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PRELIMINARY PCR-TTGE ANALYSES OF BACTERIAL COMMUNITIES ASSOCIATED WITH POLLEN FROM ANEMOPHILOUS TREES: POTENTIAL IMPACTS ON PLANTS AND HUMAN HEALTH

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ABSTRACT

Pollen from wind-pollinated plants is the predominant cause of pollinosis which corresponds mainly to allergic rhinitis and conjunctivitis. Bacterial communities associated to leaves and roots surface were analysed for many years with both culture-dependent and independent methods. However, microbiota of pollen grains was rarely investigated and never with a molecular fingerprint technique. The aim of this study was to perform a preliminary analysis of the pollen microbiota by applying PCR-TTGE method. Pollen samples were collected from various anemophilous trees genera (*Cephalotaxus*, *Cupressus*, *Pinus*, *Platanus* and *Quercus*) in Montpellier (France) in 2013. Thorough pollen preparation was essential to a successful recovery of DNA from pollen. Regarding the results, pollen microbiota was tree genus-dependent. In addition, intra-tree genus variations were also observed. The *Gammaproteobacteria* class was the most represented in pollen samples whatever the tree genus. Among this class, some bacteria were recognized as phytopathogens (*Pseudomonas*, *Erwinia*) but also opportunistic human pathogens (*Pseudomonas*, some enterobacteria). PCR-TTGE is a suitable tool to analyse the microbiota associated with pollen responsible for phytopathogenicity or affecting the human respiratory tract.

Keywords: pollinosis; anemophilous trees; pollen microbiota; health; PCR-TTGE

INTRODUCTION

Pollen grains represent a category of Primary Biological Aerosol Particles (PBAP) in addition to bacteria, virus, fungal spore, algae, cyanobacteria (**Després *et al.*, 2012**). During pollination, pollen grains come into contact with human respiratory or conjunctival mucosa and could be responsible for pollinosis which correspond mainly to allergic rhinitis and conjunctivitis. In France, pollinosis affect from 10 to 15% of the general population and are increasing in urban areas (**Ravault *et al.*, 2005**). In some cases, small pollen grains can induce asthma attacks (**Heydenreich *et al.*, 2012**). Allergic rhinitis triggered by the pollen grains of some seasonal plants is commonly named "hay fever", and appears mostly during haying season. However, atopic patients with seasonal allergies present hay fever throughout the year. The pollen grains which provoke hay fever change between individuals and from region to region. The hardly visible pollen of wind-pollinated plants such as trees, grasses, and weeds are yet the predominant cause (**D'Amato *et al.*, 2007**). In Mediterranean region, *Pinaceae* and mostly *Cupressaceae* pollen grains constitute most clinically relevant trees pollen (**D'Amato *et al.*, 2007; Yalcin *et al.*, 2013**). Moreover, pollen is regarded as a source of not only allergens but also immunomodulatory molecules, which present major roles in sensitization and/or the exacerbation of allergies (**Kamijo *et al.*, 2009**). Recently, nanovesicles containing allergens (named pollensomes) released during *in vitro* germination of pollen grains were described to contribute to allergic reaction (**Prado *et al.*, 2015**).

For many years, bacterial communities associated to plants were analysed particularly root-inhabiting (**Buée *et al.*, 2009; Bulgarelli *et al.*, 2012**), leaf-surface inhabiting (**Yashiro *et al.*, 2011; Izhaki *et al.*, 2013**) and less frequently flower-inhabiting (**Aleklett *et al.*, 2014**) bacterial microbiota. Both culture-dependent and independent methods were performed to explore bacterial microbiota. Since the diameter of pollen grains from anemophilous species varies from 17 to 58 µm (**Després *et al.*, 2012**), pollen grains may represent a support for bacteria. Moreover, many exsudated compounds of pollen grains are known to be attractive for bacterial colonizers (**Aleklett *et al.*, 2014**).

Bacterial communities associated to pollen called bacterial pollen microbiota were rarely investigated. **Colldahl and Carlsson (1968)** isolated the Gram-negative *Pseudomonas maltophilia* (currently *Stenotrophomonas maltophilia*) from pollen samples. The presence of microorganisms on surface of pollen was then confirmed by scanning electron microscopy (**Colldahl and Nilsson, 1973**). Later, **Spiewak *et al.* (1996a)** highlighted the presence of a mixed microflora consisted of Gram-positive and Gram-negative mesophilic bacteria, thermophilic actinomycetes and fungi on allergenic pollen grains. Moreover, **Spiewak *et al.* (1996b)** showed that Gram negative bacteria such as *Pantoea agglomerans* endotoxin associated to pollen should be considered as a potential factor aggravating pollinosis. More recently, **Heydenreich *et al.* (2012)** reported that grass pollen grains were colonized by Gram-negative bacteria as *Acinetobacter lwoffii* and Gram-positive bacteria belonging to the genus *Bacillus* which presents adjuvant activity inducing inflammatory T cell responses. Few studies on plant protection have been carried out in order to detect bacteria responsible for plant disease as *Pseudomonas syringae* pv *actinidiae* on the kiwifruit pollen grains (**Vanneste *et al.*, 2011**). All these studies were investigated with culture-dependent method based on the isolation of bacteria on different agar media. Molecular methods were never used.

Currently, bacterial communities associated with humans (**McCartney, 2002; Roudière *et al.*, 2009; Michon *et al.*, 2012**), animals (**Richards *et al.*, 2005; Navarrete *et al.*, 2012**), plants (**Lambais *et al.*, 2014**), foods (**Ogier *et al.*, 2004; Hamdouche *et al.*, 2015**) or environment (**Lyautey *et al.*, 2005**) are analysed by culture-independent approaches such as genetic fingerprinting methods (PCR-DGGE, PCR-TTGE). The 16S rRNA gene is the most frequently gene used for amplification. Separation of PCR products in TTGE is based on the decrease of the electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing DNA denaturants according to a temperature gradient. Molecules with various sequences will have a different melting behaviour and will stop migrating at distinct position in the gel (**Ogier *et al.*, 2002**). The aim of this study was to perform a preliminary analysis of the bacterial communities associated with pollen isolated from plants responsible for

pollinosis by applying PCR-TTGE method. A PCR anchored on the bacterial 16S rRNA gene followed by a TTGE analysis was carried out on DNA extracts from several pollen grain samples collected in Mediterranean region. On the one hand, methodological aspects were developed including a comparative study of different pollen preparation methods. On the other hand, bacterial diversity of a set of samples were analysed with a 16S rDNA based PCR-TTGE approach.

MATERIAL AND METHODS

Pollen grains sampling

Pollen samples were collected from various anemophilous trees (Tab 1) in Montpellier, France (GPS data = 43.6° N & 3.9° W) between February and April 2013, in accordance with the pollinic bulletins of the French aerobiology network RNSA (Réseau National de Surveillance Aérobiologique). Collected pollen samples belonged to the genera *Pinus* (x = 5), *Cupressus* (x = 4), *Cephalotaxus* (x = 1), *Quercus* (x = 1), and *Platanus* (x = 1). Data concerning the samples figured in Table 1. According to the type of male inflorescence, pollens were either directly collected or spikes were cut with sterile scalpel. In both cases, pollen grains were aseptically collected in sterile storage bags after shaking and sieving through sterile gauze. Careful consideration was given to collect the pollen grains in order to avoid mixed pollen samples from different plants. Pollen samples were stored at 4°C in the dark. Quantification of bacteria in pollen presented in Table 1 was performed according to the protocol described below.

Table 1 Data concerning the pollen sampling. Quantification of bacteria in pollen was performed according to the protocol described below.

Sample name	Date of sampling	Plant genus	Geographic location in Montpellier city*	Enumeration number of bacteria / g pollen
P1	2013/04/10	*Pinus*	I	$4.0\ 10^5$
P2	2013/04/10	*Pinus*	II	$1.4\ 10^4$
P3	2013/04/10	*Pinus*	I	$6.7\ 10^4$
P4	2013/04/14	*Pinus*	III	$3.9\ 10^4$
P8	2013/04/22	*Pinus*	IV	$1.9\ 10^5$
C1	2013/03/21	*Cupressus*	I	$1.4\ 10^5$
C1b	2013/03/21	*Cupressus*	I	$5.4\ 10^5$
C2	2013/02/23	*Cupressus*	V	$2.0\ 10^4$
C3	2013/03/25	*Cupressus*	I	$5.7\ 10^6$
Cx	2013/04/03	*Cephalotaxus*	VI	$5.0\ 10^5$
Q	2013/04/18	*Quercus*	VI	$7.0\ 10^3$
Pla2	2013/04/22	*Platanus*	VII	$5.7\ 10^5$

Legend:* I- Faculty of Sciences campus, II- Faculty of arts campus, III- Montmaur wood, IV- Lapeyronie hospital site, V- Faculty of Pharmacy campus, VI- Garden of plants, VII- Albert 1er square.

Preparation of pollen samples

Pollen samples were prepared by five different protocols (A, B, C.adn, C.pca and C.tsh) before DNA extraction (Fig 1).

Fig. 1

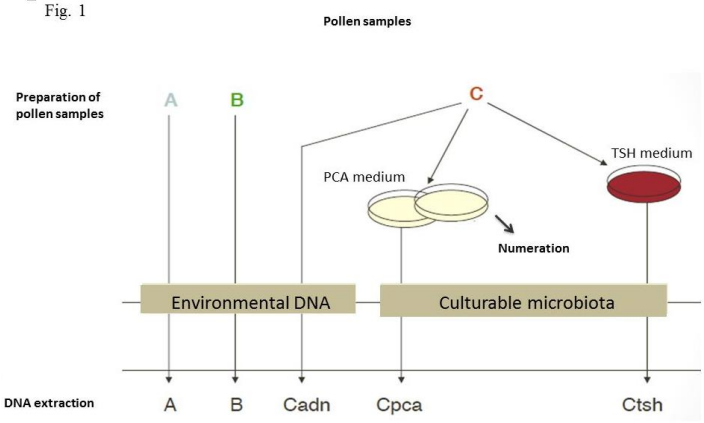

Figure 1 Presentation of the different protocols used to prepare pollen samples before DNA extraction. For protocols C.pca and C.tsh, an additional culture on PCA medium and TSH medium were performed respectively before extraction with the MasterPure™ DNA purification Kit (Epicentre).

The primary objective was to highlight the best protocol to extract effectively all bacterial DNA associated to pollen grains. For protocol A, 30 mg of pollen sample were suspended directly in 150 µl of Tris-EDTA (TE) buffer with 1 µl of lysozyme (Sigma) and incubated at 37 °C for 18 h. For protocol B, 30 mg of pollen sample were centrifuged (12,000 g for 10 min) thrice in 150 µl of TE buffer. Then, the final pellet was suspended in 150 µl of TE buffer with 1 µl of lysozyme and incubated at 37 °C for 18 h. The protocol C was based on the method described by **Vanneste *et al.*** (**2011**) including few modifications as follows: 30 mg of dry pollen suspended in 1 ml of sterile distilled water were sonicated for 5 min. Suspension was shaken with a rotary shaker for 60 min at 120 rpm. After settling, the supernatant was filtered through sterile gauze and centrifuged at 10,000 g for 20 min at 6°C. The supernatant was discarded and the pellet was rehydrated with 1 ml of sterile distilled water. This solution is referred to as the Final Concentrate (FC). From this FC, three different variations to protocol C called C.adn, C.pca and C.tsh were performed in order to optimise the recovery of DNA. For Cadn, DNA was directly extracted from 500 µl of FC. For C.pca, 100 µl of the FC were spread onto Plate Count Agar (PCA) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.tsh, 100 µl of the FC were spread onto Tryptic Soy Agar supplemented with 5 % of horse blood (TSH) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.pca and C.tsh, DNA was extracted from cultures collected at the surface of PCA or TSH plates respectively. The last step of protocol C consists of re-suspension in 150 µl of TE buffer with 1 µl of lysozyme and incubation at 37 °C for 18 h.

Bacterial enumeration

For protocol C, three decimal dilutions were carried out in saline serum from Final Concentrate (FC). Then 100 µl of FC and each dilution were plated in duplicate on PCA agar and incubated at 30 °C for 18 h.

DNA extraction

DNA was extracted from the five previous protocols of pollen preparation, following the recommendations of the MasterPure™ DNA purification kit (Epicentre). Quality of DNA extracts was evaluated using UV spectrophotometry (rate 260 nm / 280 nm) and diluted to obtain a final concentration of 50 µg/ml.

PCR –Temporal Temperature Gradient Gel Electrophoresis (TTGE) analysis

A 199 bp-fragment (from position 338 to position 536, *Escherichia coli* numbering) overlapping the V3 variable region of 16S rDNA (position 338 to position 534, *E. coli* numbering) (**Sundquist *et al.*, 2007**) was amplified using primers HDA1f-GC and HDA2 (**Ogier *et al.*, 2002**). A 40-bp GC-clamp was added to the forward primer. PCR was performed in a final volume of 50 µl containing 200 µM each dNTP (Fermentas), 200 nm each primer (Sigma), 2.5 U of Fast Start *Taq* DNA Polymerase (Roche) in the appropriate 1x reaction buffer with 1.8 mM $MgCl_2$ and 1 µl of the DNA extract. Amplification program was carried out as follows: an initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min and 72 °C for 7 min for the final extension. PCR products were checked using conventional electrophoresis in 1.5 % (w/v) agarose gel with 1x Tris Borate EDTA (TBE) buffer and then submitted to TTGE analysis using a Dcode Universal Mutation Detection System (Bio-Rad). PCR products (1 µl) were loaded into 8% (w/v) bisacrylamide (37.5:1), 7 M urea, 40 µl TEMED and 0.1 % (w/v) ammonium persulfate gels. Migrations were performed in 1x Tris Acetate EDTA (TAE) buffer with additional magnetic shaking in the electrophoresis compartment. A pre-migration for 15 min at 63 °C and 20 V was followed by migration for 16 h at 46 V with an initial temperature of 63 °C and a final temperature of 70 °C corresponding to an increase of 0.4 °C / h. Gels were stained for 15 min with 0.5 mg / ml ethidium bromide in 1x TAE buffer, rinsed for 45 min in 1x TAE buffer and then photographed on a UV transilluminator.

TTGE bands analysis and sequencing

Dominant bands were cut from TTGE gels, rinsed twice with molecular biology grade water and eluted overnight in 10 mM Tris buffer (pH 8.5) at 37 °C. Extracted DNA was re-amplified using primers HDA1 and HDA2 without GC-clamp as previously described (**Michon *et al.*, 2010**). Then PCR products were sequencing on an applied automatic sequencer (Cogenics) by using the forward primer HDA1. DNA sequences were visualized and analysed with BioEdit program version 7.0.9 (**Hall, 1999**). The 16S rRNA gene sequences were screened using GenBank's Blast program (**Altschul *et al.*, 1990**).

Fingerprinting and statistical analysis

The TGGE gel images were analyzed using Image Quant TL software v. 2003 (Amersham Biosciences). Individual lanes of gel images were aligned which permits detection and record of the relative position of each DNA band. TTGE patterns were manually scored by presence and absence of co-migrating bands between lanes. Pairwise community similarities were quantified using the Dice similarity coefficient (S_D): $S_D = 2 N_c / N_a + N_b$ (**Heyndrickx *et al.*, 1996**) where N_a represented the number of bands detected in sample A, N_b represented the number of bands in sample B, and N_c represented the number of bands common to both samples. A cluster analysis was carried out using the similarity matrix to group pollen samples according to their similarity index. A Principal Component Analysis (PCA) was also used as a multivariate technique for exploratory data analysis using Statistica (version 7) software (StatSoft, USA).

RESULTS

Bacterial communities were analysed on pollen samples from various wind-pollinated trees in order to evaluate the microbiota associated to pollen. The comparison of microbial ecology of pollen grains from various anemophilous trees was performed by PCR-TTGE analysis.

Optimization of preparation of pollen samples and bacterial numeration

Five protocols were evaluated to prepare the pollen samples. Figure 2 shows TTGE patterns obtained from pollen samples of five *Pinus* specimens, *i.e.*, P1-4 and P8 (Tab 1) prepared according to four different protocols (A, C.adn, C.pca and C.tsh).

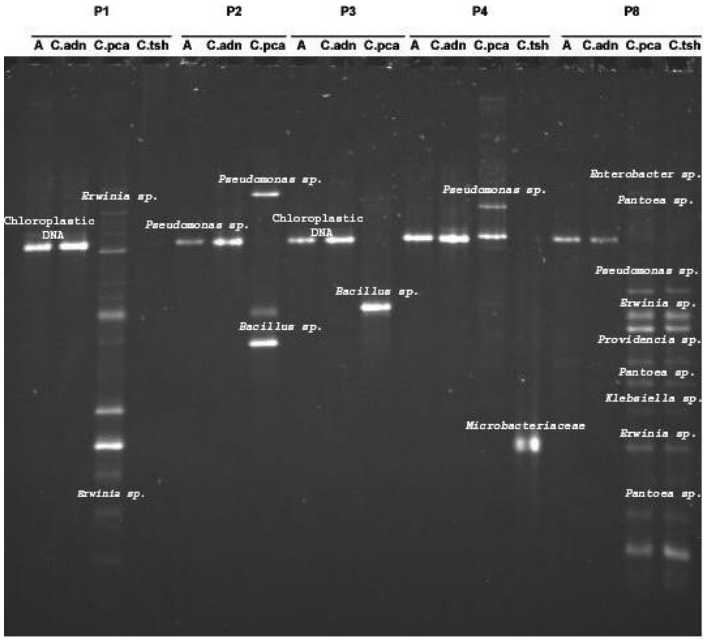

Figure 2 Bacterial 16S rDNA PCR-TTGE profiles obtained from pollen samples of five different *Pinus* (P1, P2, P3, P4 and P8), prepared according to four different protocols (A, C.adn, C.pca, C.tsh).

For P8 sample, one band was detected for protocols A and C.adn while 16 bands were detected for protocols C.pca and C.tsh. Figure 3 presents TTGE patterns obtained from pollen samples of *Cupressus* (C1, C1b, C2 and C3), *Cephalotaxus* (Cx), *Quercus* (Q) and *Platanus* (Pla2) prepared according to the five different protocols, *i.e.*, A, B, C.adn, C.pca and C.tsh.

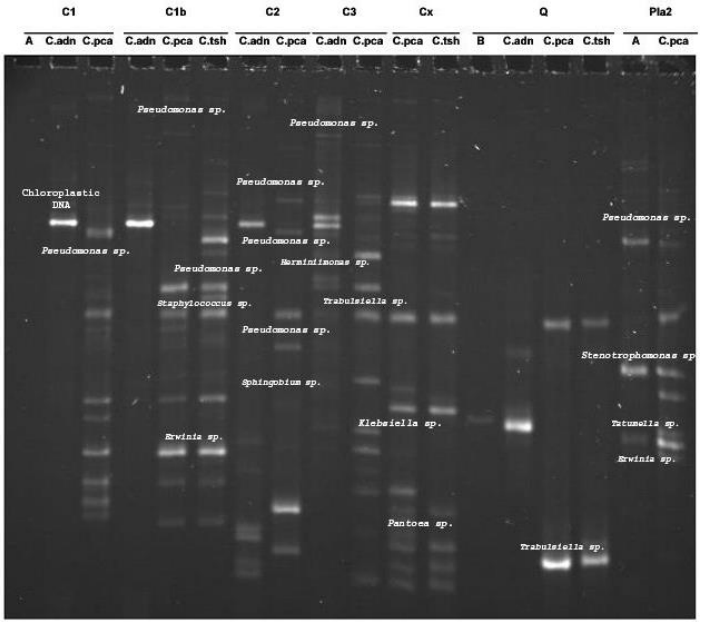

Figure 3 Bacterial 16S rDNA PCR-TTGE profiles obtained from pollen samples of *Cupressus* (C1, C1b, C2 and C3), *Cephalotaxus* (Cx), *Quercus* (Q) and *Platanus* (Pla2) prepared according to five protocols (A, B, C.adn, C.pca, C.tsh).

For C1b, one band was detected for protocol C.adn while 12 and 16 bands were respectively obtained for protocols C.pca and C.tsh. Thus, both protocols C.pca and C.tsh highlight the highest number of detected bands whatever the sample.
The cluster analysis of TTGE patterns obtained from pollen samples of the five *Pinus* specimens prepared according to four protocols showed two main dissimilar clusters: the first cluster grouped patterns obtained from protocols C.pca and C.tsh and the second cluster comprised patterns obtained from protocols A and C.adn (Fig 4).

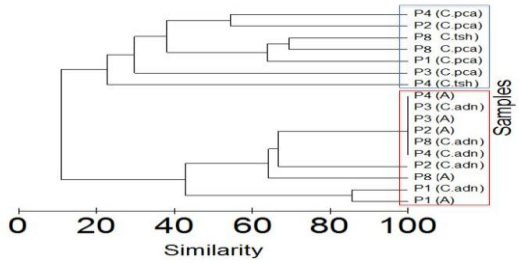

Figure 4 Cluster analysis of 16S rDNA PCR-TTGE profiles obtained from pollen samples of five *Pinus* samples, prepared according to four protocols.

The Principal Component Analysis performed on the same TTGE patterns showed 70% of the variability of samples according to four protocols with or without a step of culture before DNA extraction (Fig 5).

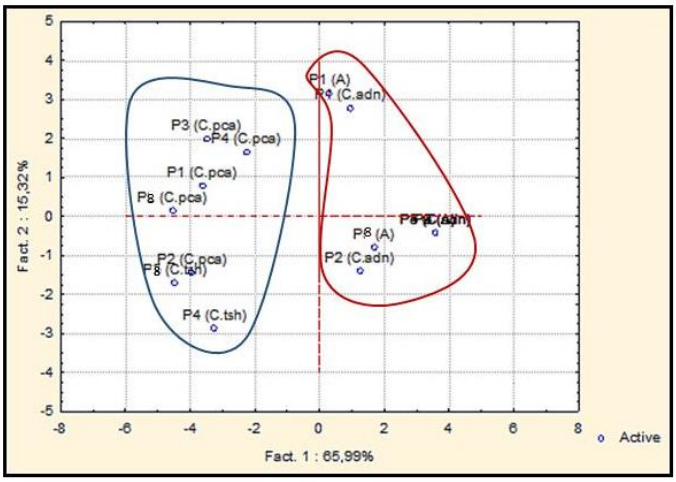

Figure 5 Principal Component Analysis (PCA) of 16S rDNA PCR-TTGE profiles obtained from pollen samples of five different *Pinus* samples, prepared according to four protocols.

Two distinct groups could be discriminated: the first group contained samples obtained from protocols with step culture (C.pca and C.tsh), a second group included samples prepared without step culture (protocols A and C.adn).
Bacterial enumeration from PCA medium varied from 1.4 10^4 to 4.0 10^5 CFU / g pollen and from 2.0 10^4 to 5.7 10^6 CFU / g pollen for *Pinus* and *Cupressus*, samples, respectively (Table 1). Bacterial numeration ranged from 7.0 10^3 to 5.7 10^5 CFU / g pollen for *Quercus*, *Cephalotaxus* and *Platanus*.

Bacterial communities associated to pollen samples

The objective of this study was to evaluate the bacterial communities associated to pollen grains from different tree genera, i.e., *Pinus*, *Cupressus*, *Cephalotaxus*, *Quercus*, and *Platanus* using PCR-TTGE.
Concerning pollen samples from *Pinus*, a total of 53 DNA bands were observed in total of the samples (Fig 2). Among the 53 observed bands, 46 bands were cut and sequenced. DNA sequences obtained from cut bands varied from 147 to 172 bp compared to the 199 bp of the V3 region. First, we demonstrated that several sequences corresponded to chloroplast DNA. Presence of chloroplast DNA was not unexpected because of the high similarity between bacterial and chloroplast 16S rRNA gene sequence in the context of endosymbiotic origin of chloroplast (**McFadden 2001**). As a result, bacterial but also chloroplast 16S rRNA genes could be amplified with primers used in this study. Then, we detected a majority of bacteria belonging to the *Enterobacteriaceae* family such as *Erwinia* sp., *Pantoea* sp., *Enterobacter* sp., *Klebsiella* sp., and *Providencia* sp. but also to *Pseudomonadaceae*, *Xanthomonadaceae*, and *Microbacteriaceae* families, and to the genus *Bacillus*. Within the genus *Pinus*, the five TTGE patterns corresponding to C.pca protocol were very different from one tree to another. Indeed, we observed that bacterial communities associated to pollen seemed to vary among the genus *Pinus*. Only bacterial strains belonging to *Pseudomonas* genus seemed to be present in four *Pinus* samples from five.
Concerning pollen samples from *Cupressus*, *Cephalotaxus*, *Quercus*, and *Platanus*, a total of 99 DNA bands were observed in total of the samples which permitted to obtain 81 DNA sequences. From *Cupressus* samples, we found bacteria belonging to *Enterobacteriaceae* family such as *Erwinia* sp., *Pantoea* sp., *Dickeya* sp., *Trabulsiella* sp., *Providencia* sp. as well as to genera *Pseudomonas* and *Staphylococcus*. *Pseudomonas* strains appeared to be common to all *Cupressus* samples. Pollen sample from *Cephalotaxus* presented also bacteria belonging to *Enterobacteriaceae* as *Erwinia* sp., *Klebsiella* sp., *Enterobacter* sp., *Trabulsiella* sp., *Pantoea* sp. and to *Pseudomonadaceae* families. For *Quercus*, we found chloroplastic DNA from *Trabulsiella* sp. Finally, for *Platanus* pollen sample, the TTGE pattern comprises bacteria from *Enterobacteriaceae* (*Tatumella* sp. and *Erwinia* sp.), *Pseudomonas* sp. and *Stenotrophomonas* sp.

DISCUSSION

In the present study, we analysed bacterial communities associated to twelve pollen samples collected from five anemophilous tree genera at different locations in Montpellier (France). We used a molecular fingerprint technique called PCR-TTGE which was already applied to determine bacterial communities associated with human, animals or plants (**Perez Pulido *et al.*, 2005; Michon *et al.*, 2012; Navarrete *et al.*, 2012**). Five pollen preparation protocols were evaluated. Preparation of pollen samples before DNA extraction was a critical step of the PCR-TTGE analyses since it appeared decisive to obtain the totality of bacterial communities associated to a sample. As concerns the number of detected DNA bands for each TTGE profile, mean values varied according to the pollen preparation protocol. Thus, the best results were obtained with the protocol adapted from **Vanneste *et al.* (2011)** followed by a culture dependent step on a non-selective medium (PCA medium) or enriched medium (TSH medium) before DNA extraction, amplification and TTGE analyses. Indeed, the previous mentioned preparation of pollen comprising sonication and a 1-hour shaking allowed to optimally recovery bacteria from exine pollen wall. Then, the culture-dependent step permitted to concentrate bacteria. We obtained TTGE patterns with a largest number of DNA bands corresponding to main but also less abundant bacteria present on pollen samples. However potential viable but nonculturable (VBNC) bacteria could not be detected. The culture-independent protocol highlighted only the main bacteria. The bacterial enumeration of pollen samples varied from 7.0 10^3 to 5.7 10^6 CFU/g pollen. These results were concordant with those obtained by **Spiewak *et al.* (1996a)** for different trees.
The bacterial communities associated to pollen samples varied among two plant genera (inter- genera variations) but also inside a same genus (intra-genus variation). Indeed, we observed very different TTGE profiles for the five pollen samples from *Pinus* and for the four pollen samples from *Cupressus*. Bacteria belonging to the class *Gammaproteobacteria* (*Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*…) were the most represented in pollen samples before bacteria belonging to the phylum *Firmicutes*, whatever the plant genus. These results were consistent with those observed by **Spiewak *et al.* (1996a)** which highlighted in particular mixed microflora consisting of Gram-positive and Gram-negative mesophilic bacteria on allergenic pollen grains. Bacteria belonging to *Gammaproteobacteria* class represented 73% of the 16S rDNA sequences obtained and were detected in most of samples. These results are in accordance with those of **Wetzel *et al.* (2010)** which showed that 81.2% of bacterial species isolated from edible flowers belonged to the class *Gammaproteobacteria*. Given the length of the amplified V3 sequence as well as the intragenomic and intraspecific heterogeneity of the 16S rRNA gene, it is very difficult to obtain identification at the species level and even at the genus level. It is a classical disadvantage of the PCR-TTGE method (**Michon *et al.*, 2012**).
Some *Enterobacteriaceae* species are commonly present in plant. We found DNAs of *Erwinia* sp. and *Pantoea* sp. in *Pinus* and *Cupressus* samples. *Erwinia* is a well-known bacterial genus associated to plants. Some *Erwinia* species could lead to damages of plant structure, wilting, dieback, yellowing or rot (**Thomson *et al.*, 1999**). The main phytopathogenic *Erwinia* species are *E. amylovora* and *E. carotovora* responsible for the fire blight of *Rosaceae* especially apple and pear (**Piqué *et al.*, 2015**), and soft rot diseases respectively (**Parent *et al.*, 1996**). *Pantoea agglomerans* (formerly *Erwinia herbicola*) could be opportunistic pathogen in cases of weakness of the plant (**Dutkiewicz *et al.*, 2016**). *Dickeya* sp., *Enterobacter* sp. and *Klebsiella* sp. DNAs were also detected in our pollen samples. The genus *Dickeya* comprises several species particularly *D. chrysanthemi* (formerly *Erwinia chrysanthemi*) known to be phytopathogens (**Toth *et al.*, 2011**). *Enterobacter asburiae*, *Enterobacter cloacae* and *Enterobacter cowanii* were already recovered from plants (**Wetzel *et al.*, 2010; Humann *et al.*, 2011**). *E. asburiae* was considered as a Plant Growth Promoting Rhizobacteria (PGPR) particularly for *Citrus reticulata* (**Thokchom *et al.*, 2014**) whereas *E. cloacae* is showed to be phytopathogenic for numerous plants such as onion, ginger, papaya and macadamia (**Humann *et al.*, 2011**). *E. cowanii* as well as *Klebsiella oxytoca* were reported to cause wilt in many plants (**Sarkar and Chaudhuri, 2015**). *K. oxytica*, *Enterobacter* sp., *Erwinia* sp., *Tatumella ptyseos* etc. were also dominant taxa in a Greek vineyard (**Nisiotou *et al.*, 2011**). To conclude on the *Enterobacteriaceae* species present on pollen grains, we observed that they were usually present in other parts of the plants where they are potentially phytopathogens.
The genus *Pseudomonas* which also belongs to the *Gammaproteobacteria* class presents various species associated to plants, e.g., *P. syringae* (**Kennelly *et al.*, 2007**), *P. rhizosphaerae* (**Peix *et al.*, 2003**) and *P. panacis* (**Park *et al.*, 2005**). Thus *Pseudomonas* spp. could be considered as phytopathogen, biological agent or PGPR (**Vanneste *et al.*, 2011; Noori and Saud, 2012; Mansfield *et al.*, 2012**). The genus *Xanthomonas* with *X. campestris* and *X. oryzae* causes also significant damage in a range of crops too (**Ryan *et al.*, 2011**).
The *Enterobacteriaceae* is a well-known family of bacteria which constitute human intestinal microbiota but several species or serotypes can also be pathogenic to human. Most of them are recognized as opportunistic pathogen and causes respiratory symptoms. In particular, *Pantoea* strains are responsible for respiratory infections in immunocompromised patients (**Flores Popoca *et al.*, 2012; Kursun *et al.*, 2012; Walterson and Stravrinides, 2015; Dutkiewicz *et al.*, 2016**). *Enterobacter asburiae* was also found in human samples particularly in sputa (**Koth *et al.*, 2012**). Klebsiellae cause various infections in humans including community-acquired pneumonia and nosocomial infections. Moreover, *Tatumella ptyseos* strains were notified to be responsible for human tracheobronchial/pulmonary infections (**Costa *et al.*, 2008**). For the *Pseudomonadaceae* family, *Pseudomonas aeruginosa* is likely to be responsible for community-acquired infections; but serious infections are predominantly hospital-acquired. For example, *P. aeruginosa* is the second most common cause of nosocomial pneumonia. *P. aeruginosa* is also recovered from respiratory tract of cystic fibrosis patients (**Winstanley *et al.*, 2016**). In addition, *P mosselii* should be taken into account as a potential human pathogen which was especially isolated from tracheal aspirate of a patient suffering from pulmonary infections (**Leneveu-Jenvrin *et al.*, 2013**). Regarding the phylum *Firmicutes* which represents 14% of the 16S rDNA sequences obtained, we underscored the presence of *Bacillus* strains in particular *Bacillus cereus* which is well-known as volatile human pathogen (**Bottone, 2010**), and *Staphylococcus pasteuri* responsible for a few cases of infections in immunocompromised patients (**Morfin-Otero *et al.*, 2012**). Pollen samples from *Cephalotaxus*, *Quercus* and *Platanus* comprised also *Gammaproteobacteria* such as *Enterobacteriaceae* and *Pseudomonas* strains.
Indeed, we detected in pollen grains from anemophilous (deciduous and coniferous) trees various bacterial genera, i.e., *Pseudomonas*, *Erwinia* and *Enterobacter* well-known as responsible for a wild range of human diseases and plant infections.

CONCLUSION

In this present study, we analysed for the first time bacterial communities associated to pollen grains in particular from *Pinus* and *Cupressus* but also from *Cephalotaxus*, *Quercus* and *Platanus* by a molecular method. PCR-TTGE is a suitable tool since bacterial microbiota was determined for each pollen samples. Bacterial communities varied inside a plant genus and from genus to genus. We highlighted here the potential presence of phytopathogens and also human potential opportunistic bacteria on allergenic pollens. Indeed, pollen grains

should be considered as vectors for these human opportunistic bacteria. In addition to be allergenic, pollen could participate to the dissemination of bacteria

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