

# Microbial Ecology

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

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<b>Corresponding Author:</b>	Alex Galanis Democritus University of Thrace School of Health Sciences GREECE	
<b>Corresponding Author Secondary Information:</b>		
<b>Corresponding Author's Institution:</b>	Democritus University of Thrace School of Health Sciences	
<b>Corresponding Author's Secondary Institution:</b>		
<b>First Author:</b>	Olga Pappa	
<b>First Author Secondary Information:</b>		
<b>Order of Authors:</b>	Olga Pappa	
	Apostolos Beloukas	
	Apostolos Vantarakis	
	Athina Mavridou	
	Anastasia Kefala	
	Alex Galanis	
<b>Order of Authors Secondary Information:</b>		
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<b>Abstract:</b>	<p>The recently described Double Locus Sequence Typing (DLST) typing scheme implemented to deeply characterize the genetic profiles of 52 resistant environmental <i>Pseudomonas aeruginosa</i> 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 (<i>oprD</i>) 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 <i>oprD</i>-typing can discriminate phenotypically and genetically related environmental <i>P. aeruginosa</i> isolates providing reliable phylogenetic analysis at a local level.</p>	
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1 **Molecular characterization and phylogenetic analysis of *Pseudomonas aeruginosa* isolates**  
2 **recovered from Greek aquatic habitats implementing the Double Locus Sequence Typing**  
3 **scheme**

4

5 **Olga Pappa<sup>1,4,5</sup>, Apostolos Beloukas<sup>2,4</sup>, Apostolos Vantarakis<sup>3</sup>, Athena Mavridou<sup>4</sup>,**  
6 **Anastasia-Maria Kefala<sup>5</sup>, Alex Galanis<sup>5\*</sup>**

7

8 <sup>1</sup>*Central Public Health Laboratory, Hellenic Centre for Disease Control and Prevention, Athens,*  
9 *Greece*

10 <sup>2</sup>*Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and*  
11 *Global Health, University of Liverpool, Liverpool, United Kingdom*

12 <sup>3</sup>*Environmental Microbiology Unit, Department of Public Health, School of Medicine,*  
13 *University of Patras, Greece*

14 <sup>4</sup>*Department of Medical Laboratories Technological Educational Institute of Athens, Athens,*  
15 *Greece*

16 <sup>5</sup>*Department of Molecular Biology and Genetics, Democritus University of Thrace,*  
17 *Alexandroupolis, Greece*

18

19 \*For correspondence:

20 Dr Alex Galanis, Department of Molecular Biology and Genetics, Democritus University of  
21 Thrace, Alexandroupolis, Greece, e-mail: agalanis@mbg.duth.gr; Tel.: +30 2551030634; Fax:  
22 +30 2551030624

23 **Abstract**

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

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

45

46 **Introduction**

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

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

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

72 DLST is a recently developed typing scheme based on the partial sequencing of three  
73 highly variable loci, *ms172*, *ms217* and *oprD* [12]. As the combination of two loci gave  
74 resolution results only slightly lower than the combination of the three loci, the authors proposed  
75 the use of only two loci in the DLST scheme for *P. aeruginosa* instead of three [12]. The new  
76 sequence-based scheme was compared to MLST in a large number of clinical and environmental  
77 *P. aeruginosa* isolates, proving that when epidemiological and phylogenetic analyses are  
78 conducted at a local level MLST can be replaced by DLST [13]. The online publicly available  
79 DLST database (<http://www.dlst.org/Paeruginosa/>) uses nucleotide sequences of the two loci  
80 (*ms172* and *ms217*) to define the DLST type [12]. The method is new and thus there is not much  
81 published information regarding both clinical and environmental isolates of *P. aeruginosa* [12-  
82 15]. Although in Basset's et al work the *oprD* locus was not selected for the final typing scheme,  
83 it has been reported as one of the important genetic markers that can be used in population  
84 studies, not only due to its contribution to carbapenem resistance but also due to its high genetic  
85 diversity [23]. It has been used for typing and for phylogenetic purposes both in clinical and  
86 environmental strains in order to reveal additional evolutionary forces that contribute to the high  
87 clonality of *P. aeruginosa* population [24].

88 At the present study all three typing schemes: the DLST scheme as it has been proposed  
89 [12], the *oprD*-typing scheme, and the combination of the three loci (*ms172*, *ms217* and *oprD*)  
90 were applied to environmental *P. aeruginosa* isolates collected from various water ecosystems in  
91 Greece. Using a bacterial population chosen as to represent various resistant profiles, different

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

97

## 98 **Material and Methods**

### 99 *Bacterial isolates*

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

111

### 112 *Isolation of genomic DNA*

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

116

#### 117 *Antibiotic Susceptibility testing*

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

127

#### 128 *Detection of Extended Spectrum Beta-Lactamases (ESBLs) and Metallo Beta-Lactamases* 129 *(MBLs)*

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

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

138

#### 139 *Double Locus Sequence Typing (DLST) and oprD-typing*

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

154

#### 155 *Analysis of the sequenced data*

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

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

165

166 *Molecular epidemiological analysis*

167 • *eBURST analysis and minimum spanning trees construction*

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

176

177 • *Maximum likelihood phylogenetic analysis of the oprD*

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

180

181 *Index of diversity and concordance of the typing methods*

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

192

## 193 **Results**

### 194 *Antimicrobial susceptibility profiles and detection of beta-lactamase-producers*

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

206 MBL genes tested. The resistant phenotypes were distributed across all geographical areas (Fig.  
207 1b), while the Peloponnese presented the highest percentage of all the resistant profiles. ESBL  
208 isolates appeared in three geographically unrelated areas of Greece together with other resistant  
209 mechanisms (Fig. 1b).

210

#### 211 *DLST analysis*

212 Fifty-four isolates (including the reference strains) were successfully typed implementing the  
213 DLST scheme. DLST was able to assign an already known allele number for 40 isolates while  
214 for 12 isolates two new loci were recognized for the *ms217* marker (allele 190 and 191,  
215 <http://www.dlst.org/Paeruginosa/ms217.txt>). The phylogenetic analysis revealed 27 types with  
216 DLST-type 90-190 (6/54; 11.1%) being the predominant one; the second new allele 191 (3/54;  
217 5.5%) was combined with three different *ms172* loci (1-191, 83-191, 10-191). Five out of the 54  
218 isolates (9.3%) presented the DLST-type 90-139; 3/54 isolates had the DLST-types 18-54, 19-  
219 33, 20-105 and 55-58 respectively (5.6% each), while the rest 28 isolates were distributed among  
220 8 different DLST-types including 2 isolates each (16/54; 29.6% in total) and 12 isolates (12/54;  
221 22.22%) presenting unique DLST-types, including the reference strains: NEQAS: 32-39 and  
222 PAO1: 16-4 (Fig. 2a; Table S1). The predominant DLST-type (90-190) was present in wild-type  
223 isolates as well as in isolates with resistant phenotypes R1 deriving from Northern and Central  
224 Greece and the Ionian islands present in a variety of water samples. ESBL isolates were  
225 distributed among 7 DLST-types; 5 of them (20-105, 90-139, 55-58 and 19-162) co-existed with  
226 wild-type, non-wild-type and R1 isolates recovered mainly from the Peloponnese with no  
227 significant correlation to the types of water samples (Fig. 2a; Table S1). The allele 190 combined  
228 with other *ms172* loci was also present in Attica and the Peloponnese in ESBL producers. The  
229 new allele 191 for the *ms217* gene was detected exclusively in isolates deriving from thermal

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

234

#### 235 *oprD*-typing

236 *oprD* locus was detected in 54 isolates (including the reference strains). BLAST analysis of the  
237 54 *oprD* genes distributed the isolates in 9 groups (G1-G9), with two groups, 1 and 4, including  
238 the majority of the isolates, with 21 and 8 isolates, respectively. BLAST search against the NCBI  
239 data base showed that the coding sequence of the group 1-*oprD* gene was identical to *P.*  
240 *aeruginosa* strain PA121617 (GenBank accession no. CP016214), while the coding sequence of  
241 the group 4-*oprD* gene was identical to *P. aeruginosa* strain MTB-1 (GenBank accession no.  
242 CP006853). The reference strain NEQAS was identical to *P. aeruginosa* strain ATCC 27853  
243 (GenBank accession no. CP015117), while the coding sequence of PAO1's *oprD* gene was  
244 identical to *P. aeruginosa* genome assembly PAO1OR, chromosome:I (GenBank accession no.  
245 LN871187), as expected. The above results and the coding sequences of the rest *oprD*-groups are  
246 shown in Table S1. The ML phylogeny revealed five major clusters -A, B, C, D and H- and cluster  
247 E with the reference strain PAO1 as outgroup; only the cluster A was consistent with the initial  
248 results containing all the group 1-*oprD* isolates, except one (167), which presented various  
249 Single Nucleotide Polymorphisms (SNPs) comparing to the major group; it was located at a  
250 distance from the major group and it was consequently considered as a singleton (C2); the  
251 reference strain NEQAS was located in Cluster A. Cluster B was divided into five subgroups  
252 where the B3 subgroup was separated from B2 and B4 with three and two SNPs respectively  
253 (isolate 137). Cluster C was separated into four sub-clusters consisted of three different *oprD*-

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

266

#### 267 *DLST\_n\_oprD*

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

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

281

### 282 *Discriminatory power and Congruence of the typing schemes*

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

296

### 297 **Discussion**

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

301 of the resistant phenotypes among the DLST-types; the discriminatory power of the three typing  
302 schemes was calculated and evaluated. The fact that the resistant *P. aeruginosa* isolates in such  
303 diverse aquatic environments are shown at a proportion as high as 34%, is considered worrying  
304 and surveillance of such resistant isolates is needed [40]. At the selected population tested, the  
305 main intrinsic resistant mechanism observed was the R1 phenotype which corresponds to AmpC,  
306 partially/fully derepressed with resistance to aztreonam (Table S1); high resistance to ATM has  
307 been previously reported in environmental isolates deriving from soil [41] or from hospital  
308 waste-water treatment [42], but never in *P. aeruginosa* isolates deriving from aquatic  
309 ecosystems. The phenotypically ESBL and MBL positive isolates did not produce positive  
310 results when tested molecularly, except in one isolate where the CTX-M group 9  $\beta$ -lactamase  
311 was present; however there is published information highlighting the emergence of ESBL genes  
312 in Greek aquatic environments [1]. *P. aeruginosa* porin-D is a 443-amino-acid protein that  
313 facilitates the uptake of basic antibiotics, imipenem, and meropenem across the outer membrane  
314 [43]. It has been extensively reported that inactivation of porin-D due to various mutations  
315 (premature stop codons, insertion / deletion or disruption of sequences) leads to the development  
316 of resistance to imipenem and sometimes to meropenem and doripenem [18, 24, 43, 44].  
317 Resistance to carbapenems can also arise from the production of MBLs but it is not as common  
318 mechanism as the mutation-driven resistance [43]; nevertheless it is possible that both  
319 mechanisms may coexist in a population. In our strain collection the 6 non-typeable isolates by  
320 *oprD*-typing presented the R3 phenotype (Loss of porin-D, 4 isolates:171,172, 263, 289) and the  
321 production of MBLs (metallo-b-lactamases, 2 isolates: 266, 267) (Table S1). However, further  
322 studies are needed to detect modifications in the protein-D and to evaluate the role of this porin  
323 in the carbapenem resistance in environmental *P. aeruginosa* isolates. The NCBI search revealed

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

336 DLST is a new and promising typing scheme, which was proposed in order to conduct  
337 epidemiological studies at a local level with low cost in a short time. It has been proved that  
338 DLST produces stable results even when it is applied on isolates recovered during studies with  
339 durations of months or even years [12]. At the present study the method was tested in 52 *P.*  
340 *aeruginosa* isolates recovered on a period of three years from various aquatic habitats of Greece  
341 representing a variety of resistant profiles. eBURST analysis of DLST data identified 14 DLST-  
342 types and 15 singletons within 52 isolates indicating that *P. aeruginosa* is a non-clonal  
343 population undergoing significant recombination events which is consistent to a number of  
344 papers in the literature [5, 17, 21]. It was characterized by high discriminatory power, while two  
345 new *ms217* loci (190 and 191) were recognized and subjected to DLST data base (Table S1, Fig.  
346 2b). The majority of the isolates belonged to a few dominant clones widespread among resistant

347 phenotypes such as DLST-type 90-190 where wild-type, atm-resistant isolates and ESBL  
348 producers hold the same allelic profile or the types 1-191, 83-191 and 10-191 with the new allele  
349 *ms217*-191, which were present in two wild-type isolates and in the CTX-M-group 9 isolate (Fig.  
350 2b) The latter outcomes suggest that, the circulation of acquired resistant mechanisms in these  
351 environments could be driven by their genetic profiles, and are enhanced by the following results  
352 where the combination of the three genetic markers is presented.

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

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

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

387           In two recently published studies regarding the typing of *P. aeruginosa* isolates recovered  
388 from the ICUs and the hospital environment, additional value on this novel typing scheme is  
389 added; the method is applied in a large bacterial population combined to Whole Genome  
390 Sequencing for epidemiological purposes highlighting the epidemic DLST-type in a short time  
391 [14, 15]; however, still there is not available any experimental work regarding exclusively  
392 aquatic *P. aeruginosa* isolates or isolates presenting significant antimicrobial resistance.

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

400

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411

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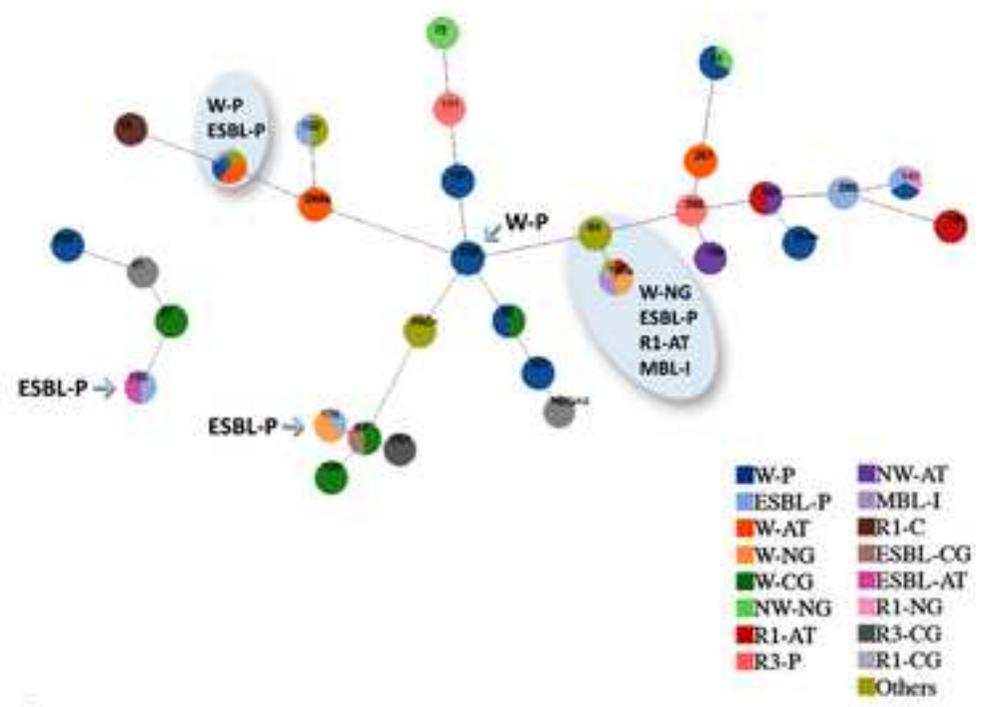
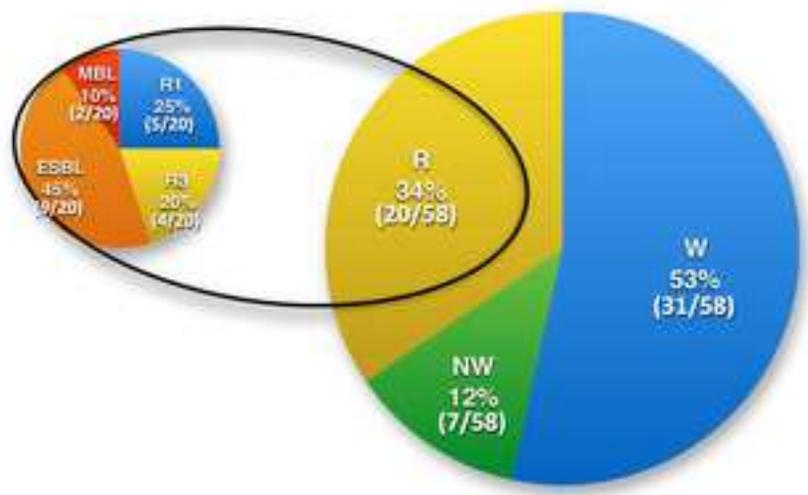
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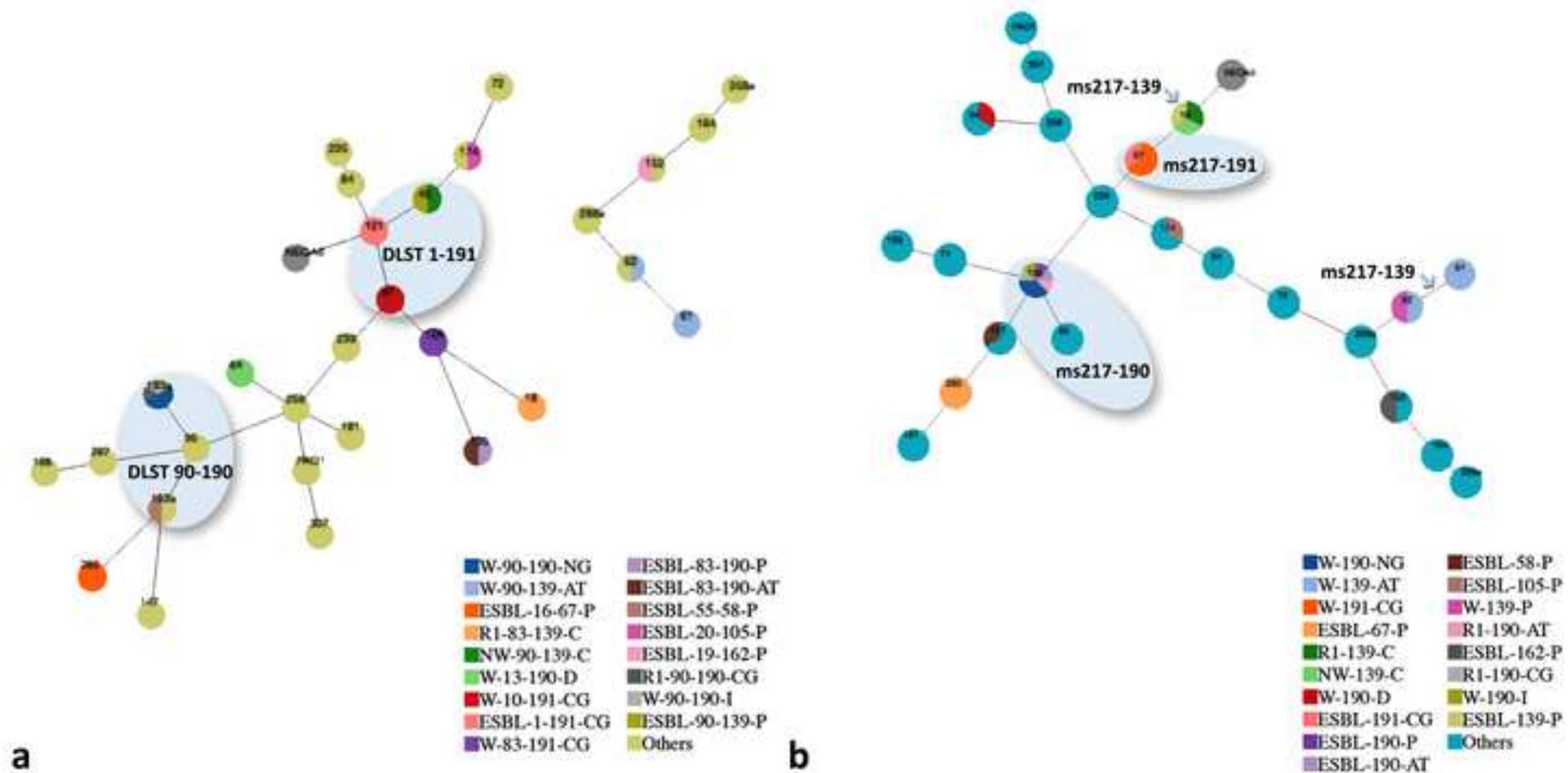
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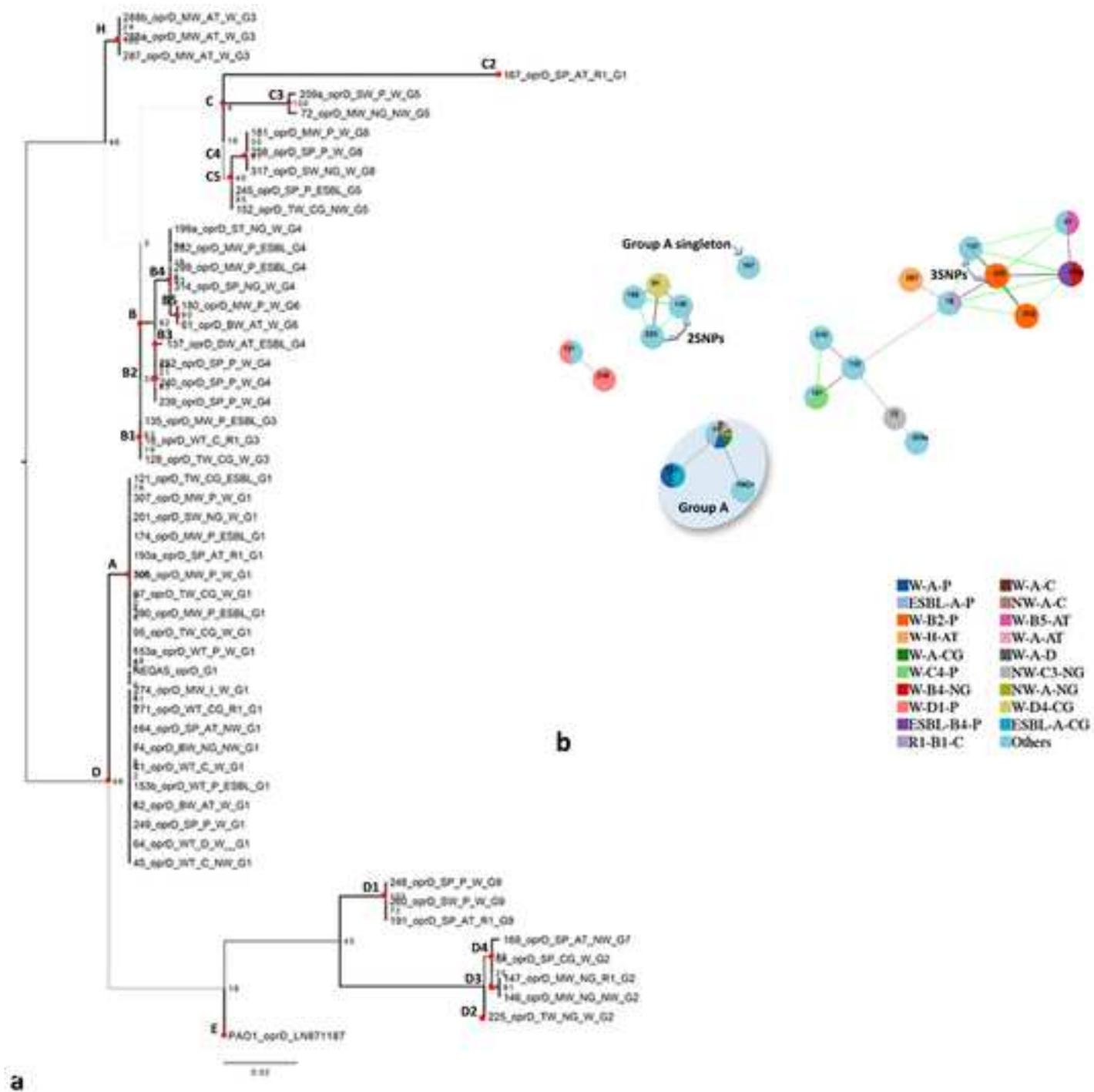
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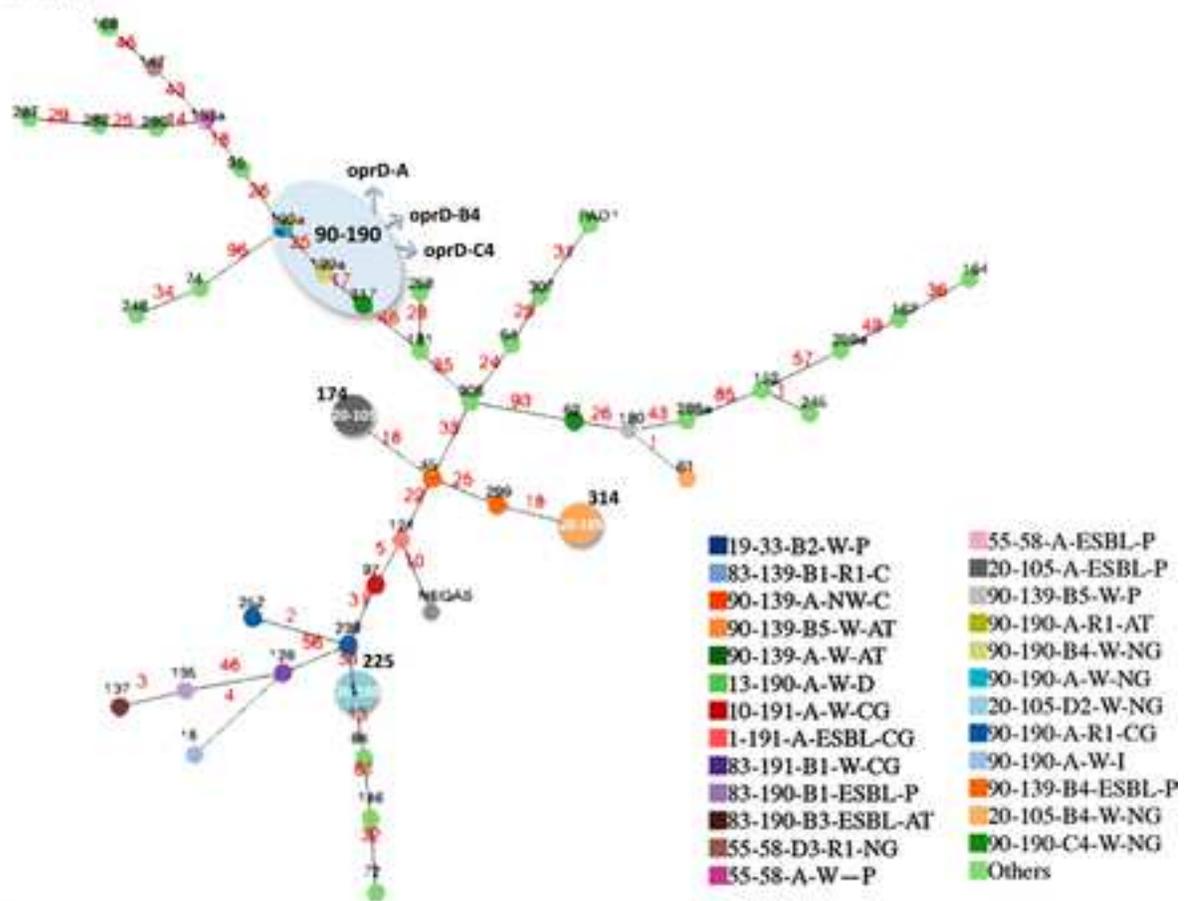
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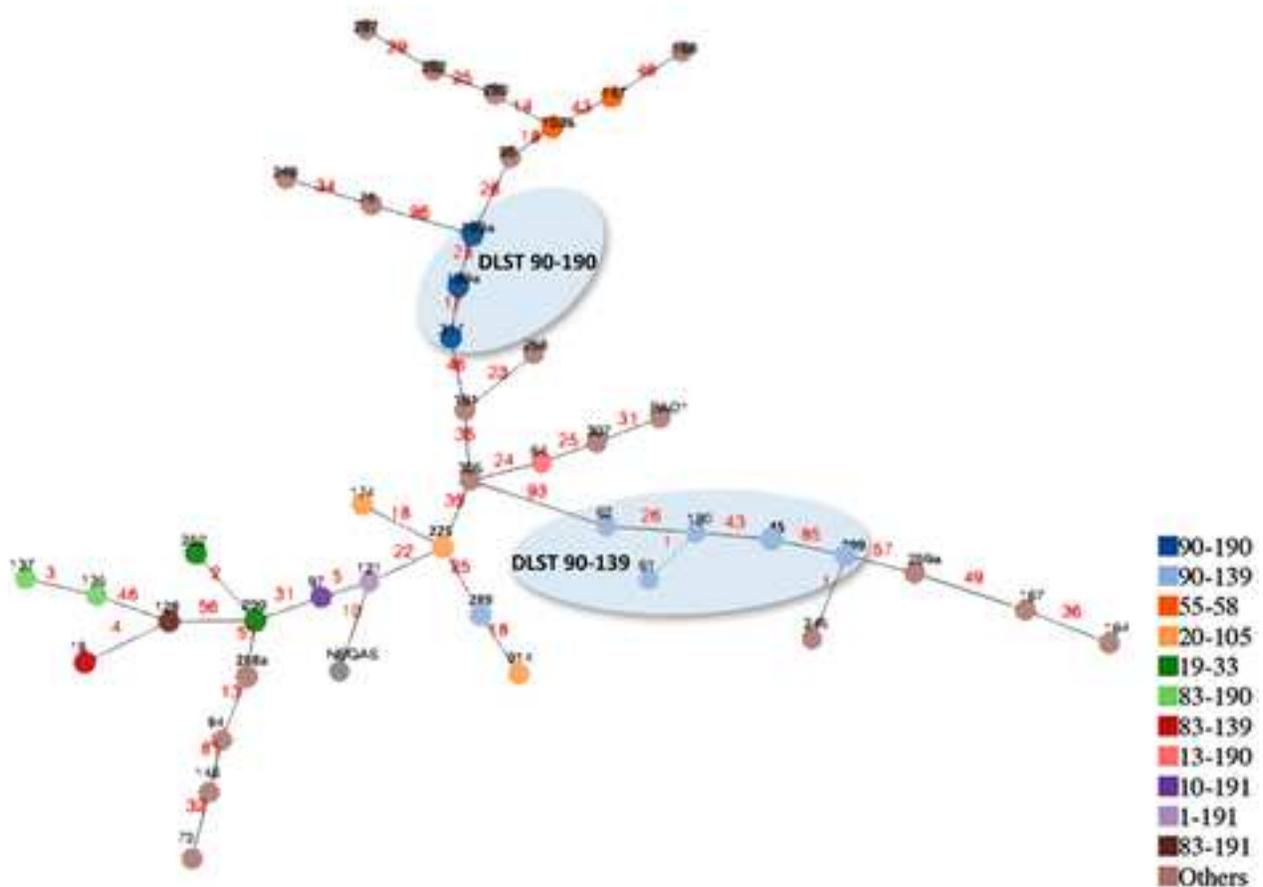




## DLST\_n\_oprD



## DLST



**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
<b>DLST</b>	0.966				0.204 (0.000-0.423)	0.326 (0.140-0.511)
<b>oprD-typing</b>	0.839	0.062 (0.000-0.157)		0.037 (0.000-0.100)		0.059 (0.000-0.120)
<b>DLST_n_oprD</b>	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)	