Genetic diversity analysis of isolates belonging to the *Photobacterium phosphoreum* species group collected from salmon products using AFLP fingerprinting☆

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Abstract :

An accurate amplified fragment length polymorphism (AFLP) method, including three primer sets for the selective amplification step, was developed to display the phylogenetic position of *Photobacterium* isolates collected from salmon products. This method was efficient for discriminating the three species *Photobacterium phosphoreum*, *Photobacterium iliopiscarium* and *Photobacterium kishitanii*, until now indistinctly gathered in the *P.phosphoreum* species group known to be strongly responsible for seafood spoilage. The AFLP fingerprints enabled the isolates to be separated into two main clusters that, according to the type strains, were assigned to the two species *P. phosphoreum* and *P. iliopiscarium*. *P. kishitanii* was not found in the collection. The accuracy of the method was validated by using *gyr*B-gene sequencing and *lux*A-gene PCR amplification, which confirmed the species delineation. Most of the isolates of each species were clonally distinct and even those that were isolated from the same source showed some diversity. Moreover, this AFLP method may be an excellent tool for genotyping isolates in bacterial communities and for clarifying our knowledge of the role of the different members of the *Photobacterium* species group in seafood spoilage.

Highlights

► AFLP fingerprinting is a reliable tool for genotyping bacterial isolates. ► Diversity of bacterial isolates is shown by AFLP genotyping. ► The method allows the species delineation in the *P. phosphoreum* species group. ► Both *P. iliopiscarium* and *P. phosphoreum* are present in salmon products.

Keywords : *Photobacterium phosphoreum*, AFLP, *gyr*B-gene, *lux*A-gene, Salmon products, Seafood spoilage

Abbreviations

- AFLP, amplified fragment length polymorphism;
- PCR, polymerase chain reaction;
- MAP, modified atmosphere packaging;
- SSO, specific spoilage organism;
- UPGMA, unweighted pair group with arithmetic mean;
- TTGE, temporal temperature gradient gel electrophoresis

Footnote ☆

Note: Nucleotide sequence data reported are available in the EMBL database under the accession numbers LN715117 to LN715169 available from the European Nucleotide Archive (ENA) browser at http://www.ebi.ac.uk/ena/data/view/LN715117-LN715169 once they are released into the public domain.

1. Introduction

Fresh fish products under modified atmosphere packaging (MAP) are sold increasingly and widely in Europe as chilled products. This type of packaging, enriched with a CO_2 concentration usually varying between 20 and 60%, extends the shelf life of these products by reducing the growth of some Gram-negative bacteria like *Pseudomonas* and *Shewanella* (Gram and Huss, 1996; Sivertsvik et al., 2002). The extended shelf life facilitates the transport, chilled distribution and marketing of fresh MAP fish. However, MAP favors the development of CO_2-resistant bacteria, such as Gram-positive lactic acid bacteria, *Brochothrix thermosphacta* and the Gram-negative *Photobacterium phosphoreum* (Dalgaard et al., 1993; De la Hoz et al., 2000; Emborg et al., 2005; Hansen et al., 2009; Macé et al., 2012; Rudi et al., 2004). *P. phosphoreum* has been identified as the main specific spoilage organism (SSO) responsible for sensory spoilage of several chilled marine fish including salmon, cod, halibut, garfish, and saithe as well as shrimp (Dalgaard et al., 1993; Dalgaard, 1995; Emborg et al., 2002; Macé et al., 2013).

The genus *Photobacterium* is a member of the *Vibrionaceae* family and comprises facultatively aerobic, motile bacteria. They typically occur in seawater and are associated with marine animals such as saprophytes and enteric commensals (Urbanczyk et al., 2011). Many bacterial isolates identified as *P. phosphoreum* have been isolated from seafood products including fish. As a facultative psychrophile, this bacterium has been isolated from the surfaces and intestinal contents of fish generally occurring in cold meso-pelagic and bentho-pelagic habitats (Dunlap and Kita-Tsukamoto, 2006; Hastings and Nealson, 1981; Wilson et al., 2008) and consequently on fish fillets and steaks (Emborg et al., 2002; Hansen et al., 2009; Hovda et al., 2007; Macé et al., 2012; Reynisson et al.,2009; Rudi et al., 2004).

For many years, there has been ambiguity regarding the phylogenetic status, species composition and correct identification of strains in the *P. phosphoreum* species group. In a phylogenetic study based on multilocus sequence analysis targeting 16S rDNA, *gyr*B and *lux* ABFE genes, Ast and Dunlap (2005) demonstrated that strains originally isolated and identified as *P. phosphoreum* can be classified into three evolutionarily distinct lineages, considered species, *P. phosphoreum*, *Photobacterium*

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iliopiscarium and *Photobacterium kishitanii*. Regarded as sister species, *P. phosphoreum* and *P. iliopiscarium* are more closely related to each other than either is to *P. kishitanii*. These results were confirmed by Urbanczyk et al. (2011), reviewing the status of the genus *Photobacterium* from the perspectives of genomics, phylogeny and symbiosis. These authors carried out a phylogenetic analysis based on multiple housekeeping genes with both previously recognized and newly described species. The analysis revealed an evolutionary divergence within *Photobacterium* with the presence of two well-supported clades. The two clades separate luminous and symbiotic species from non-luminous, non-symbiotic forms. The type species of the genus is *P. phosphoreum*, a member of Clade 1, while *P. iliopiscarium* is its closest relative in the phylogenetic tree. Species of Clade 1 are luminous with the exception of *P. iliopiscarium*, for which no luminous strains have been described to date. In order to identify these three species correctly and easily among the *P. phosphoreum* group, it is necessary to develop additional molecular methods.

Molecular genotyping tools, including amplified fragment length polymorphism (AFLP), enabling the fingerprinting of specific DNA patterns are used extensively to study microbial diversity (Cocolin and Ercolini, 2008; Giraffa and Neviani, 2001; Vos et al., 1995). For a set of bacterial isolates, phylogenetic relationships can be inferred by analyzing the AFLP data.

AFLP can be applied to DNA of any source or complexity, varying in both size and base composition and there are many applications for this universal marker system. Moreover, a high level of correlation between AFLP results and published DNA hybridization data has been shown for many bacterial genera and species, indicating that AFLP is a valuable technique for the classification of bacteria (Blears et al., 1998; Mougel et al., 2002). The method shows high discriminatory power and good reproducibility and has proved to be efficient for discriminating at the species level and below in various taxa (Janssen et al., 1996, Savelkoul et al., 1999). The development and application of AFLP as a fingerprinting method has led to significant progress in the study of the genetic diversity and taxonomy of bacteria (Cappello et al., 2008; Hamza et al., 2012; Lazzi et al., 2009; Lévesque et al., 2012; Nabhan et al., 2012). However, only a few studies have been published using AFLP to investigate the phylogeny of *Photobacterium* (Ast et al., 2007), most of them dealing with *P. damselae*

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in epidemiological surveys (Botella et al., 2002; Kvitt et al., 2002; Takahashi et al., 2008; Thyssen et al., 2000).

The aim of the present work is, including reference strains, to study the phylogenetic position of potential seafood spoiling *Photobacterium* isolates collected, during previous studies, from seafood products, especially salmon steaks (Macé et al., 2012, 2013a, 2013b) and cold-smoked salmon (Leroi et al., 1998). Here, we describe an accurate AFLP method to discriminate between *P. phosphoreum* and *P. iliopiscarium*, strains previously tentatively classified within the *P. phosphoreum* species group (Ast and Dunlap, 2005). The results are supported by investigating the *gyr*B-gene sequences and the presence or absence of the *lux*A gene in every isolate of the collection. As emphasized before (Ast and Dunlap, 2005), the taxonomic confusion surrounding *P. phosphoreum* exemplifies a fundamental problem in bacterial systematics: how can species that are phenotypically similar but evolutionarily distinct be reliably recognized and diagnosed? This work aims to contribute to resolving this problem.

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains and isolates used in this study are listed in Table 1. Ten reference strains of *Photobacterium* were obtained from culture collections. They belong to the following species: *P. angustum*, *P. damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida*, *P. phosphoreum*, *P. iliopiscarium*, *P. kishitanii*, *P. indicum* and *P. lipolyticum*. The *Photobacterium* isolates were collected from fish products, most of them from different batches of spoiled salmon steaks and a few from

smoked salmon. These isolates were tentatively identified as belonging to the *P. phosphoreum* species group as described by Macé et al. (2012, 2013a, 2013b) and Leroi et al. (1998).

2.2. DNA extraction

Bacterial isolates or strains were cultured in enriched brain heart infusion broth (BHI; Biokar Diagnostics, Beauvais, France) supplemented with 2.0% NaCl and incubated at 15°C for 24 to 48 h depending on the strain. The chromosomal DNA of all bacterial isolates was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, S.A., Courtaboeuf, France). DNA quality and quantity were determined using a NanoVue Plus™ spectrophotometer (GE Healthcare Europe, Germany) by measuring absorptions at 260 and 280 nm.

2.3. Amplified Fragment Length Polymorphism (AFLP) analysis

Total genomic DNA from all *Photobacterium* strains and isolates listed in Table 1 were subjected to the AFLP Microbial Fingerprinting kit (AFLP® Analysis System for Microorganisms, Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. The Invitrogen™ protocol was slightly modified to accommodate the non-radioactive labeling of selective primers *Eco*RI-X and capillary electrophoresis.

2.3.1. Preparation of primary template

A restriction digestion mixture containing 5X reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate), 250 ng of extracted bacterial DNA, 2.5 U of *Eco*RI and *Mse*I restriction enzymes in 2 µl of buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 50% (v/v) glycerol, 0.1 % Triton X-100) and distilled water was prepared in a total volume of 25 µl and incubated for 2 h at 37°C. Then the mixture was incubated at 70°C for 15 min to inactivate the restriction endonucleases. After inactivation, 24 µl of adapter ligation solution containing *Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate and 1 µl containing 1 U of T4 DNA ligase, 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM

KCl, 50% glycerol (v/v) were added to the inactivated digestion solution and incubated at 20°C for 2 h to generate template DNA for amplification. Subsequently, a 1:10 dilution of the ligation mixture was prepared with TE buffer.

2.3.2. Pre-amplification

The "non-selective" primers *Eco*RI-0 and *Mse*I-0 provided by the AFLP Microbial Fingerprinting kit were used for pre-amplification of the diluted primary template in a 20.4 µl total volume according to the manufacturer's instructions. Pre-selective PCR was carried out in 20.4 µl reaction mixtures containing 2 µl of the diluted primary template described previously, 1.08 µl of pre-selective *Eco*RI-0 primer (27.8 ng/µl), 4.8 µl of a mixture containing *Mse*I-0 primers (6.7 ng/µl) and dNTPs, 2 µl of 10X PCR buffer plus Mg, and 0.4 µl of Taq polymerase completed with distilled water. The reaction was subjected to the following PCR conditions: 20 cycles consisting of 30 s denaturation at 94°C, 60 s annealing at 56°C and 60 s extension at 72°C. Amplifications were performed in a Bio-Rad thermal cycler (BioRad, Marne-la-Coquette, France). Subsequently, the pre-amplification product was diluted 50:1 in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

2.3.3. Selective PCR-amplification

Fluorescent dye-labeled *Eco*RI-X primers (WellRED™, purchased from Sigma-Aldrich®, Paris, France) and unlabeled selective primers *Mse*I-X from the Invitrogen™ kit were used. These primers containing adjacent nucleotides (X = A, C, G or T) were tested for optimization and selected in preliminary tests among primers provided by the AFLP Microbial Fingerprinting kit (Invitrogen), as described in the next section "Selective primer combinations".

Selected primers were used for the selective amplification of the diluted pre-amplification product (5 µl) in a 20 µl total volume containing 4.5 µl of a mixture of *Mse*I-X selective primer (6.7 ng/µl) and dNTPs, 0.5 µl of labeled *Eco*RI-X selective primer, 2 µl of 10X PCR buffer, and 0.1 µl of Taq polymerase completed with distilled water. The selective PCR amplification profile was the following:

13 cycles (94°C - 30 s, 65°C to 56°C - 30 s, 72°C - 60 s) of touch-down amplification with a 0.7°C decrease in the annealing temperature at each cycle followed by 23 identical cycles (94°C - 30 s, 56°C - 30 s, 72°C - 60 s). Amplifications were performed in a Bio-Rad thermal cycler (BioRad, Marne-la-Coquette, France).

2.3.4. Selective primer combinations

Sixteen combinations of "selective" primers, *Eco*RI-X and *Mse*I-X, containing one additional adjacent nucleotide (X = A, C, G or T) can potentially be used. According to the kit manufacturer's recommendation for bacteria, the following pairs of selective primers were first evaluated for optimization: EA-MA (*Eco*RI-A and *Mse*I-A), EC-MA, EC-MC, EC-MT, EC-MG and ET-MA. These preliminary tests were performed on isolates 1 to 33 and strains R1 to R10 (Table 1) for EC-MG, EC-MC, EC-MA and EC-MT. EA-MA and ET-MA were tested on reference strains R1 to R10 only. Finally, three selective primer pairs, EC-MC, EC-MA and EA-MA, were selected, based on the quality of the peaks produced (peak intensity, peak distribution and reproducibility of results) to study the genetic diversity of the isolates in relation to reference strains.

2.3.5. Fragment analysis

DNA amplified fragments were separated by capillary electrophoresis in the CEQ™ 8000 DNA analysis system (Beckmann Coulter, Villepinte, France): 0.5 µl volume of selective PCR product and 0.5 µl of DNA internal size standard (CEQ DNA size standard 600, Beckmann Coulter) were added to 30 µl of sample loading solution (Beckmann Coulter). Samples were loaded onto the CEQ™ 8000 system and run under the conditions specified by Beckmann Coulter. A DNA fingerprint was obtained for each primer pair tested and each sample. On this electropherogram, DNA fragments were identified according to their size (number of nucleotides). The scoring of the peaks generated was automated by analyzing the results with the 'fragment analysis module' of the CEQ 8000 software (CEQ™ 8000 Genetic Analysis System V8).

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A minimum fluorescence threshold value of 1500 RFU (Relative Fluorescence Units) was chosen on the basis of the preliminary experiments. However, each DNA fingerprint was thoroughly reviewed, especially for high molecular weight lower peaks. If not considered by the automatic data analysis, they were included in the data set of peaks if their resolution was comparable to those of ladder fragments of similar size.

DNA fragments from 60 to 640 nucleotides were included in the study in order to generate a peak table. Peak table files, containing fragments of 60-640 bp, were transferred to the BioNumerics 7.1 software (Applied Maths NV, Sint-Martens-Latem, Belgium). For each of the three selected combinations of primer pairs, a pairwise comparison of fragment patterns was carried out by calculating a similarity matrix using the Dice coefficient. The resulting similarity matrix was regrouped by unweighted pair group clustering using arithmetic averages (UPGMA). Finally, using the same software, results from the three combinations were combined to display genotype relationships between the isolates studied. This was done by averaging the three similarity matrices and generating a single UPGMA dendrogram. The cophenetic correlation was calculated between the original similarity matrix and the similarity matrix recomposed from the UPGMA tree. This enabled the reliability of the individual branching levels in the dendrogram to be estimated. The repeatability of the AFLP methodology was investigated on three different strains of *P. phosphoreum* and three different strains of *P. iliopiscarium*, including the type strains of both species. DNA was extracted from each strain in three independent assays. The 18 AFLP patterns generated were analyzed in a single dendrogram using the Dice similarity coefficient, followed by UPGMA clustering. For each isolate, repeatability was determined as the similarity value between the three replicates. Then, the six values were averaged to obtain the mean repeatability, used as a threshold to define identical genotypes.

2.4. *lux*A-gene PCR amplification

Chromosomal DNA of isolates of the *P. phosphoreum* strain group was analyzed by PCR to detect the *lux*A gene. The absence of this gene enabled *P. iliopiscarium* to be discriminated from *P. phosphoreum* and *P. kishitanii* (Ast and Dunlap, 2005). The *P. phosphoreum*-specific *lux*A primer pair

Af/Ar was used to amplify the *lux*A region (about 750 bp) simultaneously with the *gyr*B gene using the 22f/1240r primer pairs in a duplex PCR. Both primer pairs were described by Ast and Dunlap (2004). After optimization of the annealing temperature, the following amplification protocol was performed: initial denaturing at 95°C for 3 min; 35 cycles of denaturing at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1.5 min; one cycle at 72°C for 7 min; snap cooling to 12°C. The size of the PCR products was determined in a 1.8% (w/v) agarose gel (Invitrogen) using a 50-2000 bp low range DNA Ladder (exACTGene, Fisher BioReagents, Fisher Scientific International Inc.).

2.5. *gyr*B-gene amplification, sequencing and phylogenetic analysis

The *gyr*B sequences of isolates and reference strains belonging to the *Photobacterium* genus were determined in this study. Two extra *gyr*B sequences from GenBank were included in this data set, one sequence of *Photobacterium aquimaris* BS-1 (JQ229762.1) (Urbanczyk et al., 2012) for its genetic proximity to the three species *P. phosphoreum, P. kishitanii* and *P. iliopiscarium*, and one of *Photobacterium angustum* ATCC 33975 (AY900627.1) to create an out-group. Amplification of *gyr*B (approximately 1200 nucleotides) was performed according to the protocol described by Dunlap and Ast (2005). The *gyr*B product was sequenced with an automated sequencer (Beckman Coulter Genomics, Takeley, UK) using the amplification primers 22f/1240r and the internal primers ili609f (GAAGGTTATTCAAAGAAAGC) and ili602r (CTTTGAATAACCTTCTTTATCC) designed for this study. The four resulting sequences were assembled into a unique contig with the BioEdit sequence alignment software (Hall, 1999).

A multiple alignment of the 1211 bp sequences was performed using the MEGA software package (version 6) (Tamura et al., 2013). It was verified by comparison with an alignment of the corresponding amino acids. After visual inspection, the 5' region of 671 bp was kept for further analysis and the highly conserved, less informative region in 3' was discarded. Phylogenetic analyses were also carried out with the MEGA software: phylogenetic distances were calculated using the Kimura-2 correction. A neighbor-joining dendrogram (Saitou and Nei, 1987) was constructed and a bootstrapping analysis was performed using 1000 bootstrap replicates.

3. Results

After preliminary experiments using six primer combinations (EA-MA, EC-MA, EC-MC, EC-MT, EC-MG and ET-MA), based on the number of fragments and their size distribution, three combinations were shown to be successful with all isolates or strains tested: EC-MC, EC-MA and EA-MA. Experiments performed with EA-MA displayed the largest number of fragments with an average of 67 (35 to 140) compared to the other combinations with 39 (22 to 72) and 34 (20 to 71) for EC-MA and EC-MC, respectively. The majority of the fragments (>90%) ranged in size from 60 to 350 bp. Using the Dice coefficient (optimization: 0.1%, tolerance: 0.1%) for pairwise similarity calculation and UPGMA as a clustering method, the main clusters were roughly delineated in the same way for the three primer combinations with the exception of only a few isolates (data not shown). Congruence between the three combinations estimated by Pearson correlation of the homologous pairwise similarities was 70.5, 76.1 and 75.1 for the EC-MC/EC-MA, EC-MA/EA-MA, and EC-MC/EA-MA couples, respectively. The UPGMA clustering, calculated from the overall similarity matrix obtained from the comparison of the individual matrices from the three combinations, is represented in Fig. 1.

Isolates previously identified as belonging to the *P. phosphoreum* species group were delineated in two main clusters at a cut-off level of 40% similarity: cluster I (43 isolates) and cluster II (11 isolates), respectively. These clusters were strongly delineated, with cophenetic correlation coefficients of 92 and 95%, respectively. Each of these two clusters was divided into two subclusters grouping at a cut-off level of above 60% similarity: IA (15 isolates), IB (28 isolates), IIA (8 isolates), and IIB (3 isolates). The type strain *P. phosphoreum* CIP102511[T] was included in cluster I and *P. iliopiscarium* DSM9896[T] in cluster II. Reference strains R2 (CCUG16288), previously identified as *P. phosphoreum*, R5 (*P. kishitanii*, CIP104260) and R4 (type strain of *P. kishitanii* LMG23890[T]) grouped together at 59% similarity (cophenetic correlation of 91%). With the exception of reference strain R10 (*P. lipolyticum* LMG23071[T]), all other reference strains were clearly out-grouped (similarity below 30%, cophenetic coefficient of 94%). According to these results, it can be assumed that cluster I isolates all belong to *P. phosphoreum* and cluster II isolates to *P. iliopiscarium*.

Similarity values obtained between replicates ranged between 96.3% and 82.3% with an average value of 87.1%. According to this mean value, nineteen isolates might be clonally related. Most of them were isolated from the same batch of salmon (Fig. 1): J4C12, J8C5, J4C13 (genotype a) and J7C2, J7C4, J7C1 (genotype b) from batch G, as well as J4C1, J7C5, J4C2, J7C13, J7C14 (genotype f) and J3C17, J4C4 (genotype g) from the same batch G; MIP2566, MIP2567 (genotype c) and MIP2562, MIP2563 (genotype e) from batch D; only MIP2569 and MIP2583 (genotype d) were isolated from two different batches, D and E.

To support these findings, *lux*A-gene PCR detection and *gyr*B-gene sequencing were performed. *lux*A and *gyr*B genes were co-amplified in a single multiplex PCR in which the *gyr*B gene was the internal PCR control. The *lux*A gene was missing in all 11 isolates from cluster II of the AFLP analysis (Fig. 1), while the gene was confirmed in all other isolates of cluster I and reference strains R1, R2 and R4, R5, supposedly belonging to *P. phosphoreum* and *P. kishitanii*, respectively. The *lux*A-gene amplification results are summarized in Table 1.

The *gyr*B gene of isolates and reference strains R1 (*P. phosphoreum,* CIP102511[T]), R2 (*P. phosphoreum,* CCUG16288), R3 (*P. iliopiscarium,* DSM9896[T]), R4 and R5 (*P. kishitanii,* LMG23890[T] and CIP104260, respectively) was amplified and sequenced. Sequence analysis using the neighbor-joining method, with the Kimura-2 correction, resulted in the phylogenetic tree shown in Fig. 2. The tree displays three distinct groups with high bootstrap values around the three type strains of *P. phosphoreum, P. kishitanii* and *P. iliopiscarium.* Genetic distances between the *P. kishitanii* group and the groups of *P. phosphoreum* and *P. iliopiscarium* are 12% and 12.9%, respectively. The distance between *P. phosphoreum* and *P. iliopiscarium* is 6.2%. Within the groups, similarity is 99.5% (98 – 100) for the *P. phosphoreum* group, 99.5% (98.9 – 100) for the *P. iliopiscarium* group and 98% (96.9 – 100) for the *P. kishitanii* group. These three species show a genetic distance of at least 10.1% with *P. aquimaris* and a genetic distance of at least 19.8% with *P. angustum.* As anticipated, the *P. kishitanii* reference strain CIP104260 was found to be identical to the *P. kishitanii* type strain. The strain CCUG16288 clustered with this group at around 97% similarity.

4. Discussion

According to the AFLP study, 43 isolates were identified as *P. phosphoreum* and 11 isolates as *P. iliopiscarium*, which is completely supported with the findings obtained from the *lux*A-gene analysis and *gyr*B-gene analysis. Indeed, the *gyr*B-gene sequenced *P. phosphoreum* isolates fell into cluster I, together with the type strain *P. phosphoreum* CIP102511[T]. The *gyr*B-gene *P. iliopiscarium* isolates, with their type strain DSM9896[T], could be assigned to cluster II. Both clusters corresponded to the separation observed in the AFLP analysis (Fig. 1) and also corroborated the finding of the *lux*A gene in *P. phosphoreum* and *P. kishitanii* but not in *P. iliopiscarium*. We have thus confirmed the results of Ast et al. (2005), that the absence of *lux*A distinguishes *P. iliopiscarium* from *P. phosphoreum* and *P. kishitanii* and this absence is consistent with the inability of the *P. iliopiscarium* isolates to produce luminescence.

From the AFLP results, it can be concluded that strains from the same *Photobacterium* species share at least 45% similarity, while less than 45% similarity suggests that isolates belong to different species of the genus *Photobacterium*. In addition, the reproducibility of the AFLP technology could be used to estimate the similarity between closely related isolates or strains from *Photobacterium*. The average similarity value obtained between replicates can be used as a threshold to define identical or clonaly related isolates belonging to the same AFLP genotype. Isolates included in the same AFLP genotype were shown to be 100% similar in the *gyr*B-gene sequence analysis (Fig. 2).

Reference strain R2 (CCUG16288), previously identified as *P. phosphoreum*, was found according to the *gyr*B-gene sequencing and the AFLP analysis, to belong to *P. kishitanii*. Since this strain, also named strain 455 or ATCC35081, was isolated by Baumann (Reichelt and Baumann, 1973) before the description of *P. kishitanii*, it clearly needs to be reclassified as *P. kishitanii*.

Interestingly, in a previous study, MIP encoded isolates were analyzed by temporal temperature gradient gel electrophoresis (TTGE) and the pattern of MIP2472 and MIP2478, now identified as *P. iliopiscarium* isolates was clearly different from those of the other *P. phosphoreum* isolates (data not shown). The hypothesis of their species difference was confirmed here.

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AFLP technology is generally considered a powerful technique for the identification of strains related at the species level. For *Vibrionaceae,* this method has been applied extensively to the genus *Vibrio* (Arias et al., 1997; Beaz-Hidalgo et al., 2008; Tao et al., 2012; Thompson et al., 2001) but only to two species of the genus *Photobacterium*: *P. damselae* (Botella et al., 2002; Kvitt et al., 2002; Takahashi et al., 2008; Thyssen et al., 2000) and *P. kishitanii* (Ast et al., 2007). In most of these studies, the restriction enzyme pair *Taq*I/*Hind*III was used, with only one primer set for the selective amplification. The enzymes *Eco*RI/*Mse*I, used in the present work, have previously been applied with several primer sets for selective amplification in the studies of Ast et al. (2007) and Takahashi et al. (2008).

As stated by Ast and Dunlap (2005), until recent years, due to the previous inability to distinguish the three species within the *P. phosphoreum* species group, the ecological distribution of *P. phosphoreum* and *P. iliopiscarium* was poorly known. *P. phosphoreum* is commonly isolated from the intestine and skin of marine fish, incubated at low temperatures. *P. iliopiscarium* has been recovered from the intestines of species of cold-water fish and from samples of spoiled packaged fish stored under modified atmosphere at low temperature (Urbanczyk et al., 2011). In this study, all the isolates were picked from different salmon products during studies monitoring the microbial quality of raw salmon (Macé et al., 2012, 2013b) and cold-smoked salmon (Leroi et al., 1998). *P. kishitanii* did not occur among this set of isolates studied. This species, known to be a specific and exclusive bioluminescent symbiont of many different deep-sea fish, has also been encountered on warm-water fish skin (Ast and Dunlap, 2005).

To our knowledge, *P. kishitanii* has never been listed in the literature as occurring on cold or temperate seawater fish, except in a recent work on food-specific bacterial communities associated with meat and seafood spoilage (Chaillou et al., 2014). In that study, a metagenomic approach was

used in which *P. kishitanii* was demonstrated to be part of the core seafood community i.e. occurring in cod fillet, salmon fillet and cold-smoked salmon.

The *P. phosphoreum* species group has been identified as the main SSO responsible for sensory spoilage of several chilled marine seafoods (Dagaard et al., 1993; Dalgaard, 1995; Emborg et al., 2002; Macé et al., 2013a). However, due to the inability until recent years to distinguish *P. phosphoreum* and *P. iliopiscarium*, their respective spoilage potential has never been studied. Some strains of the *P. phosphoreum* species group used in the present study have been assessed in our laboratory for their spoilage potential. Five strains previously isolated from cold-smoked salmon, identified as *P. phosphoreum* in the present study, have been shown to be spoiling bacteria when inoculated on sterile cold-smoked salmon (Joffraud et al., 2001, 2006; Stohr et al., 2001). In a recent work, Macé et al. (2013a) studied the spoilage potential of a mixture of four strains of *Photobacterium*, two *P. phosphoreum* and two *P. iliopiscarium* strains according to the present study. The mixture was spoiling but, as the strains were tested together, we cannot conclude about the respective spoilage behavior of the two species. Further studies are necessary to resolve this point.

5. Conclusion

In this work, an AFLP analysis was implemented on a collection of isolates recovered from various salmon products. In order to evaluate its robustness, three primer sets were used for the selective amplification step. As for other bacterial genera reported in the literature, AFLP was confirmed as a powerful technique for the delineation of isolates, related beyond species level, of the genus *Photobacterium*. The method also detected clonally related isolates belonging to the same AFLP genotype. The AFLP method was shown to distinguish clearly *P. phosphoreum* and *P. iliopiscarium*, two species that were previously difficult to discriminate with other than highly specialized phylogenetic studies. This discrimination was supported and validated by *gyr*B-gene sequence analysis and by the presence or absence of the *lux*A gene. Only a few isolates have previously been studied for their ability to spoil salmon products and the respective spoilage behavior of these two species was not clarified. Thus, the AFLP method presented here could be an excellent tool for genotyping isolates

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recovered from bacterial communities associated with seafood spoilage and shedding light on the role

of the different members of the *Photobacterium* species group in seafood deterioration.

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Table 1

Table 1. List of isolates and reference strains used in this study with their identification, origin and code. Results of AFLP identification including species and subcluster as well as *lux*A gene amplification are given in the right columns.

Strain/isolate ID	Previous identification	Origin	Batch	Code	AFLP identification**	*lux*A
01	*P. phosphoreum**	Salmon steak	A[1]	MIP2423	*P. phosphoreum*, IB	+
02	*P. phosphoreum**	Salmon steak	C[2]	MIP2472	*P. iliopiscarium*, IIB	-
03	*P. phosphoreum**	Salmon steak	C	MIP2473	*P. phosphoreum*, IB	+
04	*P. phosphoreum**	Salmon steak	C	MIP2478	*P. iliopiscarium*, IIB	-
05	*P. phosphoreum**	Salmon steak	D[3]	MIP2560	*P. phosphoreum*, IB	+
06	*P. phosphoreum**	Salmon steak	D	MIP2562	*P. phosphoreum*, IB	+
07	*P. phosphoreum**	Salmon steak	D	MIP2563	*P. phosphoreum*, IB	+
08	*P. phosphoreum**	Salmon steak	D	MIP2565	*P. phosphoreum*, IB	+
09	*P. phosphoreum**	Salmon steak	D	MIP2566	*P. phosphoreum*, IB	+
10	*P. phosphoreum**	Salmon steak	D	MIP2567	*P. phosphoreum*, IB	+
11	*P. phosphoreum**	Salmon steak	D	MIP2569	*P. phosphoreum*, IB	+
12	*P. phosphoreum**	Salmon steak	E[4]	MIP2583	*P. phosphoreum*, IB	+
13	*P. phosphoreum**	Salmon steak	E	MIP2585	*P. phosphoreum*, IB	+
14	*P. phosphoreum**	Salmon steak	E	MIP2586	*P. phosphoreum*, IB	+
15	*P. phosphoreum**	Salmon steak	E	MIP2588	*P. phosphoreum*, IB	+
16	*P. phosphoreum**	Salmon steak	E	MIP2591	*P. phosphoreum*, IB	+
17	*P. phosphoreum**	Salmon steak	F[5]	MIP2605	*P. phosphoreum*, IB	+
18	*P. phosphoreum**	Salmon steak	F	MIP2607	*P. phosphoreum*, IB	+
19	*P. phosphoreum**	Salmon steak	F	MIP2609	*P. phosphoreum*, IB	+
20	*P. phosphoreum**	Salmon steak	F	MIP2611	*P. phosphoreum*, IB	+
21	*P. phosphoreum**	Salmon steak	F	MIP2612	*P. phosphoreum*, IB	+
22	*P. phosphoreum**	Salmon steak	F	MIP2613	*P. phosphoreum*, IB	+
23	*P. phosphoreum**	Smoked salmon	[6]	SF955	*P. phosphoreum*, IB	+
24	*P. phosphoreum**	Smoked salmon	[6]	SF1224	*P. phosphoreum*, IB	+
25	*P. phosphoreum**	Spoiled haddock fillet	[7]	EU2181	*P. iliopiscarium*, IIB	-
26	*P. phosphoreum**	Smoked salmon	[8]	SF686	*P. phosphoreum*, IB	+
27	*P. phosphoreum**	Smoked salmon	[8]	SF684	*P. phosphoreum*, IB	+
28	*P. phosphoreum**	Lyngby, Denmark	[9]	RF44	*P. phosphoreum*, IB	+
29	*P. phosphoreum**	Smoked salmon	[8]	SF680	*P. phosphoreum*, IB	+
30	*P. phosphoreum**	Smoked salmon	[8]	SF714	*P. phosphoreum*, IB	+
31	*P. phosphoreum**	Smoked salmon	[8]	SF723	*P. phosphoreum*, IB	+
32	*P. phosphoreum**	Salmon steak	G[10]	J3C17	*P. iliopiscarium*, IIA	-
33	*P. phosphoreum**	Salmon steak	G	J4C1	*P. iliopiscarium*, IIA	-
34	*P. phosphoreum**	Salmon steak	G	J4C2	*P. iliopiscarium*, IIA	-
35	*P. phosphoreum**	Salmon steak	G	J4C3	*P. phosphoreum*, IA	+
36	*P. phosphoreum**	Salmon steak	G	J4C4	*P. iliopiscarium*, IIA	-
37	*P. phosphoreum**	Salmon steak	G	J4C5	*P. phosphoreum*, IA	+
38	*P. phosphoreum**	Salmon steak	G	J4C11	*P. phosphoreum*, IA	+
39	*P. phosphoreum**	Salmon steak	G	J4C12	*P. phosphoreum*, IA	+
40	*P. phosphoreum**	Salmon steak	G	J4C13	*P. phosphoreum*, IA	+
41	*P. phosphoreum**	Salmon steak	G	J4C14	*P. phosphoreum*, IA	+
42	*P. phosphoreum**	Salmon steak	G	J7C1	*P. phosphoreum*, IA	+
43	*P. phosphoreum**	Salmon steak	G	J7C2	*P. phosphoreum*, IA	+
44	*P. phosphoreum**	Salmon steak	G	J7C4	*P. phosphoreum*, IA	+
45	*P. phosphoreum**	Salmon steak	G	J7C5	*P. iliopiscarium*, IIA	-
46	*P. phosphoreum**	Salmon steak	G	J7C11	*P. iliopiscarium*, IIA	-

47	*P. phosphoreum**	Salmon steak	G	J7C13	*P. iliopiscarium*, II$_A$	-
48	*P. phosphoreum**	Salmon steak	G	J7C14	*P. iliopiscarium*, II$_A$	-
49	*P. phosphoreum**	Salmon steak	G	J8C4	*P. phosphoreum*, I$_A$	+
50	*P. phosphoreum**	Salmon steak	G	J8C5	*P. phosphoreum*, I$_A$	+
51	*P. phosphoreum**	Salmon steak	G	J8C11	*P. phosphoreum*, I$_A$	+
52	*P. phosphoreum**	Salmon steak	G	J8C13	*P. phosphoreum*, I$_A$	+
53	*P. phosphoreum**	Salmon steak	G	J8C14	*P. phosphoreum*, I$_A$	+
54	*P. phosphoreum**	Salmon steak	G	J8C15	*P. phosphoreum*, I$_A$	+
R1	*P. phosphoreum*	(Cohn 1878) Beijerinck 1889		CIP102511^T	*P. phosphoreum*, I$_B$	+
R2	*P. phosphoreum*	*Etelis marshi*, Hawaii		CCUG16288	*P. kishitanii*	+
R3	*P. iliopiscarium*	Pyloric ceca of herring		DSM9896^T	*P. iliopiscarium*, II$_B$	-
R4	*P. kishitanii*	Marine fish, light organ		LMG23890^T	*P. kishitanii*	+
R5	*P. kishitanii*	Cape Town, South Africa		CIP104260	*P. kishitanii*	+
R6	*P. angustum*	Seawater		CIP75.10^T		
R7	*P. damselae* ssp. *damselae*	Damsel fish, skin ulcer		CIP102761^T		
R8	*P. damselae* ssp. *piscicida*	Epizootic of white perch		CIP103910		
R9	*P. indicum*	Marine mud		LMG22857^T		
R10	*P. lipolyticum*	Intertidal sediment		LMG23071^T		

*tentatively identified *P. phosphoreum* species group as described by Macé et al., 2012, 2013b.

** species, subcluster according to AFLP analysis.

+ presence of *lux*A gene.

- absence of *lux*A gene

[1] Batch A: salmon steak packaged under modified atmosphere, processed in plant I (France) on October 2009 from atlantic salmon (*Salmo salar*) farmed in Norway.

[2] Batch C: salmon steak packaged under modified atmosphere, processed in plant II (France) on October 2009 from atlantic salmon (*Salmo salar*) farmed in Norway.

[3] Batch D: salmon steak packaged under modified atmosphere, processed in plant I (France) on Marsh 2010 from atlantic salmon (*Salmo salar*) farmed in Ireland.

[4] Batch E: salmon steak packaged under vacuum, processed in plant I (France) on Marsh 2010 from atlantic salmon (*Salmo salar*) farmed in Norway.

[5] Batch F: salmon steak packaged under modified atmosphere in plant II (France) on Marsh 2010 from atlantic salmon (*Salmo salar*) farmed in Norway.

[6] strains isolated from cold-smoked salmon processed at IFREMER (1996) with different salting and smoking conditions (Leroi et al., 1998).

[7] EU2181: strain isolated from spoiled haddock fillet in Iceland (2003).

[8] strains isolated from cold smoked salmon and identified as described by Leroi et al. (1998) and studied for their spoilage potential by Joffraud et al. (2001) and Stohr et al. (2001).

[9]: strain provided by DTU Aqua, Technical University of Denmark.

[10] Batch G: salmon steak packaged under modified atmosphere purchased from a local supermarket (Macé et al., 2013b).

Figure captions:

Fig. 1. Cluster analysis of the AFLP patterns of isolates and strains using the UPGMA dendrogram generated by the averaged similarity matrix inferred from the three matrices, obtained with the Dice coefficient, corresponding to the three combinations. Cophenetic correlation coefficients and error flags figure at each branch. Main clusters defined at different similarity levels are indicated by brackets. Vertical line corresponding to cut off at 87.1% similarity delineates clonally distinct isolates.

Genotypes: (isolate code) a= J4C12; J8C5; J4C13. b= J7C2; J7C4; J7C1. c=MIP2566; MIP2567.

d=MIP2569; MIP2583. e=MIP2562; MIP2563. f= J4C1; J7C5; J4C2; J7C13; J7C14. g=J3C17;

J4C4.

* species identification according to *gyr*B gene sequencing.

Fig. 2. Unrooted phylogenetic tree based on neighbor-joining analysis of the *gyr*B gene sequence similarities of the *Photobacterium* strains and *Photobacterium* isolates. Strain type sequences of *Photobacteriumphosphoreum, Photobacterium kishitanii* and *Photobacterium iliopiscarium* are indicated by a small black triangle. The numbers at branch nodes are bootstrap values shown as percentages of 1000 bootstrap replicates. Only values > 80% are shown. Scale bar represents 2% estimated sequence divergence.

Species identification according to AFLP analysis and presence (+) or absence (-) of the *lux*A gene are indicated on the right-hand side of the brackets.
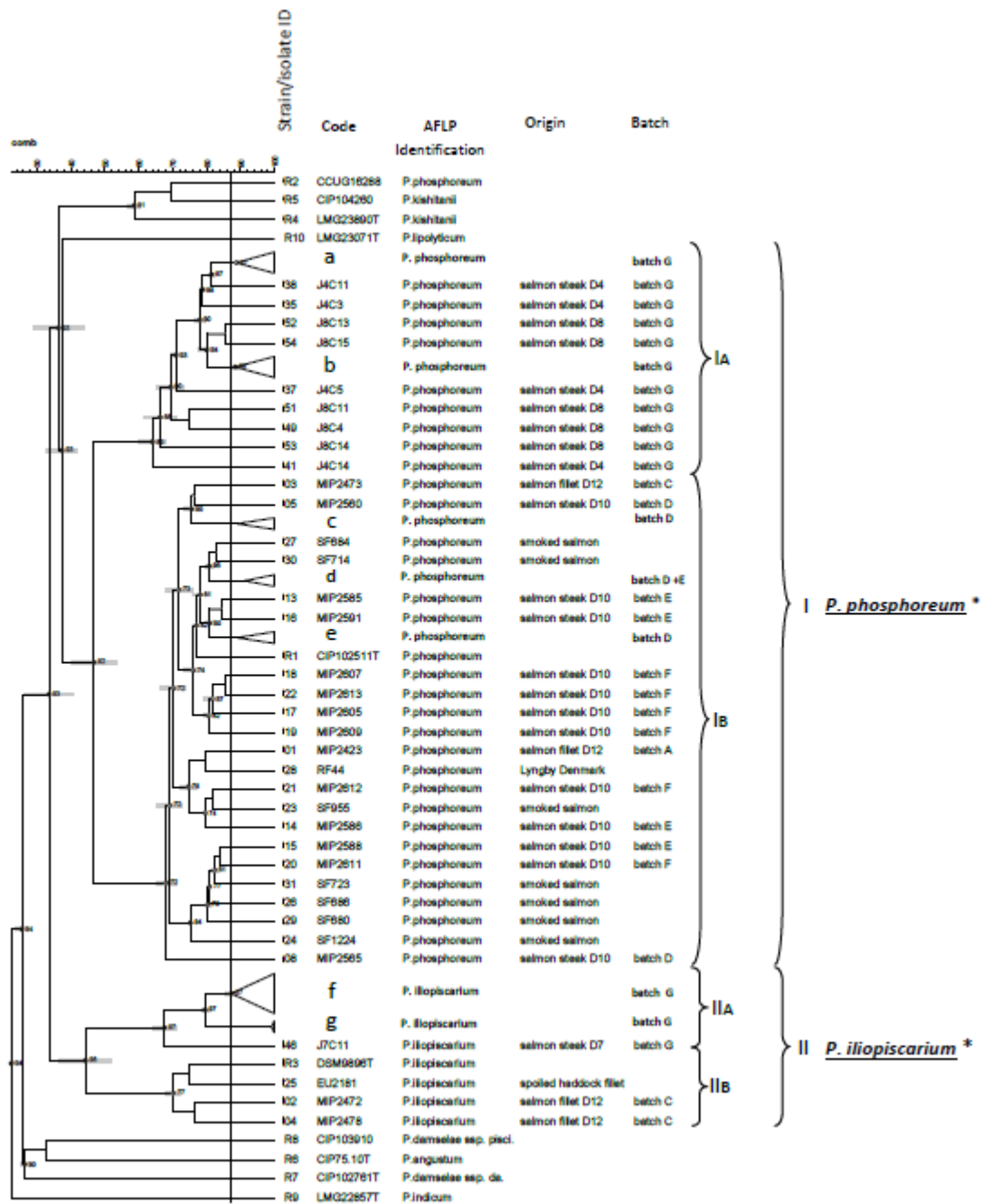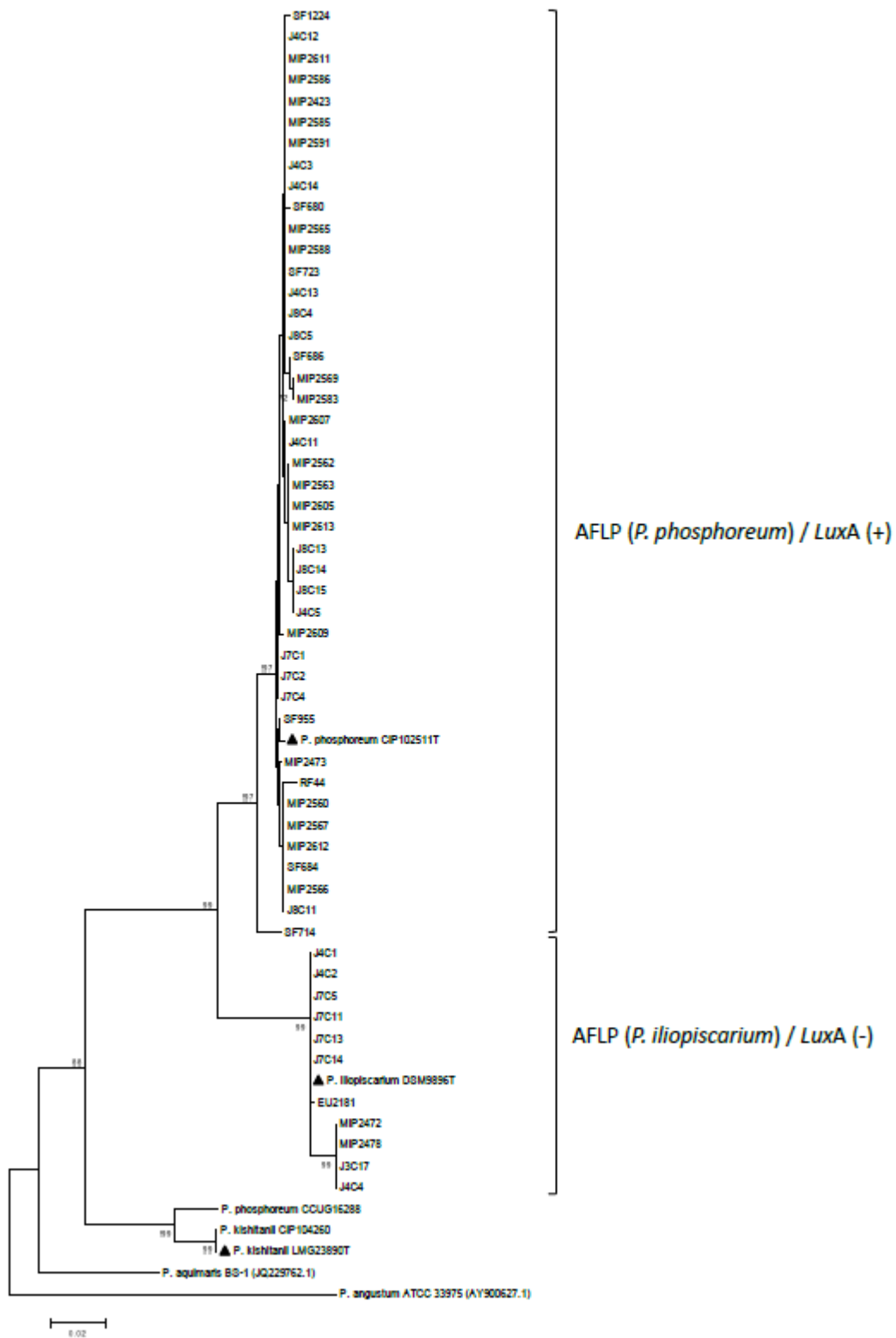
Figure 1.

Figure 2.