2 to 10^7 bacteria per cm^2 (4). Some of these bacteria live in a symbiotic relationship with their host, and protect against invasion by pathogenic microorganisms (5, 6). The physical and chemical features of various parts of the skin form unique bacterial community structures, that are adapted to the niche that they inhabit (7). Colonization by bacteria is dependent on the physiology of the skin site, with specific bacteria being associated with their microenvironments (8).

The scalp surface also provides a distinct microenvironment to the microbiome, primarily arising from the host skin's physiological conditions, which include sebum content, moisture, pH, and topography of the hair. In general, skin sites are roughly classified into three groups, based on their microenvironments, which are: moist, dry, and sebaceous groups. Among these skin site groups, scalp belongs to the sebaceous group. Sebaceous glands of the scalp produce a large amount of oily sebum (9). Furthermore, several studies on scalp microbiome in various countries have revealed the association of dandruff with bacterial community structure (10-13). These studies showed that the major bacterial genera on the scalp are *Cutibacterium (Propionibacterium)* and *Staphylococcus*. When compared with

normal scalp, the scalp with dandruff had a decreased population of *Cutibacterium* and an increased population of *Staphylococcus*. As dandruff is one of the disorders of the scalp, it is also a problem of the hair. Despite these facts, the relationship between the microbiomes on human hair (scalp hair) and scalp is yet unknown, and studies to elucidate this relationship and would be essential for a better understanding of both hair and scalp health.

Several studies have reported observations on the bacteria present on hair, using fluorescence light microscopy or scanning electron microscopy (SEM) (14, 15). Existence of bacteria was reported not only on hair shafts but also on hair follicles (16). Analysis of bacterial community structures on hair by terminal restriction fragment length polymorphism (T-RFLP) (17) indicated that these bacterial community structures are specific for each individual, and when collected from any human body part including hand (18), could be a tool for identifying a suspect. Bacterial community structures on specific parts of hair shaft and hair root were further compared by 16S rRNA gene amplicon sequencing analysis, and it was reported that the bacteria on hair shaft are indigenous and derived from the hair root, having similar number and structure from the top to the base parts of a long hair shaft (19). The report also suggested that bacterial community structures on scalp hair are distinct from those on other body sites, including various parts of the skin (20). However, the differences of bacterial community structures found on scalp hair and scalp were not studied, which would be essential for a better understanding of hair and scalp health, as well as bacterial ecology of hair.

In this study, we analyzed bacterial community structures, by 16S rRNA gene amplicon sequencing, to elucidate characteristics of the structures and to analyze the relationship between bacterial community structures of human scalp hair and human scalp within the same individual.

Materials and methods

This research was performed with permission from the research ethics committee of the Graduate School of Bioscience and Biotechnology at Kyushu University.

Samples and collection

Hair shaft samples and scalp swab samples were collected from 18 healthy Japanese and Chinese adults of both sexes (9 males and 9 females), ranging in age from 21 to 62 years, who consented to take part in this study (Supplemental Table 1). None of the volunteers were taking any medication during the experimental period. All of the volunteers washed their hair 6 hours prior to sample collection. Also, samples at both sites were collected on the same day. Samples of hair shafts and scalp swabs were collected using nitrile gloves. Scalp swab samples were directly taken from the crown of the head using cotton swabs (Mentip for hospital, Nihon Menbou Corporation, Saitama, Japan) pre-moistened with 50 μL of sterile distilled water. Cotton swabs were rubbed onto the scalp surface (between the hair strands) to

97 cover a total surface area of 2.5 cm^2. The head of each swab was cut from the handle and
98 placed into an Eppendorf tube. Samples of hair shaft were cut using sterilized scissors, and
99 chopped into pieces of 5 mm length with the scissors before use.
100

101 ***Extraction of bacterial DNA from hair shaft and scalp swab samples***

102 Bacterial DNA was extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL,
103 Düren, Germany) according to the manufacturer's instructions, with a slight modification.
104 First, samples of scalp swabs and hair shafts were immersed in 100 µL of lysozyme solution
105 (20 mg/mL lysozyme derived from egg white [Wako Pure Chemical Industries, Osaka, Japan]
106 in 20 mM Tris-HCl and 0.2 mM EDTA, pH 8.0) for 30 min at 37 °C, as previously reported
107 (19), and the DNA extracts obtained (100 µL) were stored at –20 °C until use.
108

109 ***Quantification of the bacterial cell number on hair and scalp by qPCR***

110 The bacterial cell number on the hair and scalp of 18 volunteers was quantified by estimation
111 of 16S rRNA gene copy number using real-time PCR (CFX Connect™ System, BIO-RAD
112 Laboratories, Inc., CA, USA) with universal primers for a portion of the bacterial 16S rRNA
113 gene. We have previously shown that the values estimated by quantitative PCR (qPCR)
114 correspond well with the values obtained by the direct SEM observation (19).

Each 10 μL reaction mixture consisted of 2 μL of KOD SYBR® qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan), 0.1 μL of each primer [357F (5′-CCT ACG GGA GGC AGC AG-3′) (21) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (22)], and 2 μL of bacterial DNA. The amplification program included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles each of denaturation at 95 °C for 5 s, annealing at 64 °C for 20 s, and elongation at 72 °C for 20 s. DNA extract from *Escherichia coli* DH5α was used as a standard to generate a calibration curve. After amplification, the copy numbers of the 16S rRNA genes per hair sample were calculated per cm of hair and converted to per cm^2 of hair. For the calculation, the following equation was used:

$$\text{Cells/cm}^2 = \text{qPCR copies / hair length (cm)} \times \text{hair diameter (cm)} \times \pi$$

The diameter of the hair was measured using a stereo microscope (Stemi 305, ZEISS, Oberkochen, Germany).

Analysis of the bacterial community structures on scalp and hair by 16S rRNA gene sequencing

To analyze the bacterial community structures of scalp and hair from 18 volunteers using the MiSeq™ platform (Illumina Inc., CA, USA), a three-step PCR method was performed using the extracted DNA samples. In the first-step PCR amplification, a universal primer set for the V4 region of the bacterial 16S rRNA gene (515F, 5′-GTG CCA GCM GCC GCG GTA A-3′

and 806R, 5′-GGA CTA CHV GGG TWT CTA AT-3′) (23) was used. The 25 μL reaction mixture consisted of 12.5 μL of Kapa HiFi HotStart Ready Mix (Kapa Biosystems Inc., Wilmington, MA, USA), 0.5 μL of each primer (10 pM), and 11.5 μL of extracted bacterial DNA. The amplification program included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles each of denaturation at 98 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. After electrophoresis through a 1.5% (w/v) agarose gel, the targeted bands were excised from the gel with sterilized cutters, and the DNA was extracted using the FastGene® Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). In preparation of 16S rRNA amplicon sequencing with MiSeq, the templates are given tail, adapter, and index sequences in a two-step PCR. Therefore, long-tailed primers are required for the preparation, which makes the amplification difficult. We were unable to perform direct amplification in the two-step PCR, probably because the amount of bacterial DNA obtained from a 3 cm hair shaft is very small. Therefore, we first performed PCR using the universal primer set without any additional sequences. As a result, we succeeded in obtaining enough template fragments with a minimum number of reaction cycles.

For the second-step PCR, a universal primer set for the V4 region of the bacterial 16S rRNA gene and tailed sequences for MiSeq sequencing were used (1-515F, 5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG CCA GCM GCC GCG GTA A-3′

and 1-806R, 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACH VGG GTW TCT AAT-3′) (24). Although it is reported that this primer set would poorly amplify *Propionibacterium* of human skin (25), the results in this study showed good amplifications of *Cutibacterium acnes* (previous name is *Propionibacterium acnes*) of not only scalp but also hair using this primer set as the predominant species. The 25 μL reaction mixture consisted of 1.0 μL of each primer (5 μM) which was heat-shocked at 95 °C for 5 min, 12.5 μL of Kapa HiFi HotStart Ready Mix, 12.5 ng of DNA obtained from the first-step PCR amplicon, and sterilized ultrapure water. The amplification program included an initial denaturation step at 95 °C for 3 min, followed by 20 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. PCR products were purified using the FastGene® Gel/PCR Extraction Kit according to the manufacturer's instructions.

For the third-step PCR, a primer set with flow cell adapter sequences, index sequences, and tailed sequences was used (Forward primer, 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-Index sequence-TCG TCG GCA GCG TC-3′ and Reverse primer, 5′-CAA GCA GAA GAC GGC ATA CGA GAT-Index sequence-GTC TCG TGG GCT CGG-3′). The third-step PCR mixture (25 μL) was composed of 12.5 μL of Kapa HiFi HotStart Ready Mix, 0.5 μL of each primer (10 pM), 11.5 μL of the second-step PCR amplicon. The amplification program included an initial denaturation step at 95 °C for 3 min, followed by 8 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. After electrophoresis in a 1.5% (w/v) agarose gel, the target

bands were excised with sterilized cutters, and the DNA was extracted using the FastGene® Gel/PCR Extraction Kit as described above. The DNA concentration of the third-step PCR amplicons was quantified by using a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The purified PCR products from each sample were mixed, denatured, and sequenced with an MiSeq System (Illumina) using MiSeq Reagent Kit v3 (300 bp ×2 cycles with pair-end; Illumina), according to the manufacturer's instructions. We obtained Good's coverage values (> 95%) for all hair samples using the DNA extraction kit and PCR conditions described above, indicating that the results obtained contain meaningful information (Supplemental Table 2). Good's coverage values were estimated using QIIME™ 1.9.1 software (26).

Bioinformatics and statistical analysis

The index and universal sequences of each read were checked, and reads with complete index sequences were selected as valid sequences. USEARCH V8.1.1861 (27) software was used to merge paired-end reads and remove chimeric sequences. After the chimera check, the reads were grouped into operational taxonomic units (OTUs) at > 97% similarity. Alpha diversity (observed OTUs and Shannon index) was evaluated at a 1% OTU distance using the QIIME™ software package (26). In the taxonomy-based analysis, representative sequences for each OTU were analyzed with the EzBioCloud platform (28). Statistical analysis in quantification of bacterial cell numbers was done using ANOVA, and statistical analysis in

bacterial community structure was done using Kruskal-Wallis test. Both statistical analyses were run under XLSTAT software ver. 2014 (http://www.xlstat.com/en/).

Accession number

Illumina raw read sequences and the top 13 most abundant OTU sequences were deposited in the DDBJ/ENA/ GenBank database under accession numbers LC557428-LC557440.

Results

Quantification of bacterial cell number on hair and scalp

Bacterial cell number on hair and scalp from 108 samples derived from 18 volunteers was analyzed separately (Supplemental Table 1). The average bacterial cell number on a hair sample was 1.6 (±1.6) × 10^5 cells/cm^2, while that on a scalp sample was 3.8 (±3.7) × 10^4 cells/cm^2, which was lower than the number on hair by one order of magnitude (Fig. 1). In spite of that, analysis of correlation between these numbers, at the level of an individual, showed that there was statistically no correlation between the numbers on both sites (Supplemental Fig. 1).

211 ***Alpha diversity of the bacterial community structures on hair and scalp***

212 Fig. 2 shows the two indexes of alpha diversity: observed OTUs, and the Shannon index.
213 Both indexes were significantly higher ($p < 0.0001$) on hair than on scalp. It is noted that the
214 average OTU numbers on hair and scalp were 46.2 and 19.2, respectively. On the other hand,
215 there was weak correlation of observed OTUs, and no correlation of Shannon indexes at
216 individual levels (Supplemental. Figs. 2A and 2B, R = 0.38, and -0.04, respectively). This
217 suggests that the bacterial communities on hair and scalp were rather independent in alpha
218 diversity formation.
219

220 ***Bacterial community structures on hair and scalp at phylum level***

221 Three major phyla, Actinobacteria, Proteobacteria, and Firmicutes were commonly found on
222 both sites, and abundances of the three phyla added up to 97.9% and 99.4% on hair and on
223 scalp respectively (Fig. 3). The Kruskal-Wallis test revealed no statistically significant
224 difference in the abundance of Actinobacteria between the sites. On the other hand, the
225 abundance of Proteobacteria was significantly higher ($p < 0.01$) on hair (36.9 ± 20.1%) than
226 on scalp (9.5±9.4%), whereas the abundance of Firmicutes was significantly lower ($p < 0.01$)
227 on hair (11.0 ± 9.3%) than on scalp (33.8 ± 12.1%). These results indicate that the bacterial
228 communities on hair and on scalp had distinguishable structures at the phylum level.
229 Correlation analysis at individual level showed that abundances of Firmicutes and

Actinobacteria between the sites were correlated (R = 0.58 and 0.63 respectively), whereas the abundance of Proteobacteria between the sites was not correlated (R = 0.18) (Supplemental Fig. 3).

Bacterial community structures on hair and scalp at OTU level

We extracted OTUs present in samples of all individuals, and found that 13 OTU sequences existed either on hair or scalp of each individual. Of these, eight OTUs (OTU 1, 2, 3, 4, 7, 9, 15, 16) were present on both sites in all individuals, and the other five OTUs (OTU 5, 18, 26, 42, 131) were present only on hair in some individuals. The total relative abundances of the eight common OTUs were 70.9% and 94.6% on hair and on scalp, respectively, being much lower on hair. This observation corresponded with the results of alpha diversity analysis (Fig. 2).

These 13 OTUs were assigned to a most closely related species (Phylum, pairwise similarity) (Fig. 4). The five major OTUs showing abundance higher than 5% were: OTU1 related to *Cutibacterium acnes* (Actinobacteria, 99.3%), OTU2 related to *Staphylococcus epidermidis* (Firmicutes, 99.3%), OTU3 related to *Lawsonella clevelandensis* (Actinobacteria, 99.3%), OTU4 related to *Pseudomonas lini* (Proteobacteria, 99.3%) and OTU15 related to *Pseudomonas antarctica* (Proteobacteria, 99.0%). Other three OTUs found in all individuals were OTU 7, 9, and 16. These show rather low total abundance at 3.2% and 1.0% in total on

hair and on scalp, respectively. It is noticeable that the five OTUs (OTU 5, 18, 26, 42, 131), found only on hair, added up to 6.0% in total abundance, and were all assigned to phylum Proteobacteria.

Relative abundances of the five major OTUs present at both sites, and their statistical correlations between hair and scalp are shown in Fig. 5 and Fig. 6, respectively. OTU1 related to *Cutibacterium acnes* was the most predominant species on both sites. Its relative abundance was not significantly different between the sites (Fig. 5A). On the other hand, its individual abundance was statistically correlated between the two sites (R = 0.66) (Fig. 6A). OTU2 related to *Staphylococcus epidermidis* was less abundant on average, on hair than on scalp (Fig. 5B), and its individual abundance was also statistically correlated between the two sites (R = 0.69) (Fig. 6B). OTU3 related to *Lawsonella clevelandensis* showed no significantly difference in its relative abundance between the sites (Fig. 5C), while its individual abundance was statistically correlated between the two sites (R = 0.79) (Fig. 6C). On the other hand, the relative abundances of OTU4 related to *Pseudomonas lini* and OTU15 related to *Pseudomonas antarctica* were higher on hair than on scalp (Fig. 5D, 5E). These two, OTU4 and OTU15 showed correlation between individual abundance on hair and on scalp (R = 0.60, R = 0.60) (Fig. 6D, 6E). The five OTUs (OTU 5, 18, 26, 42, 131), sometimes found only on hair showed relatively lower correlation between their abundance on the hair and on the scalp of the same individuals (Fig. S4). These results suggest that bacterial community structures on each site were partly formed with skin-resident bacteria including

269 *Cutibacterium, Staphylococcus,* and *Lawsonella*, and partly formed with some hair-specific
270 bacteria belonging to phylum Proteobacteria.
271

272 ***Beta diversity of the bacterial community structures on hair and scalp***

273 Fig. 7A shows the beta diversity of bacterial community structures on hair and scalp samples
274 obtained by principal coordinates analysis (PCoA) based on weighted UniFrac distances.

275 The plots were roughly grouped into two clusters on each site, although some plots were
276 positioned at similar areas. We further performed biplot analysis at phylum level and the
277 result corresponded well with the relative abundances of major phyla in bacterial community
278 structures on each site shown in Fig. 2 As seen in Fig. 7A, biplot of Actinobacteria was
279 located in the middle of the two clusters; biplot of Firmicutes was located in the scalp cluster.
280 On the other hand, biplot of Proteobacteria was located in the hair cluster. This clustering
281 tendency was confirmed by comparison of average weighted UniFrac distance (Fig. 7B): the
282 distances within hair and within scalp were significantly lower ($p < 0.0001$) than those
283 between hair and scalp. These results indicated that the bacterial community structure on hair
284 was specified by a comparably higher abundance of Proteobacteria, although major OTUs
285 were commonly present on both sites.
286

Discussion

Recent studies of bacterial community structures on scalp elucidated its relationships with health and disease of hair (scalp hair) and scalp (9-13). Also, relatively stable and individually unique bacterial communities were found on hair (17, 19). In this study, we provide the first overview of characteristics and relationship of the bacterial community structures on hair and scalp using real-time PCR and 16S rRNA amplicon sequencing. As a result, it was found that major bacterial species were commonly present at both sites, but the bacterial community structure on hair was specific and distinguishable from that on the scalp. Important relationships obtained are summarized in Table 1. Significant differences between the bacterial community structures on hair and scalp were found in terms of cell density, alpha diversity, and on relative abundances of Firmicutes and Proteobacteria, while no difference was found in terms of relative abundance of Actinobacteria. There was correlation between hair and scalp of an individual person in relative abundance of Actinobacteria and Firmicutes, while there was no correlation in terms of cell density, alpha diversity, and relative abundance of Proteobacteria. We will discuss each result one by one.

Average bacterial cell number on hair was significantly higher ($p<0.05$) than on scalp (Fig. 1). Bacterial cell number on hair was similar to that seen in previous reports (19, 29). On the other hand, there were no correlation between hair and scalp on individual bacterial cell number. It should be notable that there was almost no variation in the three samples per person, showing that error due to sampling method was relatively low. In spite of

that, taking into account that the sampling methods to collect bacterial DNA were different at each site (whole extraction for hair shaft and swabbing for scalp), further structural analysis was conducted by comparing relative abundances of a highlighted bacterial group, instead of the cell densities. Grice et al. (30) reported that major OTUs were overlapping (97.2%) in bacterial skin samples collected using three different methods: swab, scrape, and punch biopsy.

We showed two indexes of alpha diversity on each site: observed OTUs and Shannon Index. Both indexes were significantly higher on hair than on scalp (Fig. 2). This could explain why the eight OTUs, commonly present on both sites of all individuals, show higher abundance on scalp (94.6%) than on hair (70.9%) (Fig. 4). These results suggest that various kinds of minor bacterial species inhabited only hair. It was reported that physical conditions affect the formation of skin bacterial communities, and thus, alpha diversity of bacterial community structures on sebaceous skin was lower than that on dry skin (1, 12, 31). In a previous study, we also reported that alpha diversity was higher on dry hair shaft than on sebaceous hair root (19). Higher hydrophobicity of hair would cause its higher alpha diversity, compared to a sebaceous scalp. On the other hand, there was no correlation between hair and scalp in terms of the individual alpha diversity of their bacterial community structures. This suggests that the bacterial community structures on the two sites are independent in alpha diversity formation.

At phylum level, average relative abundance of Actinobacteria were similar on hair

and scalp (50.0 ± 22.5% and 56.1 ± 21.0%, respectively), but not similar for Firmicutes (11.0 ± 9.3% and 33.8 ± 12.1%), and Proteobacteria (36.9 ± 20.1% and 9.5±9.4%) (Fig. 3). On the contrary, correlation between hair and scalp on individual relative abundances was found both in Actinobacteria and Firmicutes, but not in Proteobacteria. For more detailed analysis of bacterial community structure, we assigned the OTU sequences to the most closely related bacterial species. Four genera - *Cutibacterium*, *Staphylococcus*, *Lawsonella*, and *Pseudomonas* were present with more than 5% abundance in both sites for every sample (Fig. 4). These bacterial genera are also reported to be present in other human body sites. *Cutibacterium* is the major bacterial genus present in areas with sebaceous glands, such as the forehead (32). *Staphylococcus* widely inhabits various parts of human skin and the nasal area (33). In case of *Lawsonella,* the genus has been isolated from various kinds of human abscesses (34). Some of the common OTUs, *Cutibacterium acnes* (previous name is *Propionibacterium acnes*) and *Staphylococcus epidermidis*, were also found other studies, as you indicated. These bacteria protect the host from pathogenic bacteria (32). They are considered to proliferate using sebum and sweat as nutrient, and playing role of barrier function on hair and scalp against pathogenic bacteria (32). On the other hand, the genus *Proteobacteria* generally inhabits the natural environment such as soil and river water, and has been recognized as being transient on human skin (35). Correlations between hair and scalp were found for individual relative abundances of five major OTUs (Fig. 6) but not in some secondary OTUs related to Proteobacteria including *Pseudomonas* (Fig. S4). Our results indicated that *Pseudomonas* is the second major genus in hair, showing stable and non-

348 transient habitation on hair. In a previous study, we reported that major bacterial OTUs
349 including *Pseudomonas* were common on hair shaft and hair root, and it was possible that
350 these bacteria on hair shaft were indigenous and not transient due to circumstances (19).
351 *Cutibacterium* was the most abundant bacterial genus on hair, even though the physical
352 condition of hair seems dry and different from other sebaceous human skin zones.
353 *Cutibacterium acnes* carries the genes for biosynthesis of biotin, which is an essential nutrient
354 for hair growth and scalp health (11, 36, 37). On the other hand, *Pseudomonas*, the second
355 major bacterial genus present on hair, was not identified in a key role. Further detailed studies
356 are required to clarify the bacterial ecology of hair.

357 Finally, we analyzed beta diversity and biplots at phylum level, to compare
358 bacterial community structures between hair and scalp (Fig. 7A). It was confirmed that
359 phylum Proteobacteria (to which *Pseudomonas lini* and *Pseudomonas antarctica* belong)
360 contributed to the formation of bacterial community structure specific for hair, and phylum
361 Firmicutes (to which *Staphylococcus epidermidis* belongs) contributed to that on scalp.
362 Although some reports analyzed bacterial community structures on scalp (9-13), or on hair (1,
363 19, 38), there was no study on the correlation between these sites at the same individual level.
364 Also, Klerk et al. (39) evaluated bacterial adherence and colonization on hair by SEM
365 observation and reported that *Pseudomonas aeruginosa* (belonging to Proteobacteria) adhered
366 to and colonized hair surfaces, while *Staphylococcus epidermidis* (belonging to Firmicutes)
367 showed inhibited growth. This inhibition of *S. epidermidis* may be attributable to hair-derived

368 antimicrobial proteins or peptides (40). Therefore, it was suggested that hair contained
369 specific bacterial community structures, in that the relative abundances of *Pseudomonas*
370 (Proteobacteria) was higher on hair than on other skin sites.

371 In conclusion, it was suggested that bacterial community structure on hair was
372 formed from both hair-specific and skin-derived bacteria, which were different from other
373 skin microbiomes, including forearms, nostrils, and forehead (20). In particular, *Pseudomonas*
374 was one of the most hair-specific bacteria, but the genus has been recognized as being
375 transient on skin and not much attention has been paid to it. Further studies on hair-specific
376 bacteria, particularly genus *Pseudomonas* would clarify their roles and interaction with hair.
377

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382

383 **Author contribution**

384 Kota Watanabe processed the experimental data, performed the analysis and wrote the manuscript.
385 Azusa Yamada and Yuri Nishi carried out the part of experiments. Yukihiro Tashiro and Kenji Sakai

supervised the project from experimental design to submission of the manuscript. All authors agreed and approved the manuscript to be published.

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485 Table 1: Correlation of bacterial community structures between hair and scalp

	Significant difference between hair and scalp ($p<0.05$)	**Hair**	**Scalp**	**Correlation between hair and scalp on individual abundance ($R \geqq 0.4$)**
Cells /cm^2	Yes	High $1.6(\pm1.6)\times10^5$	Low $3.8(3.7)\times10^4$	No
Alpha diversity				
Observed OTUs	Yes	High 46.2(±15.9)	Low 19.2(±8.8)	No
Shannon Index	Yes	High 3.0(±0.8)	Low 1.8(±0.5)	No
Relative abundance (%)				
Actinobacteria	No	50.5(±22.5)	56.1(±21.0)	Yes
Firmicutes	Yes	Low 11.0(±9.3)	High 33.8(±12.1)	Yes
Proteobacteria	Yes	High 36.9(±20.1)	Low 9.5(±9.4)	No

Supplemental Table 1: Details of hair and scalp samples in 18 volunteers and quantification of bacterial cell number at each site by qPCR of the bacterial 16S rRNA gene

Volunteer	Gender	Age	Hair length (mm)	Hair diameter (μm)	Cell number / cm^2 of hair	Cell number / cm^2 of scalp
F001	Female	22	263(±57)	89	$5.7(\pm2.4)\times10^4$	$3.2(\pm2.5)\times10^4$
F002	Female	22	417(±31)	63.3	$1.7(\pm0.4)\times10^5$	$1.1(\pm0.3)\times10^5$
F003	Female	21	287(±40)	88.9	$5.5(\pm2.7)\times10^4$	$4.5(\pm3.1)\times10^4$
F004	Female	27	335(±12)	87.2	$7.1(\pm1.9)\times10^4$	$3.4(\pm0.7)\times10^4$
F005	Female	23	332(±20)	75.6	$1.1(\pm0.3)\times10^5$	$4.2(\pm1.3)\times10^4$
F006	Female	22	371(±27)	73.9	$3.0(\pm0.5)\times10^4$	$5.1(\pm1.5)\times10^4$
F007	Female	41	193(±1)	88	$5.9(\pm1.4)\times10^4$	$1.2(\pm0.4)\times10^4$
F008	Female	27	402(±19)	79.7	$1.4(\pm0.07)\times10^5$	$4.3(\pm1.1)\times10^3$
F009	Female	23	236(±32)	99.7	$6.0(\pm0.7)\times10^4$	$1.9(\pm0.8)\times10^4$
M001	Male	25	83(±2)	98.4	$5.6(\pm2.9)\times10^5$	$2.6(\pm0.3)\times10^4$
M002	Male	26	29(±5)	70.8	$4.1(\pm1.0)\times10^5$	$3.0(\pm0.7)\times10^4$
M003	Male	22	88(±7)	68.7	$1.3(\pm0.6)\times10^5$	$9.2(\pm9.2)\times10^4$
M004	Male	22	46(±4)	76.6	$1.6(\pm0.6)\times10^5$	$2.2(\pm0.4)\times10^4$
M005	Male	22	111(±10)	102	$1.0(\pm0.1)\times10^5$	$2.6(\pm0.4)\times10^4$

M006	Male	29	74(±11)	109.8	$2.6(\pm0.3)\times10^5$	$5.9(\pm2.2)\times10^4$
M007	Male	62	65(±3)	61.8	$3.5(\pm0.5)\times10^5$	$4.9(\pm1.8)\times10^4$
M008	Male	40	72(±2)	111.4	$1.0(\pm0.3)\times10^5$	$1.5(\pm1.0)\times10^4$
M009	Male	23	65(±2)	104	$2.2(\pm0.6)\times10^5$	$1.5(\pm0.1)\times10^4$

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505 Supplemental Table 2: Details of MiSeq analysis of 108 hair and scalp samples

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Hair					Scalp			
Gender	**Volunteer ID**	**Hair length (mm)**	**Row read number**	**Good's coverage value (%)**	**Gender**	**Volunteer ID**	**Row read number**	**Good's coverage value (%)**
Female	F001	180	18,918	99.1	Female	F001	34,119	99.5
	F001	290	7,085	99.4		F001	24,464	99.3
	F001	316	16,292	99.0		F001	13,093	99.3
	F002	373	15,218	98.8		F002	12,473	98.9
	F002	437	23,647	98.2		F002	21,808	99.2
	F002	442	24,693	98.0		F002	26,899	98.5
	F003	258	4,753	98.8		F003	36,584	99.4
	F003	260	15,589	98.4		F003	24,193	99.5
	F003	343	42,776	98.7		F003	30,594	99.8
	F004	320	31,116	98.5		F004	22,270	99.3
	F004	335	36,188	98.8		F004	27,600	99.2
	F004	350	37,979	98.8		F004	14,740	99.5
	F005	315	25,346	97.8		F005	31,619	99.4

	F005	320	23,023	98.0		F005	19,455	99.4
	F005	360	22,073	97.9		F005	8,328	99.7
	F006	386	32,067	98.3		F006	21,400	99.5
	F006	333	23,162	97.9		F006	29,124	99.0
	F006	394	4,283	98.1		F006	20,064	99.4
	F007	192	17,769	99.1		F007	17,274	99.2
	F007	193	18,219	98.9		F007	16,555	99.5
	F007	195	28,974	98.4		F007	23,044	99.5
	F008	378	34,007	98.6		F008	28,647	99.5
	F008	405	26,627	99.3		F008	6,453	99.6
	F008	424	2,780	99.2		F008	19,118	99.8
	F009	200	21,399	97.8		F009	20,378	98.2
	F009	230	35,982	97.3		F009	8,360	99.1
	F009	277	32,199	97.8		F009	16,522	99.0
Male	M001	81	47,895	98.3	Male	M001	30,257	99.1
	M001	82	15,069	97.7		M001	29,871	99.2
	M001	85	45,660	97.6		M001	33,081	98.7
	M002	25	31,574	99.3		M002	17,969	99.5
	M002	26	26,218	99.4		M002	25,807	99.1
	M002	36	13,497	99.2		M002	18,835	99.5

	M003	78	7,673	98.9		M003	26,571	99.3
	M003	91	27,818	99.0		M003	23,967	99.8
	M003	95	4,179	98.0		M003	22,234	99.7
	M004	42	32,069	98.8		M004	29,714	99.4
	M004	44	9,282	98.6		M004	24,510	99.2
	M004	52	4,946	98.7		M004	31,295	98.7
	M005	102	38,698	98.8		M005	23,583	99.6
	M005	105	14,144	98.8		M005	42,952	99.4
	M005	125	28,906	98.6		M005	10,726	99.5
	M006	73	19,200	98.0		M006	24,508	99.5
	M006	61	23,549	98.3		M006	23,135	99.6
	M006	88	9,293	98.1		M006	14,694	99.3
	M007	61	24,335	98.1		M007	13,234	98.6
	M007	67	22,557	98.2		M007	3,388	98.9
	M007	68	23,936	98.2		M007	23,693	98.7
	M008	70	16,657	99.4		M008	35,365	99.5
	M008	72	38,767	99.2		M008	20,841	99.3
	M008	74	39,555	99.2		M008	13,439	99.3
	M009	62	26,924	99.0		M009	12,713	99.6
	M009	65	22,408	99.0		M009	19,288	99.4

	M009	67	30,353	99.1		M009	31,819	99.2

Figure legends

Figure 1 Bacterial cell number (copies/ cm^2) on hair and scalp.

Figure 2 Alpha diversity of bacterial community on the hair and scalp,

based on (A) observed OTU and (B) Shannon index. The values are obtained from clustering of 1,000 reads per sample.

Figure 3 The average relative abundances of the major phyla in bacterial community structure on hair and scalp. The different alphabets mean that there are significant differences at comparison of each bacterial phyla between the sites (Kruskal-wallis test, $p<0.01$).

Figure 4 Phylogenetic tree and abundance heatmap of the major 13 OTUs commonly found in all 18 individuals.

Figure 5 Comparison of relative abundance of most major five OTUs between hair and scalp. (A) relative abundance of OTU1 related to *Cutibacterium acnes*, (B) OTU2 related to *Staphylococcus epidermidis*, (C) OTU3 related to *Lawsonella clevelandensis*, (D) OTU4 related to *Pseudomonas lini*, and (E) OTU15 related to *Pseudomonas antarctica*.

Figure 6 Correlation of relative abundance of most major five OTUs between hair and scalp. (A) OTU1 related to *Cutibacterium acnes*, (B) OTU2 related to *Staphylococcus epidermidis*, (C) OTU3 related to *Lawsonella clevelandensis*, (D) OTU4 related to *Pseudomonas lini*, and (E) OTU15 related to *Pseudomonas antarctica*.

543 Figure7A Beta diversity of bacterial community structure on hair and scalp in 108 samples
544 from eighteen volunteers and their biplot analysis

545 analysis at phylum level. The PCoA plots based on the weighted UniFrac analysis are shown.
546 (B) Comparison of weighted Unifrac distances within and between hair and scalp samples.

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549 Suppl Figure 1 Correlation of bacterial cell number between hair and scalp

550 Suppl Figure 2 Correlation of alpha diversity between hair and scalp. (A) Observed OTUs,
551 (B) Shannon.

552 Suppl Figure 3 Correlation of relative abundance of major three phylum between hair and
553 scalp. (A) Actinobacteria, (B) Firmicutes, and (C) Proteobacteria.

554 Suppl Figure 4 Correlation of relative abundance of second major OTUs between hair and
555 scalp. (A) relative abundance of OTU131 related to *Pseudomonas endophytica*, (B) OTU9
556 related to *Pseudomonas alcaliphila*, (C) OTU18 related to *Rhodanobacter ginsengiterrae*, (D)
557 OTU7 related to *Rahnella aquatilis*, (E) OTU26 related to *Escherichia coli*, (F) OTU5 related
558 to *Moraxella osloensis*, (G) OTU42 related to *Acinetobacter bereziniae*, and (H) OTU16
559 related to *Carnobacterium gallinarum*.

560

561 Table. 1 Summary of differences in abundance and correlation of bacterial community

562 between hair and scalp

563 Suppl Table1 Details of hair and scalp samples in eighteen volunteers and quantification of

564 the bacteria cell number at each site by qPCR of the bacterial 16S rRNA gene.

565 Suppl Table 2 Details of MiSeq analysis of 108 hair and scalp samples.

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Fig. 1 Watanabe et al.

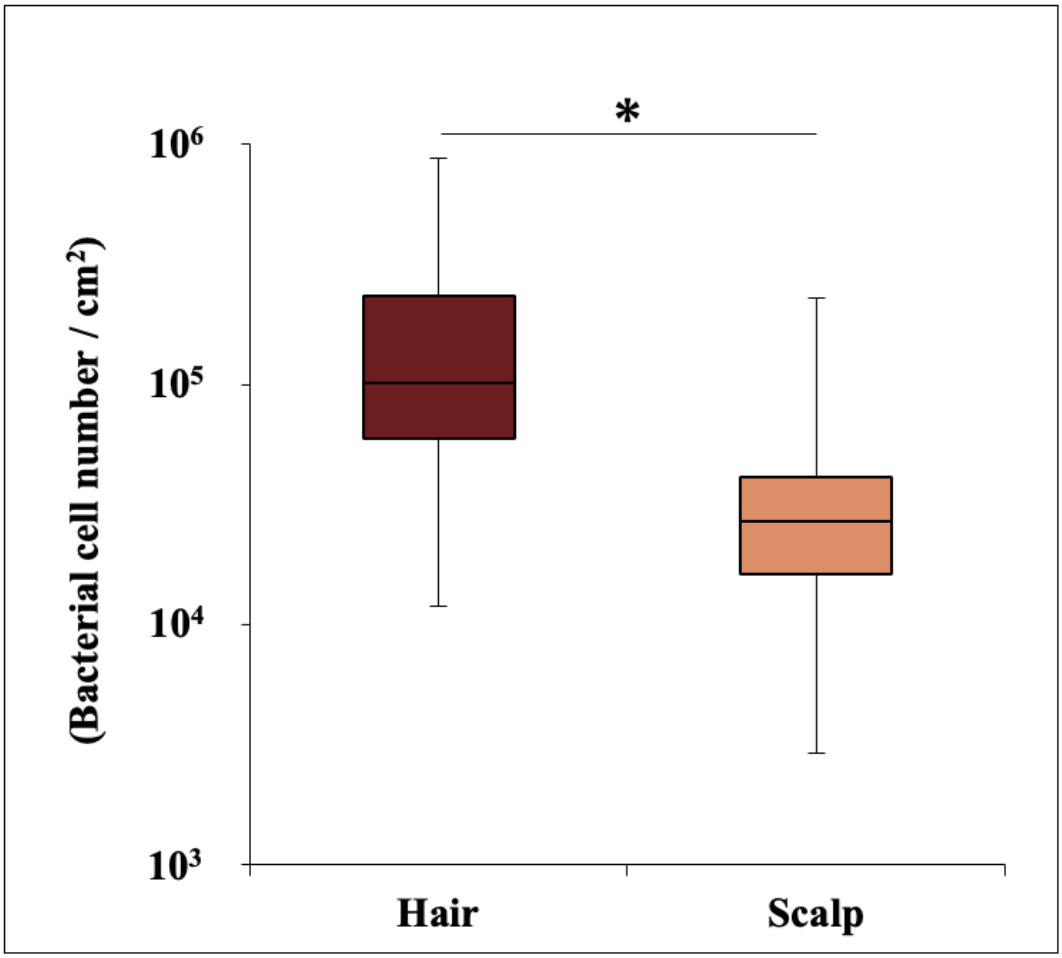
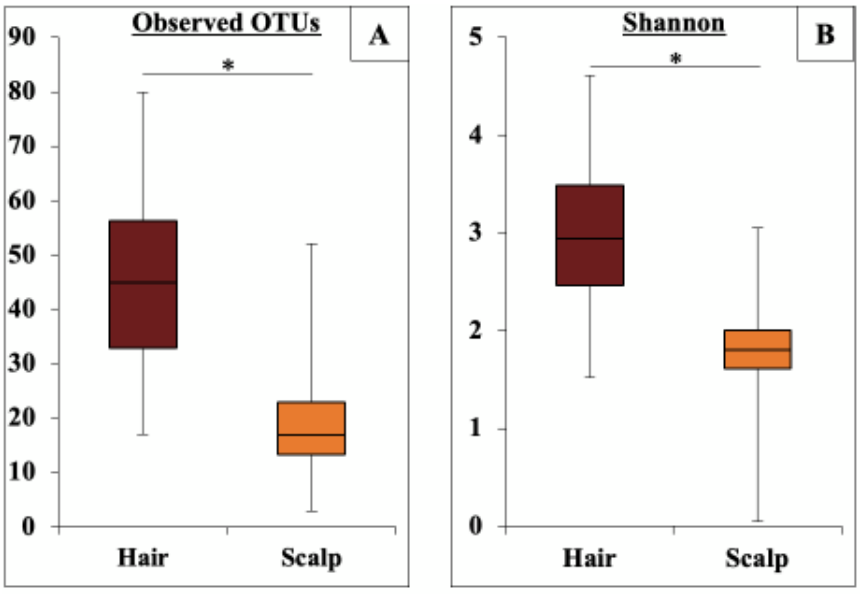


Fig. 2 Watanabe et al.



*Kruskal-wallis test, $p<0.0001$

Fig. 3 Watanabe et al.

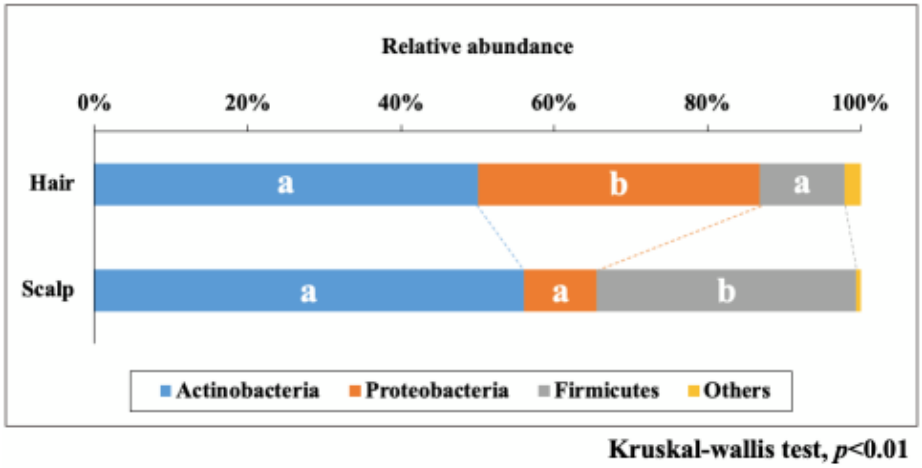


Fig. 4 Watanabe et al.

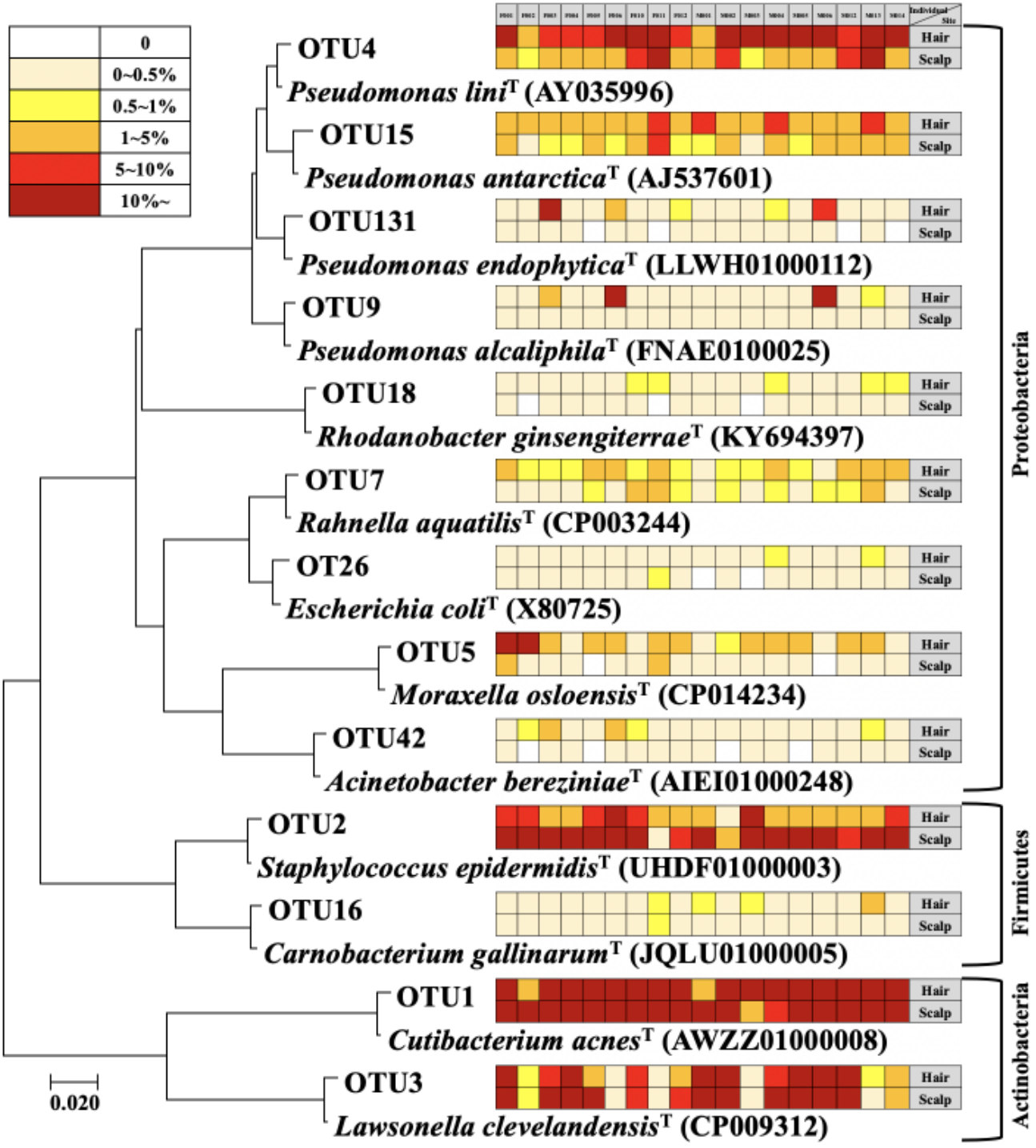


Fig. 5 Watanabe et al.

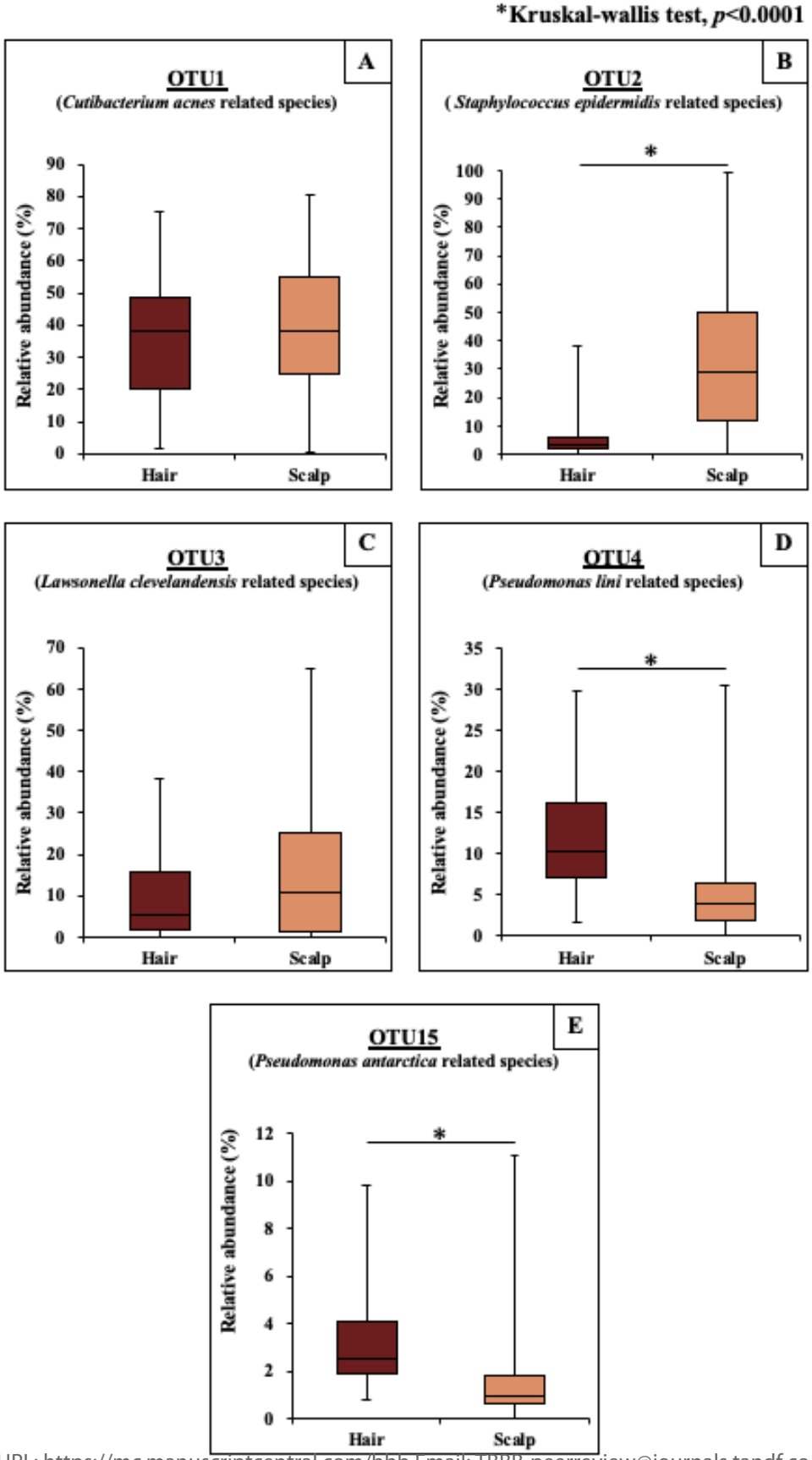
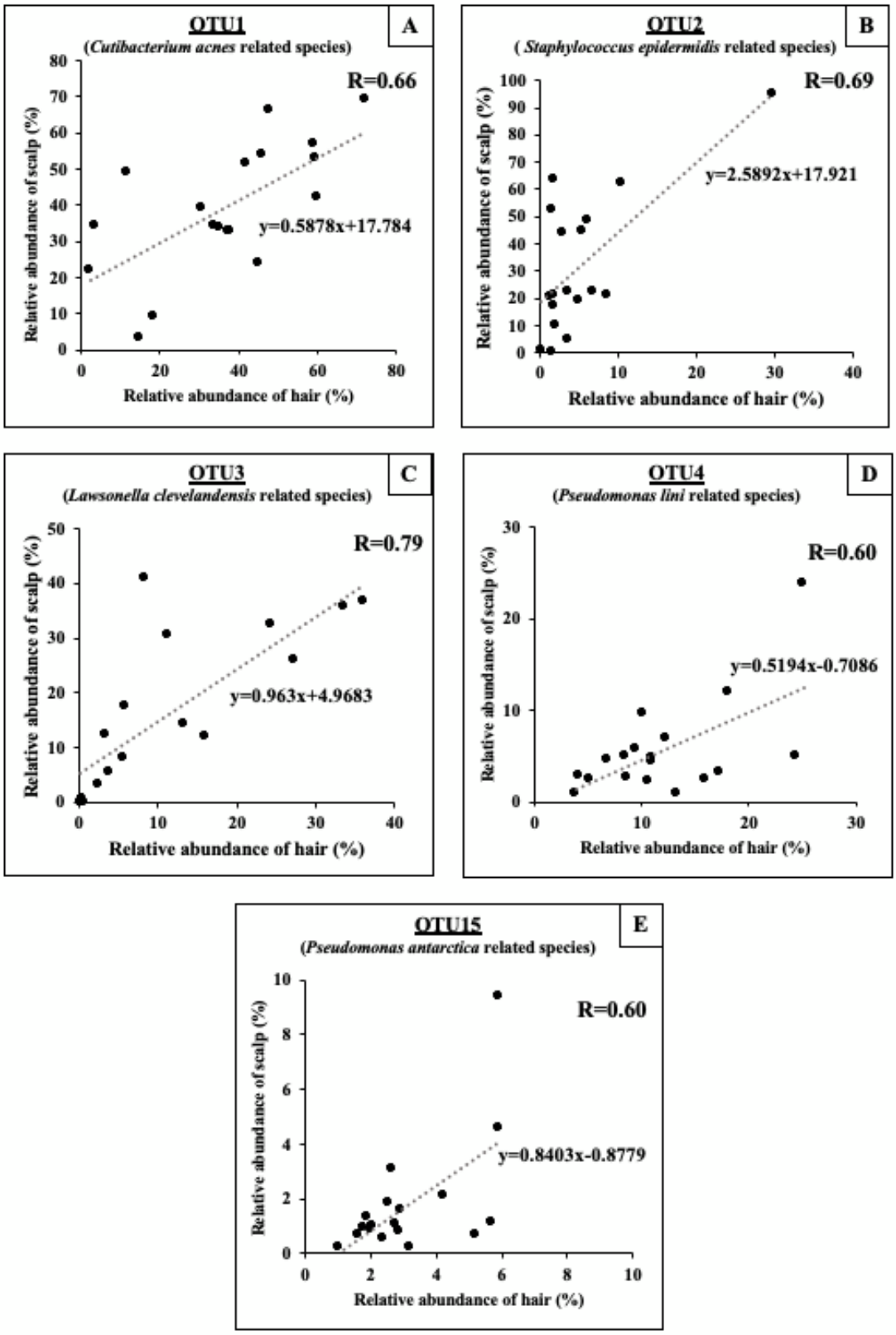
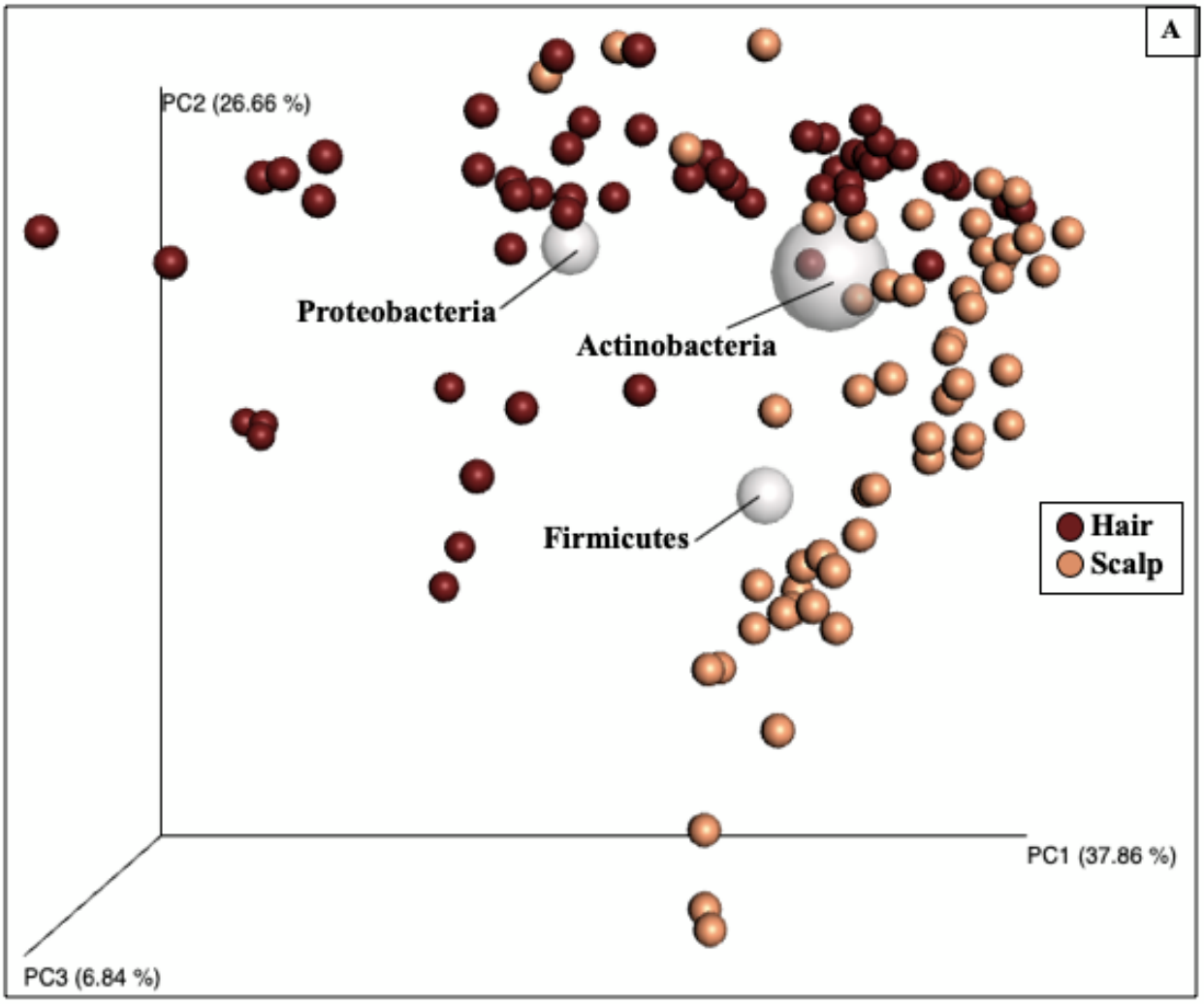


Fig. 6 Watanabe et al.




A
PC2 (26.66 %)
Proteobacteria
Actinobacteria
Firmicutes
Hair
Scalp
PC1 (37.86 %)
PC3 (6.84 %)

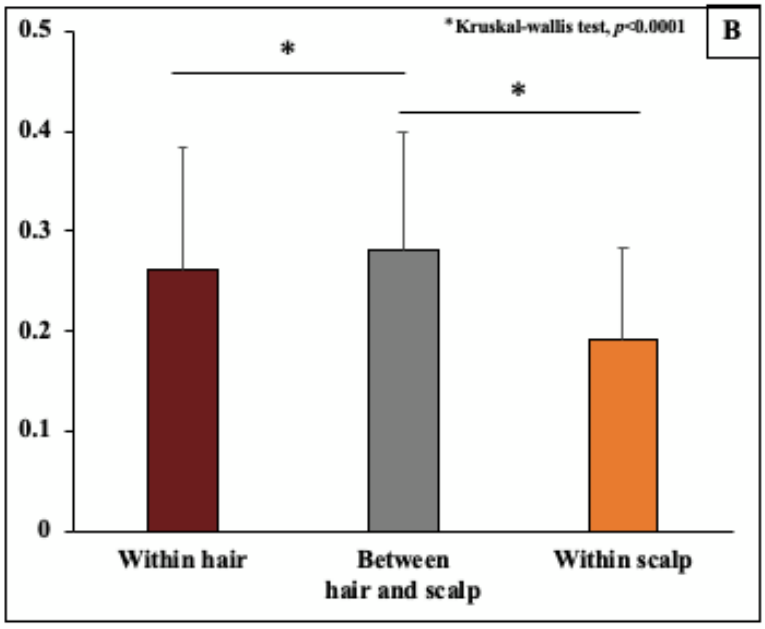

B
*Kruskal-wallis test, p<0.0001
*
*
0.5
0.4
0.3
0.2
0.1
0
Within hair
Between hair and scalp
Within scalp

Fig. S1 Watanabe et al.

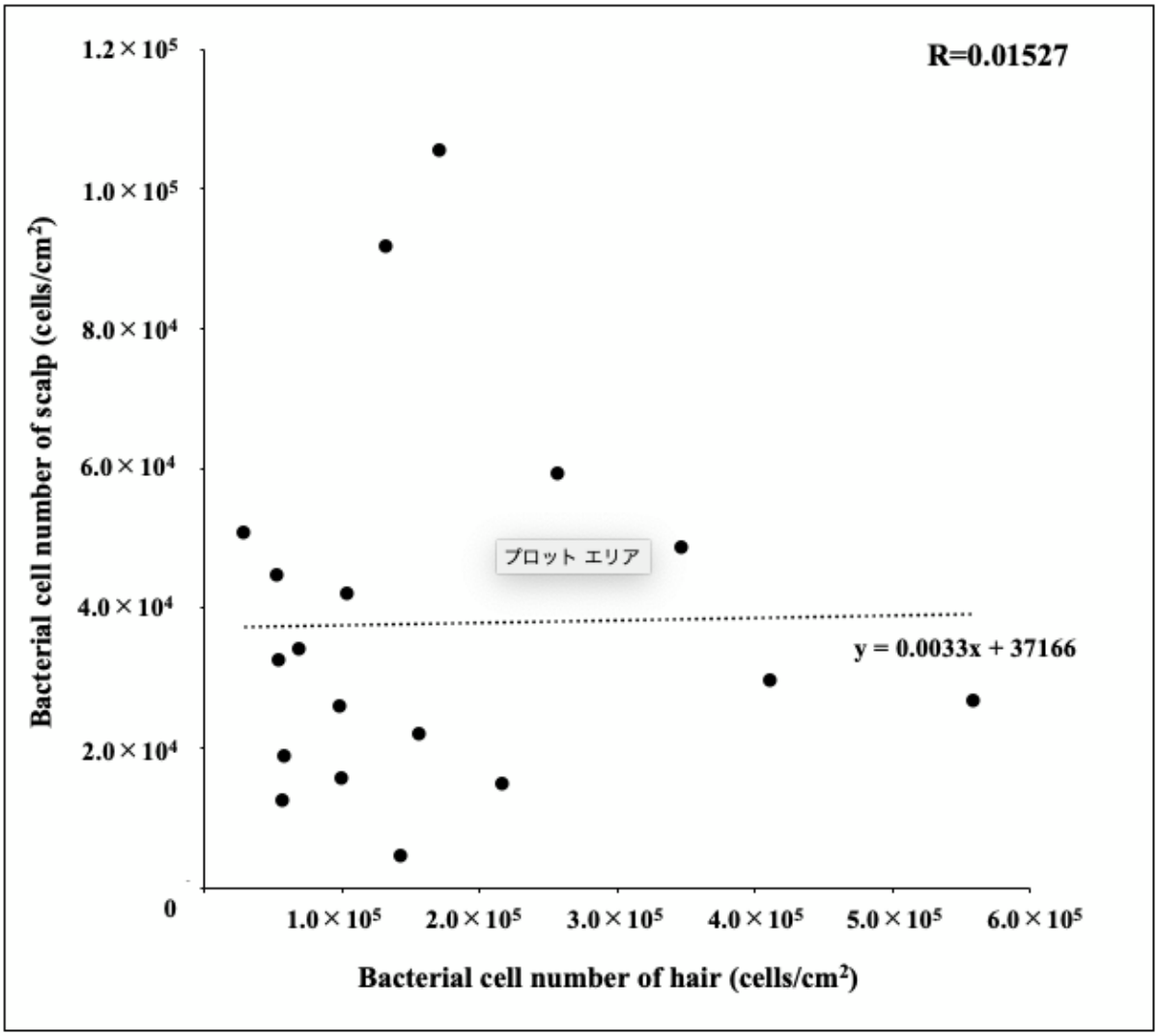


Fig. S2 Watanabe et al.

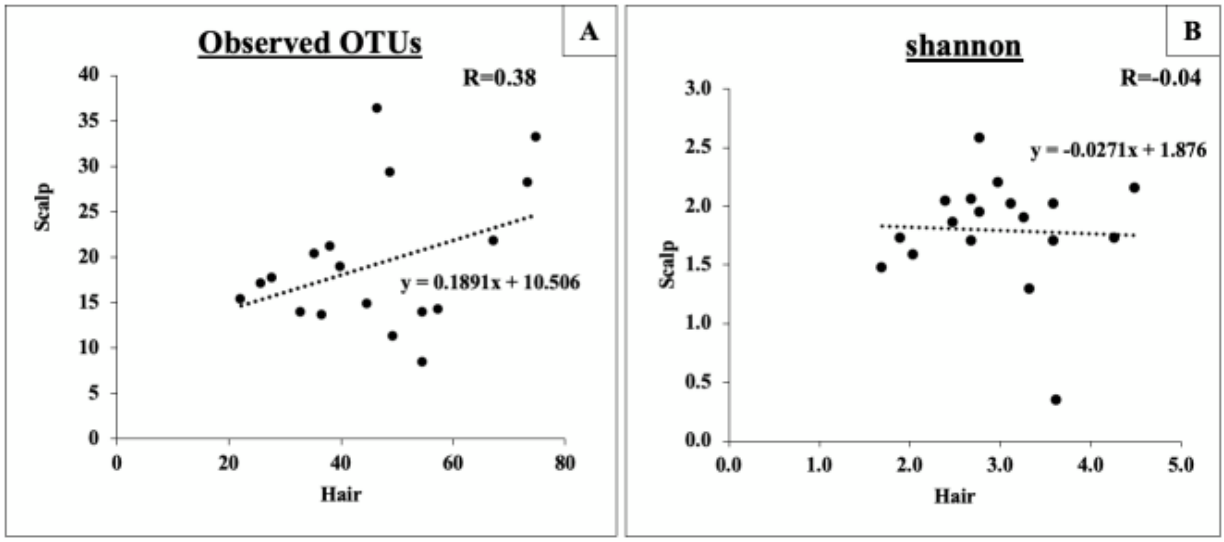
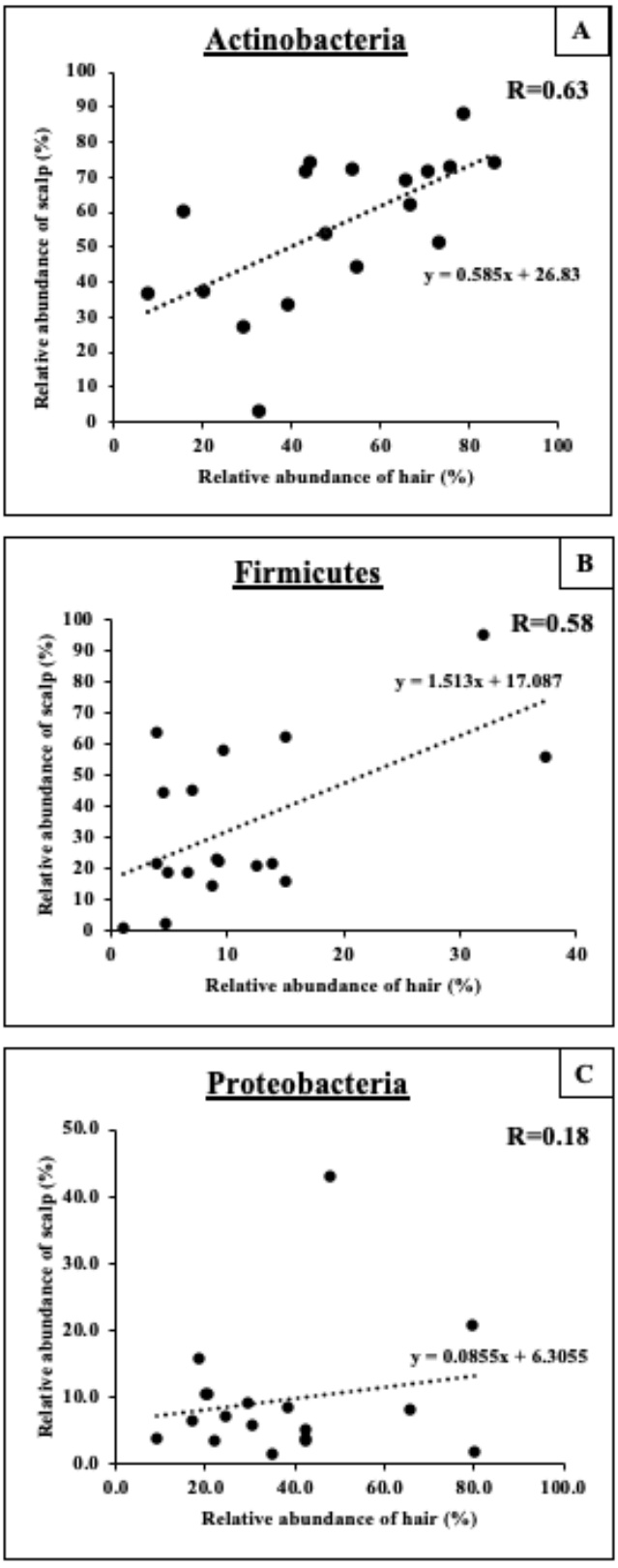
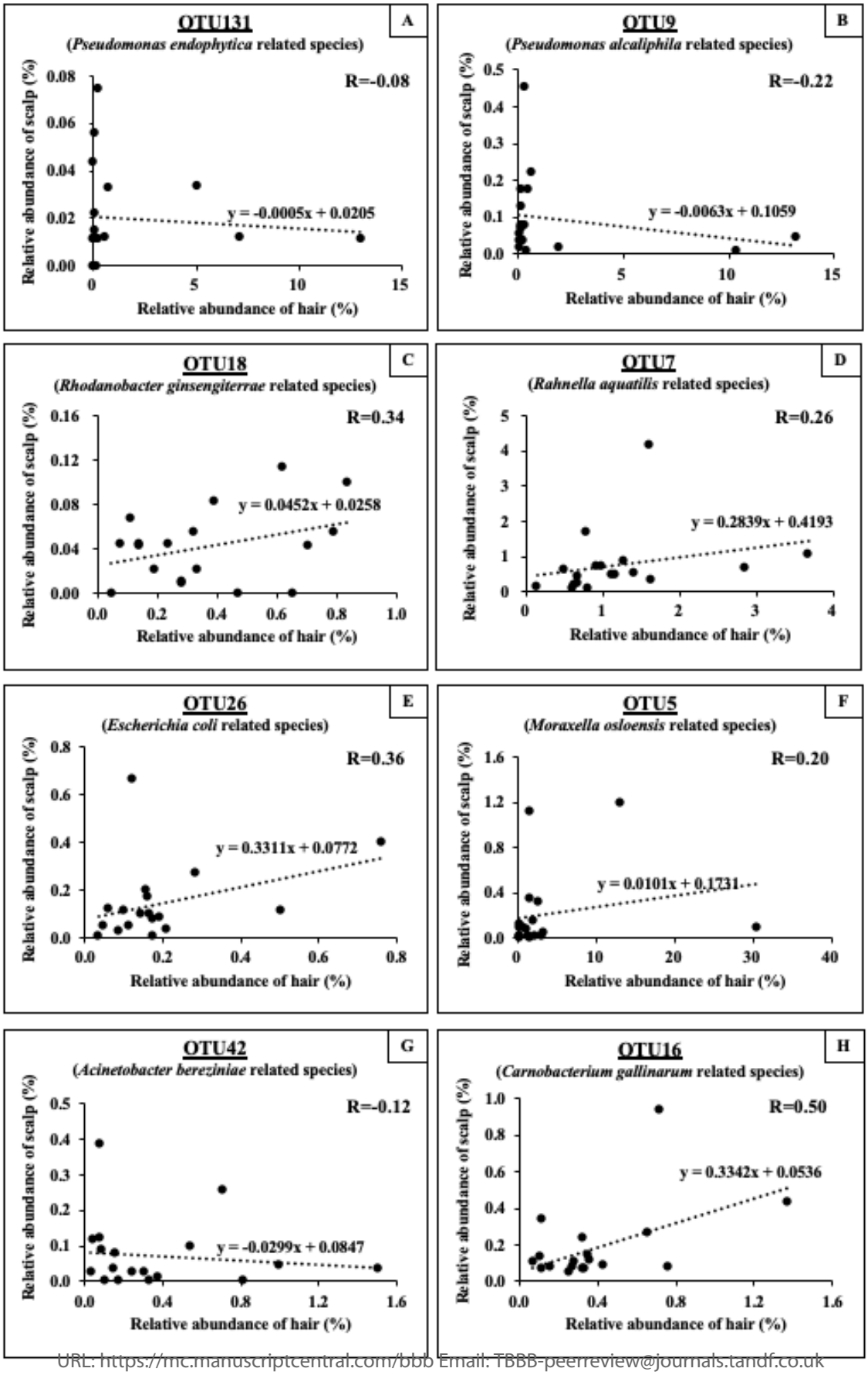
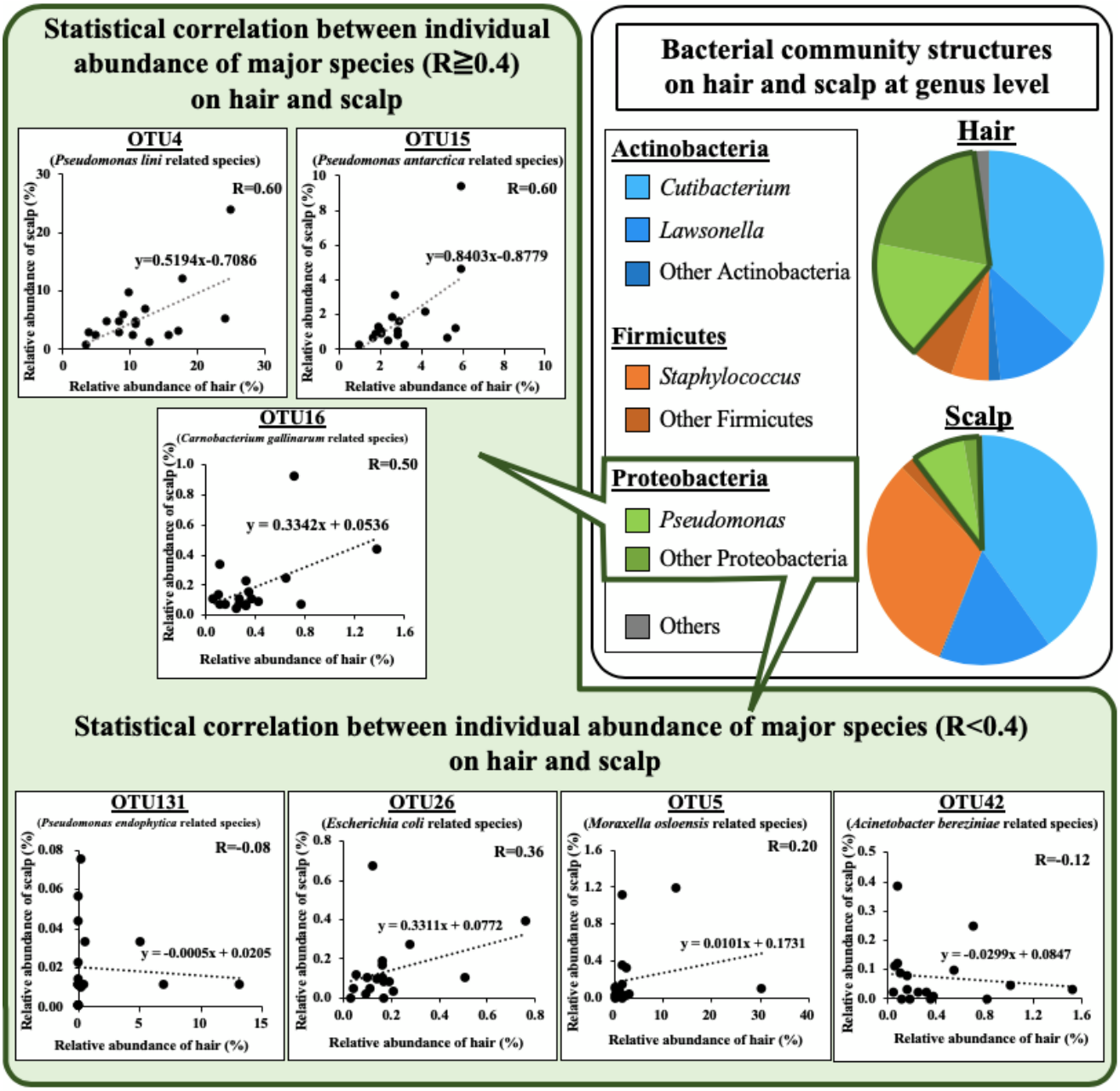


Fig. S3 Watanabe et al.




A
OTU131
(Pseudomonas endophytica related species)
R=-0.08
y = -0.0005x + 0.0205
B
OTU9
(Pseudomonas alcaliphila related species)
R=-0.22
y = -0.0063x + 0.1059
C
OTU18
(Rhodanobacter ginsengiterrae related species)
R=0.34
y = 0.0452x + 0.0258
D
OTU7
(Rahnella aquatilis related species)
R=0.26
y = 0.2839x + 0.4193
E
OTU26
(Escherichia coli related species)
R=0.36
y = 0.3311x + 0.0772
F
OTU5
(Moraxella osloensis related species)
R=0.20
y = 0.0101x + 0.1731
G
OTU42
(Acinetobacter bereziniae related species)
R=-0.12
y = -0.0299x + 0.0847
H
OTU16
(Carnobacterium gallinarum related species)
R=0.50
y = 0.3342x + 0.0536
Relative abundance of scalp (%)
Relative abundance of hair (%)


Statistical correlation between individual abundance of major species (R≧0.4) on hair and scalp
OTU4
(Pseudomonas lini related species)
R=0.60
y=0.5194x-0.7086
Relative abundance of scalp (%)
Relative abundance of hair (%)
OTU15
(Pseudomonas antarctica related species)
R=0.60
y=0.8403x-0.8779
Relative abundance of scalp (%)
Relative abundance of hair (%)
OTU16
(Carnobacterium gallinarum related species)
R=0.50
y = 0.3342x + 0.0536
Relative abundance of scalp (%)
Relative abundance of hair (%)
Bacterial community structures on hair and scalp at genus level
Actinobacteria
Cutibacterium
Lawsonella
Other Actinobacteria
Firmicutes
Staphylococcus
Other Firmicutes
Proteobacteria
Pseudomonas
Other Proteobacteria
Others
Hair
Scalp
Statistical correlation between individual abundance of major species (R<0.4) on hair and scalp
OTU131
(Pseudomonas endophytica related species)
R=-0.08
y = -0.0005x + 0.0205
Relative abundance of scalp (%)
Relative abundance of hair (%)
OTU26
(Escherichia coli related species)
R=0.36
y = 0.3311x + 0.0772
Relative abundance of scalp (%)
Relative abundance of hair (%)
OTU5
(Moraxella osloensis related species)
R=0.20
y = 0.0101x + 0.1731
Relative abundance of scalp (%)
Relative abundance of hair (%)
OTU42
(Acinetobacter bereziniae related species)
R=-0.12
y = -0.0299x + 0.0847
Relative abundance of scalp (%)
Relative abundance of hair (%)