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Molecular characterization and phylogenetic analysis of Pseudomonas aeruginosa isolates recovered from Greek aquatic habitats implementing the Double Locus Sequence Typing scheme

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Corresponding Author:	Alex Galanis Democritus University of Thrace School of Health Sciences GREECE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Democritus University of Thrace School of Health Sciences
Corresponding Author's Secondary Institution:	
First Author:	Olga Pappa
First Author Secondary Information:	
Order of Authors:	Olga Pappa
	Apostolos Beloukas
	Apostolos Vantarakis
	Athina Mavridou
	Anastasia Kefala
	Alex Galanis
Order of Authors Secondary Information:	
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Molecular characterization and phylogenetic analysis of *Pseudomonas aeruginosa* isolates recovered from Greek aquatic habitats implementing the Double Locus Sequence Typing scheme

Olga Pappa[1,4,5], Apostolos Beloukas[2,4], Apostolos Vantarakis[3], Athena Mavridou[4], Anastasia-Maria Kefala[5], Alex Galanis[5*]

[1]*Central Public Health Laboratory, Hellenic Centre for Disease Control and Prevention, Athens, Greece*

[2]*Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom*

[3]*Environmental Microbiology Unit, Department of Public Health, School of Medicine, University of Patras, Greece*

[4]*Department of Medical Laboratories Technological Educational Institute of Athens, Athens, Greece*

[5]*Department of Molecular Biology and Genetics, Democritus University of Thrace, Alexandroupolis, Greece*

*For correspondence:

Dr Alex Galanis, Department of Molecular Biology and Genetics, Democritus University of Thrace, Alexandroupolis, Greece, e-mail: agalanis@mbg.duth.gr; Tel.: +30 2551030634; Fax: +30 2551030624

Abstract

The recently described Double Locus Sequence Typing (DLST) typing scheme implemented to deeply characterize the genetic profiles of 52 resistant environmental *Pseudomonas aeruginosa* isolates deriving from aquatic habitats of Greece. DLST scheme was able not only to assign an already known allelic profile to the majority of the isolates but also to recognize two new ones (*ms217*-190, *ms217*-191) with high discriminatory power. A third locus (*oprD)* was also used for the molecular typing, which has been found to be fundamental for the phylogenetic analysis of environmental isolates given the resulted increased discrimination between the isolates. Additionally, the circulation of acquired resistant mechanisms in the aquatic habitats according to their genetic profiles was proved to be more extent. Hereby, we suggest that the combination of the DLST to *oprD*-typing can discriminate phenotypically and genetically related environmental *P. aeruginosa* isolates providing reliable phylogenetic analysis at a local level.

Keywords: DLST, *P.aeruginosa*, oprD, aquatic habitats, phylogenesis

Introduction

In recently published studies *Pseudomonas aeruginosa* has been introduced as a potential reservoir of resistance genes in a variety of aquatic habitats such as swimming pools, water-tanks, mains waters [1], freshwaters and waste-waters [2, 3]. The bacterium besides its intrinsic antimicrobial resistance due to low outer membrane permeability (*oprD* Loss), chromosomally encoded *AmpC*, as well as an extensive efflux pump system, holds a prominent place in the development of acquired resistance mechanisms [4]. Understanding the genetic structure of resistant environmental *P. aeruginosa* isolates is of paramount importance in order to get insight into the genetic complexity and ecological versatility of this opportunistic pathogen [5, 6]. The extensive diversity of *P. aeruginosa* has given rise to the evolutionary study of the bacterium using various typing methods such as Multi Locus Variable number of tandem repeats Analysis (MLVA) [7], Pulsed Field Gel Electrophoresis (PFGE) [8, 9], Multi Locus Sequence Typing (MLST) [10, 11] and recently, Double Locus Sequence Typing (DLST) [12-15].

MLST is one of the major 'typing successes' of the past decade. It has been widely used in studies focusing on microbial population structure and molecular typing of clinical isolates, representing specific phenotypic and genotypic characteristics of the bacterium [16-18; http://pubmlst.org/paeruginosa]. However, the application of MLST in environmental isolates is still quite limited and it is questionable whether this method is only suitable for occasional isolates or for the entire spectrum [10, 19]. Novel sequences have been identified for the seven housekeeping genes of environmental isolates and were submitted to the MLST database, but the new ST-types could not be identified by the initial protocol [3, 16, 20]. This led to modification of the protocol, which added extra time and cost to an already expensive and time-consuming method, while at the same time the sensitivity and the reproducibility were reduced [11, 21, 22].

Therefore, the development of alternative methods was required in order to facilitate epidemiological and phylogenetic studies and to enable faster and cost effective, large-scale bacterial genotypic analysis.

DLST is a recently developed typing scheme based on the partial sequencing of three highly variable loci, *ms172*, *ms217* and *oprD* [12]. As the combination of two loci gave resolution results only slightly lower than the combination of the three loci, the authors proposed the use of only two loci in the DLST scheme for *P. aeruginosa* instead of three [12]. The new sequence-based scheme was compared to MLST in a large number of clinical and environmental *P. aeruginosa* isolates, proving that when epidemiological and phylogenetic analyses are conducted at a local level MLST can be replaced by DLST [13]. The online publicly available DLST database (http://www.dlst.org/Paeruginosa/) uses nucleotide sequences of the two loci (*ms172* and *ms217)* to define the DLST type [12]. The method is new and thus there is not much published information regarding both clinical and environmental isolates of *P. aeruginosa* [12-15]. Although in Basset's et al work the *oprD* locus was not selected for the final typing scheme, it has been reported as one of the important genetic markers that can be used in population studies, not only due to its contribution to carbapenem resistance but also due to its high genetic diversity [23]. It has been used for typing and for phylogenetic purposes both in clinical and environmental strains in order to reveal additional evolutionary forces that contribute to the high clonality of *P. aeruginosa* population [24].

At the present study all three typing schemes: the DLST scheme as it has been proposed [12], the oprD-typing scheme, and the combination of the three loci (*ms172, ms217* and *oprD)* were applied to environmental *P. aeruginosa* isolates collected from various water ecosystems in Greece. Using a bacterial population chosen as to represent various resistant profiles, different

sampling sites and many water types, the aims of the study were a) to evaluate the application of the DLST method in the selected environmental *P. aeruginosa* isolates and to elucidate the predominant clone in these habitats, b) to study the distribution of the resistant phenotypes among the DLST-types and c) to estimate the discriminatory power of the novel DLST method when a third locus was added to the initial proposed scheme.

Material and Methods

Bacterial isolates

A well-characterized repository of 245 confirmed *P. aeruginosa* strains isolated during the period 2011-2014 [official monitoring sampling schedule of the "Water Analysis Department, Central Public Health Laboratory (CPHL), Hellenic Center for Disease Control and Prevention (HCDCP)] [1] was used as the pool for the tested strains. A subset of fifty-eight (58/245; 23.7%) isolates was chosen by Simple Random Sampling method (SAS 9.3) so that the final number of the isolates was representative of the total population. Criteria for the collection of isolates were a) the type of water sample, b) the isolates' geographical distribution, c) the isolates' resistant phenotype and d) the year of the isolation. The characteristics of the 58 isolates are presented in detail in Table S1. Two reference strains were used as control strains: a) a clinical control provided by HPA/NEQAS (the HPA External Quality Control Scheme) and b) *P. aeruginosa* PAO1 (Collection of Institute Pasteur CIP104116, www.crbip.pasteur.fr).

Isolation of genomic DNA

P. aeruginosa genomic DNA was extracted using the Purelink Genomic DNA mini kit (Invitrogen, UK) following the manufacturer's instructions after 48 hours growth in Nutrient broth and Nutrient agar.

Antibiotic Susceptibility testing

All isolates were tested for susceptibility to 14 commonly used antibiotics belonging to four different classes: non-carbapenem b-lactams: ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), cefepime (FEP; 30 µg), piperacillin (PIP; 75 µg), ticarcillin (TIC; 75 µg), piperacillin/tazobactam (TZP; 100 µg/10 µg), ticarcllin/clavulanate (TCC; 75 µg/10 µg), aztreonam (ATM; 30 µg), carbapenems: imipenem (IPM; 10 µg) and meropenem (MEM; 10 µg), aminoglycosides: amikacin (AN; 30 µg), tobramycin (TOB; 30 µg) gentamicin (GM 30 µg), fluoroquinolones: ciprofloxacin (CIP; 5 µg) according to guidelines of the Clinical and Laboratory Standards Institute Guidelines 2011/M100S21 (http://clsi.org). The interpretation of the resistant phenotypes was performed according to published literature [25].

Detection of Extended Spectrum Beta-Lactamases (ESBLs) and Metallo Beta-Lactamases (MBLs)

ESBL isolates were phenotypically detected by a modified Double Disk Synergy Test (DDST) with the addition of boronic acid to the antibiotic disks, as previously described [26]; MBL detection was performed according to Giakkoupi and et al [27]. Consequently, isolates phenotypically positive for ESBL and MBL production were subjected to PCR for the detection of 10 different ESBL and 6 MBL genes (PER-1, OXA-2, VEB-1A, GES-1A, TEM-A, SHV-A, CTX-M-groups 1,2, 8/25 and 9; VIM-2, IMP, SIM-1, GIM-1, SPM-1 and NDM). PCR

conditions and the specific primers used for the above genes were selected from published literature [28-33] (Table S2).

Double Locus Sequence Typing (DLST) and oprD-typing

DLST and oprD-typing were implemented in 52 isolates of *P. aeruginosa* and in the selected reference strains [12]. Six isolates with resistant phenotypes R3 {Loss of oprD} and MBL {Metallo b-lactamase} were excluded from the typing procedure, as these isolates did not express the *oprD*-gene. However, they were included in Table S1 in order to present their significant antibiotic profile. Briefly, DNA extracts were used for PCR amplification of the three loci, *ms172*, *ms217* and *oprD* using specific primers (Table S2). Standard gel electrophoresis was applied and gels stained with Gel Red (Gel Red nucleic acid gel stain 10,000x in water; BIOTIUM) were examined under UV light for the presence of one visible clear band per PCR; as it was expected, the length of DNA sequences was variable among isolates. PCR products were purified (NucleoSpin, Gel and PCR clean-up, MACHEREY-NAGEL) and were sequenced by CeMIA SA (http://cemia.eu/sangersequencing.html) using the amplification primers for the three loci (Table S2). The procedure was repeated a second time when the sequence quality was too low or no sequence was obtained. If no sequence of good quality was obtained after the second step, the result for the isolate was considered a null allele [12].

Analysis of the sequenced data

All chromatograms were imported, edited and trimmed in Sequencer 5.3 (https://www.genecodes.com) using the start signatures of the trimmed pattern for the three loci, *ms172*, *ms217* and *oprD*, according to the protocol [12]. Trimmed sequences were subjected to BLAST for the identification of the appropriate product and then to the DLST database

(http://www.dlst.org/Paeruginosa/) for allele assignment of the genetic markers *ms172* and *ms217*; if there was no identification for the submitted locus, the procedure for submission new alleles in the DLST data base was followed and a new locus number was assigned; the *oprD* sequences were searched against the NCBI data base and compared to the *oprD* sequence of the reference strain PAO1.

Molecular epidemiological analysis

• *eBURST analysis and minimum spanning trees construction*

DLST markers are considered highly stable in the case of local phylogenetic studies [12, 34]; however, during a long-term investigation they probably undergo genetic changes [34]. In studies, as the present one, it is important to use the suitable model for analyzing sequences obtained from environmental *P. aeruginosa* isolates, deriving from a specific region in a three-year period [34, 35]. The Global optimal eBURST analysis [35; http://www.phyloviz.net/goeburst/ accessed on 01/08/2016], proposed in the literature for analysis of DLST data of *S. aureus* isolates [34, 36], was chosen and the same rules and definitions in analysis were implemented.

• *Maximum likelihood phylogenetic analysis of the oprD*

Maximum likelihood (ML) phylogeny was obtained with RaxML-HCP2 v8 [37] using GTR+I+G that was identified as the best fitted model using jModelTest2 [38].

Index of diversity and concordance of the typing methods

The index of diversity and the degree of congruence of the three typing schemes used were calculated using an online tool (http://www.comparingpartitions.info/ accessed on 01/09/2016). The discriminatory power of the typing methods described in the current work was evaluated using the Simpson's index of diversity, where an index >0.90 is considered ideal indicating that the typing method is able to distinguish each isolate from all others. The concordance between the methods was estimated using the Wallace and Rand coefficients; the Rand index (R) estimates the proportion of agreement taking into account that the agreement between the partitions could arise by chance; the Wallace coefficient (W) estimates the probability that two isolates grouped in the same type by one method will be grouped in the same type using another typing technique [39].

Results

Antimicrobial susceptibility profiles and detection of beta-lactamase-producers

The fifty-eight (58) isolates presented various resistant phenotypes (Fig. 1a). A substantial portion of the resistant isolates (9/20; 45%) was characterized as Extended Spectrum Beta Lactamases (ESBL) producers according to DDS-test (synergy between amoxicillin+clavunalic acid (AMC) and ceftazidime (CAZ) or cefotaxime (CTX)), presenting multi-drug resistant patterns (e.g. isolates 121, 174, 299, Table S1). Two isolates (266, 267, Table S1) presented the characteristic synergy between meropenem (MEM)/imipenem (IPM) and the disk with EDTA, and were characterized as Metallo Beta Lactamases (MBL) producers (2/20; 10%). The 9 ESBL and the 2 MBL producers were screened for the presence of b- lactamase genes. Out of the ten ESBL genes tested, the CTX-M-group 9 was detected in only one isolate (Table S1). None of the remaining ESBL genes was detected in any of the 9 isolates tested with the primer sets used in this study. The 2 phenotypically MBL positive isolates did not produce positive results for the 6

MBL genes tested. The resistant phenotypes were distributed across all geographical areas (Fig. 1b), while the Peloponnese presented the highest percentage of all the resistant profiles. ESBL isolates appeared in three geographically unrelated areas of Greece together with other resistant mechanisms (Fig. 1b).

DLST analysis

Fifty-four isolates (including the reference strains) were successfully typed implementing the DLST scheme. DLST was able to assign an already known allele number for 40 isolates while for 12 isolates two new loci were recognized for the *ms217* marker (allele 190 and 191, http://www.dlst.org/Paeruginosa/ms217.txt). The phylogenetic analysis revealed 27 types with DLST-type 90-190 (6/54; 11.1%) being the predominant one; the second new allele 191 (3/54; 5.5%) was combined with three different *ms172* loci (1-191, 83-191, 10-191). Five out of the 54 isolates (9.3%) presented the DLST-type 90-139; 3/54 isolates had the DLST-types 18-54, 19-33, 20-105 and 55-58 respectively (5.6% each), while the rest 28 isolates were distributed among 8 different DLST-types including 2 isolates each (16/54; 29.6% in total) and 12 isolates (12/54; 22.22%) presenting unique DLST-types, including the reference strains: NEQAS: 32-39 and PAO1: 16-4 (Fig. 2a; Table S1). The predominant DLST-type (90-190) was present in wild-type isolates as well as in isolates with resistant phenotypes R1 deriving from Northern and Central Greece and the Ionian islands present in a variety of water samples. ESBL isolates were distributed among 7 DLST-types; 5 of them (20-105, 90-139, 55-58 and 19-162) co-existed with wild-type, non-wild-type and R1 isolates recovered mainly from the Peloponnese with no significant correlation to the types of water samples (Fig. 2a; Table S1). The allele 190 combined with other *ms172* loci was also present in Attica and the Peloponnese in ESBL producers. The new allele 191 for the *ms217* gene was detected exclusively in isolates deriving from thermal

water samples from Central Greece presenting wild-type and ESBL resistant phenotypes including the CTX-M-group 9 isolate (Fig. 2b). Finally, the R3 resistant phenotype, which was present exclusively in mains water samples mainly from the Peloponnese, presented unique DLST-types (21-96, 19-91, 59-21) (Table S1).

oprD-typing

oprD locus was detected in 54 isolates (including the reference strains). BLAST analysis of the 54 *oprD* genes distributed the isolates in 9 groups (G1-G9), with two groups, 1 and 4, including the majority of the isolates, with 21 and 8 isolates, respectively. BLAST search against the NCBI data base showed that the coding sequence of the group 1-oprD gene was identical to *P. aeruginosa* strain PA121617 (GenBank accession no. CP016214), while the coding sequence of the group 4-oprD gene was identical to *P. aeruginosa* strain MTB-1 (GenBank accession no. CP006853). The reference strain NEQAS was identical to *P. aeruginosa* strain ATCC 27853 (GenBank accession no. CP015117), while the coding sequence of PAO1's oprD gene was identical to *P. aeruginosa* genome assembly PAO1OR, chromosome:I (GenBank accession no. LN871187), as expected. The above results and the coding sequences of the rest oprD-groups are shown in Table S1.The ML phylogeny revealed five major clusters -A,B,C, D and H- and cluster E with the reference strain PAO1 as outgroup; only the cluster A was consistent with the initial results containing all the group 1-oprD isolates, except one (167), which presented various Single Nucleotide Polymorphisms (SNPs) comparing to the major group; it was located at a distance from the major group and it was consequently considered as a singleton (C2); the reference strain NEQAS was located in Cluster A. Cluster B was divided into five subgroups where the B3 subgroup was separated from B2 and B4 with three and two SNPs respectively (isolate 137). Cluster C was separated into four sub-clusters consisted of three different oprD-

groups; finally, Cluster D was divided into four sub-clusters where the D2 subgroup was separated from subgroup D3 with two SNPs (isolate 225). Interestingly, group 3-oprD, as defined by ML analysis, was located in two different clusters (Clusters B1 and H) very distant from each other, while the rest oprD-groups tended to cluster together into small subgroups. The reference strain PAO1 was located separately from all other clusters as expected (Fig. 3a). The major oprD group-1 (cluster A) was present in all geographical sampling sites, water sample types and resistant phenotypes. Isolates in G4 (clusters B2, B3, B4) derived from four different water sample types mainly from sampling sites of the Peloponnese presenting wild-type and ESBL resistant isolates; group-3 oprD (clusters B1 and H) was present mainly in resistant and wild-type isolates deriving from mains water samples in the Attica region. For the remaining groups there was no significant correlation to the three parameters considered (geographical areas, water sample types and resistant phenotypes) (Fig. 3).

DLST_n_oprD

The three loci (*ms172, ms217, oprD*) were combined in order to examine the impact of the third loci on the discriminatory power. The DLST_n_oprD analysis revealed 43 types with a) the combination 90-190-A being the predominant one with 4 isolates, b) followed by the combination 19-33-B2 with three isolates. The e-burst analysis for the DLST and DLST_n_oprD types showed that the use of the *oprD* loci increased the discrimination between genetically related isolates and their phylogenetic distance (Fig. 4). DLST-types 90-139 and 90-190 were divided into three smaller clusters representing three different oprD groups. Isolates with the new allele 191 were clustered phylogenetically distant as they presented various *ms172* and *oprD* alleles. Three isolates (174, 225, 314), which belonged to 20-105 DLST-type, now constitute three different combinations, 20-105-A, 20-105-D2 and 20-105-B4, according to their *oprD*

sequence (Fig. 4). Wild-type and Non-wild-type isolates tended to appear together as it was expected, while R1 isolates and ESBL producers were scattered throughout the phylogenetic tree; the same distribution was observed when the criterion was the sampling site (Fig. 4).

Discriminatory power and Congruence of the typing schemes

The index of discrimination, the AR and AW coefficients of congruence between DLST, oprD-typing and DLST_n_oprD are shown in Table 1. The combination of the three genes increased the discrimination between the isolates tested as it was expected, while the oprD-typing presented the lowest discrimination power. The AR coefficient when DLST and DLST_n_oprD were compared was equal to 0.491, which indicates a satisfactory match between partitions. The coefficient was lower when oprD-typing was compared to DLST or to DLST_n_oprD. The fact that the AW for DLST_n_oprD ↔ DLST= 1.000 and DLST ↔ DLST_n_oprD=0.326 means that if 2 strains are in the same cluster by DLST_n_oprD, they have 100% chance of having the same DLST type, while conversely, the chance is only about 33%. This indicates that at least in the population tested, the DLST_n_oprD-typing was more discriminatory than the DLST. This was also enhanced by the AW coefficients of the {DLST_n_oprD ↔ oprD-typing vs oprD-typing ↔ DLST_n_oprD} and {DLST ↔ oprD-typing vs oprD-typing ↔DLST} combinations (Table 1).

Discussion

To the best of our knowledge, this is the first time that an attempt has been made to elucidate the predominant *P. aeruginosa* clones in Greek aquatic environments using the new DLST scheme as proposed and combined with *oprD*-typing. The study also sought to consider the distribution

of the resistant phenotypes among the DLST-types; the discriminatory power of the three typing schemes was calculated and evaluated. The fact that the resistant *P. aeruginosa* isolates in such diverse aquatic environments are shown at a proportion as high as 34%, is considered worrying and surveillance of such resistant isolates is needed [40]. At the selected population tested, the main intrinsic resistant mechanism observed was the R1 phenotype which corresponds to AmpC, partially/fully derepressed with resistance to aztreonam (Table S1); high resistance to ATM has been previously reported in environmental isolates deriving from soil [41] or from hospital waste-water treatment [42], but never in *P. aeruginosa* isolates deriving from aquatic ecosystems. The phenotypically ESBL and MBL positive isolates did not produce positive results when tested molecularly, except in one isolate where the CTX-M group 9 *β*-lactamase was present; however there is published information highlighting the emergence of ESBL genes in Greek aquatic environments [1]. *P. aeruginosa* porin-D is a 443-amino-acid protein that facilitates the uptake of basic antibiotics, imipenem, and meropenem across the outer membrane [43]. It has been extensively reported that inactivation of porin-D due to various mutations (premature stop codons, insertion / deletion or disruption of sequences) leads to the development of resistance to imipenem and sometimes to meropenem and doripenem [18, 24, 43, 44]. Resistance to carbapenems can also arise from the production of MBLs but it is not as common mechanism as the mutation-driven resistance [43]; nevertheless it is possible that both mechanisms may coexist in a population. In our strain collection the 6 non-typeable isolates by *oprD*-typing presented the R3 phenotype (Loss of porin-D, 4 isolates:171,172, 263, 289) and the production of MBLs (metallo-b-lactamases, 2 isolates: 266, 267) (Table S1). However, further studies are needed to detect modifications in the protein-D and to evaluate the role of this porin in the carbapenem resistance in environmental *P. aeruginosa* isolates. The NCBI search revealed

that the majority of the *oprD* sequences were highly conserved and identical to *P. aeruginosa* strain PA121617, which were present in wild-type isolates and in ESBL producers, as well. The group 4-oprD sequence was identical to *P. aeruginosa* strain MTB-1, a strain which was reported to co-exist with *Sphingomonas* spp MM-1 in environments polluted by γ-HCH, an organic insecticide that has caused serious environmental problems including surface and groundwater in Greece [45, 46]. The fact that the group 4-oprD isolates derived from various habitats presenting wild-type and ESBL resistance phenotype (Table S1; Fig. 2A) requires further investigation including more water samples from the specific habitats and detailed sequencing of the *oprD* gene. Phylogenetic analysis was able to divide the initial 9 oprD-groups into 17 types distinguishing some isolates with various SNPs (Fig. 2b); however it was characterized by low discriminatory power and congruence compared to DLST and when combined with the DLST at the DLST_n_oprD analysis (Table 1).

DLST is a new and promising typing scheme, which was proposed in order to conduct epidemiological studies at a local level with low cost in a short time. It has been proved that DLST produces stable results even when it is applied on isolates recovered during studies with durations of months or even years [12]. At the present study the method was tested in 52 *P. aeruginosa* isolates recovered on a period of three years from various aquatic habitats of Greece representing a variety of resistant profiles. eBURST analysis of DLST data identified 14 DLST-types and 15 singletons within 52 isolates indicating that *P. aeruginosa* is a non-clonal population undergoing significant recombination events which is consistent to a number of papers in the literature [5, 17, 21]. It was characterized by high discriminatory power, while two new *ms217* loci (190 and 191) were recognized and subjected to DLST data base (Table S1, Fig. 2b). The majority of the isolates belonged to a few dominant clones widespread among resistant

phenotypes such as DLST-type 90-190 where wild-type, atm-resistant isolates and ESBL producers hold the same allelic profile or the types 1-191, 83-191 and 10-191 with the new allele *ms217*-191, which were present in two wild-type isolates and in the CTX-M-group 9 isolate (Fig. 2b) The latter outcomes suggest that, the circulation of acquired resistant mechanisms in these environments could be driven by their genetic profiles, and are enhanced by the following results where the combination of the three genetic markers is presented.

To increase the discriminatory power of the DLST method, a third polymorphic marker such as *oprD* was used. The number of types and the discrimination was increased where the isolates were clustered into 8 groups and 35 singletons (Fig. 4; Table 1). Although in Basset's et al work [12] the *oprD* gene was removed from the final typing scheme, when *P. aeruginosa* environmental isolates are analyzed the addition of a third locus is proved to be useful for confirming or rejecting a link between pairs of isolates. Genetically closely related isolates were distinguishable by one or more events in their *oprD* sequence (Fig. 4), while the distribution of the resistant mechanisms among various genetic profiles was more extent.

It has been previously stated that even a single polymorphism can influence the bacterium's fitness from a drug resistance point of view [21], while there is still a large number of intrinsic resistant mechanisms in *P. aeruginosa* genome that have not been described [5]. The results of the present study indicate that the variety of the DLST and DLST_n_oprD genetic profiles can act as a driving force in this extensive distribution of the resistant phenotypes in the aquatic sampling sites. This hypothesis certainly needs further study; perhaps, Whole Genome Sequencing of some resistant isolates will provide significant information regarding the relationship of the three genetic markers (*ms172*, *ms217* and *oprD)* to the development and transmission of intrinsic and acquired resistant mechanisms.

Understanding the population structure and the genetic relatedness among *P. aeruginosa* strains present in natural habitats is crucial for gaining insight into the ecology and wide distribution of this bacterium. The development of a typing method which will provide reliable results in a short time at low cost is essential; papers in the literature have dealt with this issue widely [19, 24, 47]. In general it is difficult to find the optimal genetic markers establishing a real phylogenetic history; ideally SNPs that are relatively rare and scattered through the genome could be more informative compared to other markers [21]. However, it seems that combined sequence based techniques support a polyphasic approach to reveal extensive variability in some genes or in a whole population [24]. In the present study, implementing a combination of the new DLST typing scheme to a typing method using a more stable genetic marker such as *oprD* was proved to be reliable and informative as recent events of transmission were distinguished and clusters of isolates belonging to the same clone were discriminated. The congruence calculations for the three typing schemes indicated that at least in the population tested the DLST_n_oprD-typing was more discriminatory than the DLST method. The dissemination of new mechanisms of resistance in a variety of environmental *P. aeruginosa* genetic profiles was observed with wild-type and resistant isolates presenting the same DLST and DLST_n_oprD types.

In two recently published studies regarding the typing of *P. aeruginosa* isolates recovered from the ICUs and the hospital environment, additional value on this novel typing scheme is added; the method is applied in a large bacterial population combined to Whole Genome Sequencing for epidemiological purposes highlighting the epidemic DLST-type in a short time [14, 15]; however, still there is not available any experimental work regarding exclusively aquatic *P. aeruginosa* isolates or isolates presenting significant antimicrobial resistance.

This work strongly suggests that the DLST scheme is valuable in typing a carefully chosen sub-population of aquatic *P. aeruginosa* isolates, reducing significantly the time and the cost of the molecular analysis and providing a reliable phylogenetic study at a local level. The addition of the third loci (*oprD*) should be taken into consideration when the phylogenetic analysis is combined with epidemiological data such as antimicrobial sensitivity. These findings, hopefully, will have considerable impact on the study of the origin, the antimicrobial resistance and the genetic characteristics of this well-established bacterium in the Greek aquifer.

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Figure 1

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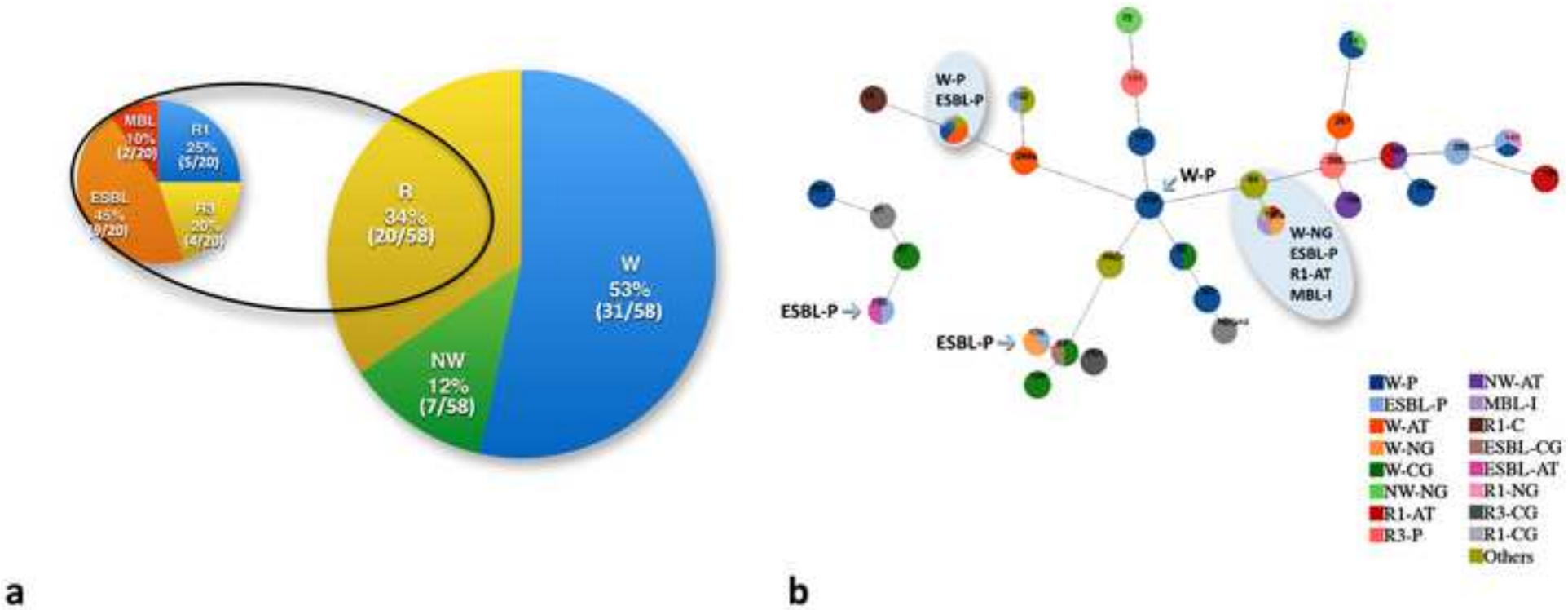

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Figure 2

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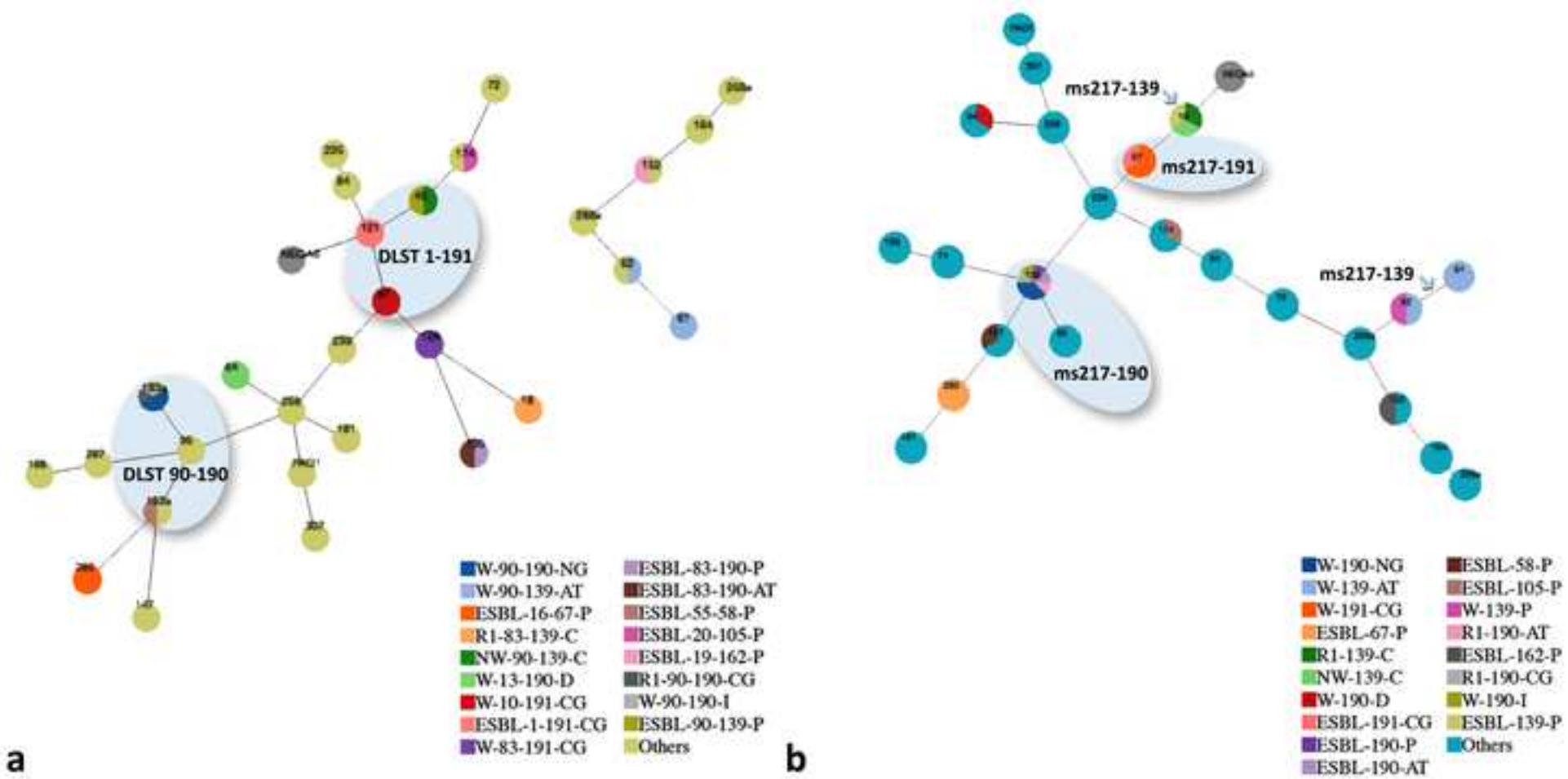

Figure 3

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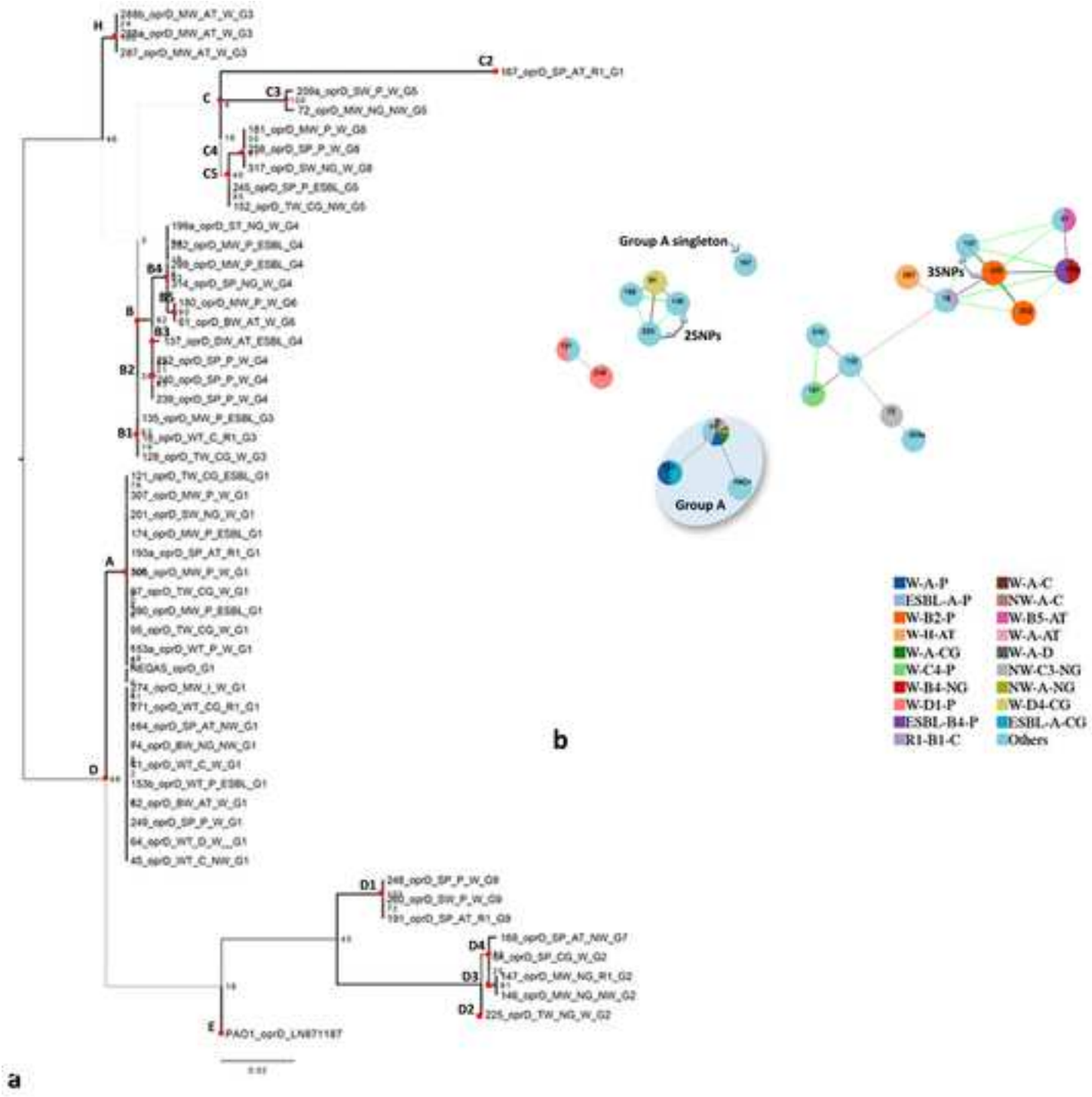

Figure 4

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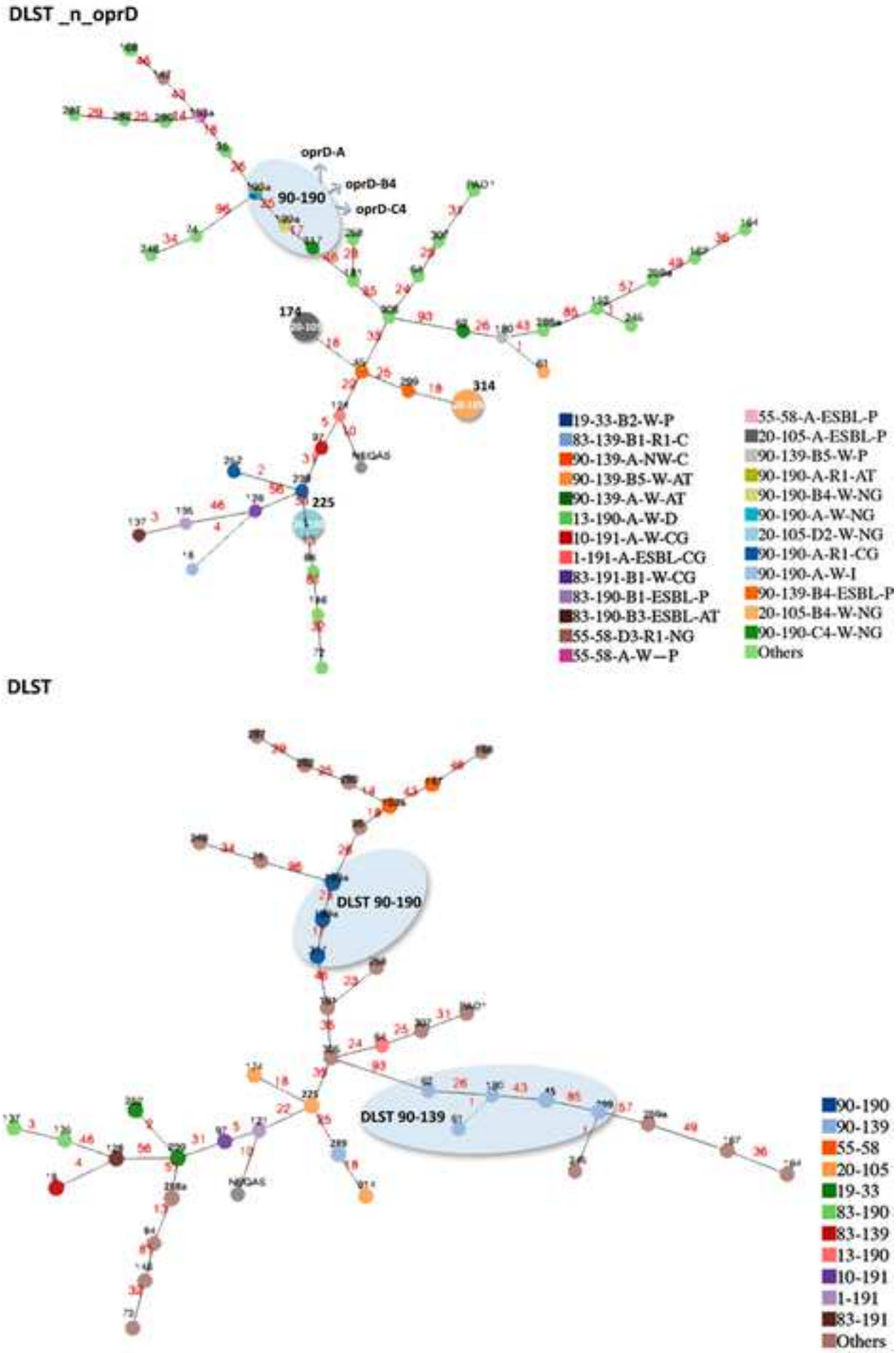

Table 1

Table 1: Index of discrimination (Simpson's ID), AR (Adjusted Rand) and AW (Adjusted Wallace) coefficients between DLST, oprD and DLST_n_oprD (95%CI)

	Simpson's ID	**AR**		**AW**		
		DLST	**oprD-typing**	**DLST**	**oprD-typing**	**DLST_n_oprD**
DLST	0.966				0.204 (0.000-0.423)	0.326 (0.140-0.511)
oprD-typing	0.839	0.062 (0.000-0.157)		0.037 (0.000-0.100)		0.059 (0.000-0.120)
DLST_n_oprD	0.989	0.491 (0.230-0.780)	0.111 (0.000-0.211)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	