Complete, closed and curated genome sequences of *Photobacterium damselae* subsp. *piscicida* isolates from Australia indicate mobilome-driven localized evolution and novel pathogenicity determinants

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**Abstract**

Despite the recent advances in sequencing technologies, the complete assembly of multi-chromosome genomes of the Vibrionaceae, often containing several plasmids, remains challenging. Using a combination of Oxford Nanopore MinION long reads and short Illumina reads, we fully sequenced, closed and curated the genomes of two strains of a primary aquatic pathogen *Photobacterium damselae* subsp. *piscicida* isolated in Australia. These are also the first genome sequences of *P. damselae* subsp. *piscicida* isolated in Oceania and, to our knowledge, in the Southern hemisphere. We also investigated the phylogenetic relationships between Australian and overseas isolates, revealing that Australian *P. damselae* subsp. *piscicida* are more closely related to the Asian and American strains rather than to the European ones. We investigated the mobilome and present new evidence showing that a host specialization process and progressive adaptive evolution to fish are ongoing in *P. damselae* subsp. *piscicida*, and are largely mediated by transposable elements, predominantly in chromosome 2, and by plasmids. Finally, we identified two novel potential virulence determinants in *P. damselae* subsp. *piscicida* – a chorismate mutase gene, which is ubiquitously retained and co-localized with the AIP56 apoptogenic toxin-encoding gene on the pHDP10 plasmid, and transfer-messenger RNA gene ssrA located on the main chromosome, homologous to a critical-to-virulence determinant in *Yersinia pseudotuberculosis*. Our study describes, to our knowledge, the only fully closed and manually curated genomes of *P. damselae* subsp. *piscicida* available to date, offering new insights into this important fish pathogen and its evolution.

**DATA SUMMARY**

Complete, closed and curated genome and plasmid assemblies for *Photobacterium damselae* subspecies *piscicida* strains QMA0505 and QMA0506 have been deposited at the National Center for Biotechnology Information under BioProject number PRJNA662633, with links to sample metadata and sequence reads from all platforms used. Accession numbers for assembled chromosomes and plasmids for strains QMA0505 and QMA0506 are CP061854–CP061860 and CP061861–CP061867, respectively.

**INTRODUCTION**

*Photobacterium damselae* subsp. *piscicida* is a Gram-negative marine bacterium found in warm-temperate waters [1]. Unlike its sister subspecies *P. damselae* subsp. *damselae*, which is a generalist, opportunistic pathogen [2, 3], *P. damselae* subsp. *piscicida* is a highly virulent fish-specialized pathogen with a widespread geographical distribution. In the Northern hemisphere, photobacteriosis caused by *P. damselae* subsp. *piscicida* leads to mortality events in high-value aquaculture species such as gilthead sea bream...
The pathogenicity of *P. damselae* subsp. *piscicida* has not yet been fully elucidated, but the unique exotoxin AIP56 is well characterized and likely critical for virulence as it can trigger apoptosis in fish macrophages, neutrophils and epithelial cells [5]. This A-B metalloprotease is encoded on the plasmid pPHDP10 and neither homologues nor its phylogenetic origin are known at present. Interestingly, this plasmid is highly stable, and it is almost always present in isolates collected over the last 30 years [6]. A second virulence factor, the siderophore piscibactin, is involved in iron uptake. The system is encoded by a gene cluster on the plasmid pPHDP70, and curing of this plasmid reduces *P. damselae* subsp. *piscicida* virulence in fish [7]. Other potential virulence factors are involved in adhesion, such as the lipoprotein PDP_0080 [8], defence against oxidative stress, such as superoxide dismutase [9], and secretion of effector proteins via a type 3 secretion system [6].

Despite the apparent serological homogeneity of *P. damselae* subsp. *piscicida* [10], there is good evidence for phylogenetic diversification. For example, Japanese strains are consistently different from European strains by RAPD analyses [11]. In addition to broad-scale genetic evidence of discrete evolutionary lineages, there are also key differences in the mobilome; in the European strains, two plasmids, 20 MDa (~30 kb) and 7 MDa (~10 kb, possibly pPHDP10), have been found, with a third 50 MDa (~75 kb, possibly pPHDP70) that carries the piscibactin genes cluster) plasmid widely present, while the Japanese isolates harbour a 37 MDa (~56 kb) plasmid [12]. Strains isolated in the USA also carry plasmids of 20 and 7 MDa but, in contrast to the European and Japanese strains, no pPHDP70 plasmid has been identified to date [6]. Furthermore, the pPHDP70 plasmid of the European strains shares four sequence blocks with the Japanese *P. damselae* subsp. *piscicida* 204 kb pAQU1 plasmid [13]. Additional transferable antibiotic-resistance plasmids have also been identified and sequenced in *P. damselae* subsp. *piscicida*, and many complete plasmid assemblies have been submitted to the National Center for Biotechnology Information (NCBI) (Table S1, available with the online version of this article).

In contrast to the plasmid characterization, chromosomal data are very limited and only contig- and scaffold-level genome assemblies are available (Table 1), with only the USA isolate 91-197 from hybrid striped bass being closed (PRJDB5709). In contrast to the majority of eubacteria, in the *Vibrionaceae*, it is common to find two chromosomes of different sizes, with the larger being generally around 3–3.3 Mb and the smaller ranging from 0.8 up to 2.4 Mb [14]. Lack of complete genomic information impairs both epidemiological pathogen surveillance and the development of effective preventative strategies in aquaculture. Vaccines against intracellular pathogens like *P. damselae* subsp. *piscicida* need to elicit not only humoral but also cellular immune responses and, to date, DNA vaccines are considered a relatively safe option for obtaining cellular immune protection in fish, which is not offered by killed whole-cell vaccines traditionally used in aquaculture [15]. This strategy has been successfully employed in experimental vaccines against *P. damselae* subsp. *piscicida* in flounder in Japan [16]. However, identification of candidate DNA vaccine immunogens relies heavily on the availability of accurate genetic information. Such information is limited for *P. damselae* subsp. *piscicida* isolates, reflecting the cost and complexity of sequencing and closing mobilome-rich multi-chromosome genomes in isolates that also carry several plasmids that are critical for virulence. For long-read sequencing appropriate for accurate genome closure, PacBio single molecule real-time sequencing technology is well tested but expensive, and samples require time-consuming preparation before submission to a dedicated facility for sequencing. In contrast, robust and cost-effective Illumina short-read platforms provide high-accuracy base calling, but are unsuited to closing complex genomes. As a consequence, most current *P. damselae* subsp. *piscicida* assemblies comprise very high numbers of short contigs (Table 1).

Recently, photobacteriosis outbreaks have been recorded in yellowtail kingfish (*Seriola lalandi*) farms in South Australia and Western Australia. Here, we sequenced and assembled complete genomes for two Australian isolates of *P. damselae* subsp. *piscicida* using Oxford Nanopore Technology (ONT) sequencing. Nanopore sequencing provides low-cost long-read data suited to closure
of complex genomes including plasmids [17]. Moreover, the preparation of the libraries is quick and easy, and the samples can be sequenced in a short time directly in the same laboratory, without the need for external facilities. As ONT is currently inferior to Illumina in terms of base-calling accuracy, closed long-read draft assemblies were polished with Illumina MiSeq short reads. For both strains, polished Nanopore assemblies were compared to polished PacBio assemblies and, when possible, mismatches were manually resolved using mapped Illumina reads. We closed both chromosomes and five plasmids belonging to the Australia outbreak isolates and manually curated the annotations providing high-quality reference sequences for *P. damselae* subsp. *piscicida*.

Using these data, we then explored the evolutionary relationships between Australian and overseas isolates, with a focus on the role of plasmids and other mobile genetic elements (MGEs).

**METHODS**

**Bacterial strains**

*P. damselae* subsp. *piscicida* strains QMA0505 (AS-16-0540 #1) and QMA0506 (AS-16-0555 #7) were isolated at the Department of Primary Industries and Regional Development (DPIRD) in Western Australia from yellowtail kingfish (*Seriola lalandi*) farms in South Australia and Western Australia, respectively, during 2016 disease outbreaks and kept frozen at −80 °C. The identity of the strains was confirmed by PCR with *P. damselae* subsp. *piscicida* specific primers [18], and by phenotypic assays with API20E tests (BioMérieux), following the manufacturer’s instructions, conventional biochemical tests incubated for 48 h at 24 °C according to Buller (2014) [19] and MALDI-TOF MS, BioTyper and the MBT 7854 MSP library (Bruker). Micro-organisms were routinely grown on tryptone soy agar 2 (Oxoid) for 24 h at 27 °C or blood agar (BA) for 48 h at 24 °C.

**DNA extraction and library preparation**

DNA was extracted from fresh cultures using the N-cetyl-N,N,N-trimethylammonium bromide (CTAB) method [20] and quantified by Qubit fluorimetry (Invitrogen). An aliquot (1 ng) of DNA from each strain was then used to prepare Nextera XT libraries for MiSeq v2 Illumina sequencing (Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia). For Nanopore sequencing, a 12-sample barcoded library was prepared using rapid sequencing kit SQK-RBK004. The library was sequenced on an Oxford Nanopore MinION device equipped with a FLO-MIN106D (R9) flow cell.

For PacBio long-read sequencing, DNA was extracted from 10 ml 48 h tryptone soy broth with 2% NaCl (TSB2) cultures of *P. damselae* subsp. *piscicida* using the Genomic-Tip G/20 kit and associated buffers (Qiagen). Aliquots (100 µl) containing 62 µg of DNA were used to prepare libraries using the Genomic-tip G/20 kit and associated buffers (Qiagen) and sequenced on a PacBio RS II instrument.

**Table 1.** *P. damselae* subsp. *piscicida* assemblies and related information available to date from the NCBI

| Strain   | Date (M/YY), level | GenBank acc. no. | Assembly method | Coverage (×) | Technology          | Host                        | Isolation (year/location) |
|----------|--------------------|------------------|-----------------|--------------|---------------------|-----------------------------|----------------------------|
| PP3      | 4/19, contig       | GCA_004683985.1  | Megahit v. 1.0  | 100          | Illumina MiSeq     | *Seriola quinqueradiata*    | NA, Japan                  |
| DI21*    | 7/15, scaffold     | GCA_000300355.4  | Newbler v. 2.6  | 60           | 454                 | *Sparus aurata*             | 1990, Spain                |
| 91-197   | 5/17, complete genome | GCA_002356235.1 | HGP v. 3        | 343.5        | PacBio RS II       | *Morone chrysops × Morone saxatilis* | 1991, USA                  |
| MT1415   | 5/19, contig       | GCA_005048985.1  | Megahit v. 1.0  | 200          | Illumina MiSeq     | *Dicentrarchus labrax*      | 1993, Italy                |
| OT-51443 | 4/17, contig       | GCA_002157645.1  | HGP v. 3        | 343.5        | PacBio RS II       | *Seriola quinqueradiata*    | 1995, Japan                |
| ATCC 29688 | 3/18, contig    | GCA_003026775.1  | SPAdes v. 3.11.0 | 88           | Illumina MiSeq     | *Seriola quinqueradiata*    | 1995, Japan                |
| ATCC 29689 | 3/18, contig    | GCA_003026245.1  | SPAdes v. 3.11.0 | 70           | Illumina MiSeq     | *Seriola quinqueradiata*    | 1995, Japan                |
| SNW-8.1  | 4/19, contig       | GCA_004684005.1  | SPAdes v. 3.6   | 50           | Illumina MiSeq     | *Salmo salar*               | 2014, Spain                |
| L091106-03H | 1/17, scaffold    | GCA_001751565.2  | CABOT vs JAN-2015; SOAPdenovo vs JAN-2015; SSPACE vs JAN-2015 | 50 | 454 | *Solea senegalensis* | 2017, Spain |

NA: Isolation date not available

*Strain not included in the phylogenetic analyses. Only pPHDP70 assembly (NZ_KP100338.1) was included.*
DNA for QMA0505 and 20.7 µg for QMA0506 were sent for PacBio 15–20 kb SMRT Bell library preparation and Blue Pippin size selection prior to sequencing on four SMRT cells using the P6-C4 chemistry on the RSII platform (Ramaciotti Centre, University of New South Wales, Sydney, Australia). In each case, DNA was quantified by Qubit fluorimetry (Invitrogen), and the quality and purity checked by gel electrophoresis, followed by amplification and sequencing of the almost complete 16S rRNA gene using universal 27F and 1492R primers [21]. Derived 16S sequences were assembled in Sequencher v5.2.2 (Gene Codes), and then analysed by BLASTN to confirm species identity before genome sequencing.

**Nanopore–Illumina hybrid genome assembly and annotation**

The quality of short Illumina reads was assessed using FastQC v.0.11.8 [22] and then trimmed using FastP v.0.20.0 [23], using the flags --trim_front=15 and --trim_tail=5. Long Nanopore reads were demultiplexed and trimmed with Porechop v0.2.3_seqan2.1.1 [24]. Draft genomes were assembled with long reads using Flye v.2.6 [25], using arguments --nano-raw, --genome-size=4.8 m and --plasmids. For assembly of chromosomes 1 and 2 from QMA0505 the default parameters were used, while for plasmids the -min-overlap was set at 8000. For QMA0506, --min-overlap was set at 7000, while only for plasmid 5 (pPHDP10) --min-overlap was set to 6200. The quality of draft assemblies was assessed by inspecting the assembly graph in Bandage v.0.8.1 [26]. These draft backbones were then polished to a coverage depth of ~60–70× with Illumina short reads using Unicycler_polish in Unicycler v.0.4.8 [27]. The bacterial origin of replication (dnaA) was set at position 0 with the fixstart command in Circlator [28]. The genome QMA0505 was annotated using Prokka [29] and further curated manually by BLASTX of all hypothetical proteins against the NCBI database and accepting results with identity and coverage greater or equal to 93%. The curated annotated genome for QMA0505 was considered a reference and used to construct a database in Prokka for the annotations of QMA0506. Finally, operon predictions were performed with FgenesB tool in SoftBerry, using Vibrio parahaemolyticus RIMD 2210633 as a reference (http://www.softberry.com/berry.phtml).

**Validation and curation of Nanopore chromosome assemblies**

The validation of chromosomal assemblies using Nanopore reads was performed on strain QMA0505. Chromosomes 1 and 2 were assembled from PacBio sequencing reads from two SMRT cells using SMRT Portal version 2.3.0 (Pacific Biosciences) and the hierarchical genome assembly process (hgap3), with default parameters for a coverage of 30-fold and based on estimated genome size 4.8 Mb, then polished with Quiver [30]. Following draft assembly, contigs were analysed for terminal overlaps with Circlator. Overlapping regions were trimmed and the resulting circular chromosomes reoriented and cut such that the origin of replication was at the start. Nanopore assemblies using Flye and PacBio hgap3 assemblies were then polished using the Illumina short reads with Unicycler_polish in Unicycler v.0.4.8 [27]. Polished assemblies were aligned in Geneious Prime v.2020.1.2 and SNPs detected using default settings. Short Illumina reads were mapped onto the polished Nanopore assemblies with medium sensitivity. For each SNP in the PacBio/Nanopore alignment that was not a tRNA nor rRNA coding region, the consensus in Illumina reads was investigated manually. When at least 90% of Illumina data indicated the same base, the SNP was corrected accordingly. tRNA and rRNA coding sequences were not included in this process, because the high similarity between multiple copies of these genes made the read-mapping step inconsistent and coverage uneven. Once manually polished, the chromosomal assemblies of QMA0505 were annotated again in Prokka using the previously manually curated annotations as a database. All the locus tags refer to QMA0505, but the loci are also present in QMA0506, unless otherwise stated.

**Curation of the Australian plasmids**

Illumina reads of both QMA0505 and QMA0506 were mapped on the polished Nanopore plasmid assemblies. For each SNP found between the pairs of plasmids aligned with MAFFT in Geneious Prime v.2020.1.2, the consensus in the mapped Illumina reads was examined. When at least 90% of Illumina data indicated the same base, the SNP was corrected accordingly.

**Phylogenetic analyses**

For phylogenetic reconstruction, available P. damselae subsp. piscicida genomes were retrieved from the NCBI (Table 1). For P. damselae isolates with no indication of the subspecies, a BLAST search for the ureC gene was performed and its absence was accepted as evidence that the subspecies was P. damselae subsp. piscicida [18]. For draft assemblies at the contig level, sequences were mapped to the reference chromosomes QMA0505 and consensus generated in Geneious Prime v.2020.1.1. Reordered genomes were aligned with Parsnp in the HarvestTools suite v.1.1.2 [31], and regions of recombination in the resulting core-genome alignments were then inferred and removed using Gubbins v.2.4.1 [32]. Phylogenies based on 30484 non-recombinant core-genome SNPs were reconstructed by maximum-likelihood method using RAxML v.8.2.11 in Geneious Prime v.2020.1.1, with the general time reversible nucleotide substitution model (GTR) and bootstrap of 1000 iterations. As the dataset contained only variable sites, an ascertainment bias correction was included with the flags --m ASC_GTRGAMMA and --asc-corr=felsenstein with the number of invariant sites specified. As outgroup, the complete curated genome sequence of P. damselae subsp. damsela strain QMA0509 (AS-15-3942 #7) from the laboratory collection was included to guide the rooting of the phylogeny. The tree was annotated with metadata using Evolview v.2 [33]. Finally, for strain OT-51443, the contigs deposited in the NCBI database were reordered with Mauve Contig Mover, using QMA0505 as a reference.
RESULTS

Case diagnosis and observations

The MALDI-TOF scores for QMA0505 (AS-16-0540 #1) and QMA0506 (AS-16-0555 #7) were significant to the species level at 2.0 and 2.1, respectively. Both isolates were positive for arginine dihydrolase, fermentation of 1% glucose and 1% mannose, and growth on agar containing 3% NaCl. The result for methyl red was weak. There was no growth on thiosulphate-citrate-bile salts-sucrose (TCBS) agar after 48 h incubation at 24 °C. Both isolates were negative for the following: β-haemolysis on BA containing 5% horse red blood cells, swarming on BA and marine salt agar (MSA) containing 5% horse red blood cells, ornithine decarboxylase, lysine decarboxylase, nitrate reduction, indole, urease, Voges–Proskauer reaction (acetoin production), hydrolysis of aesculin, gelatin and DNA, ONPG (o-nitrophenyl-β-galactoside), growth on MacConkey agar, and negative fermentation of l-arabinose, d-glucose, inositol, lactose, maltose, mannitol, mannose, salicin, sorbitol, sucrose, trehalose and xylose. The isolates were sensitive to vibriostatic agent 0/129 discs of 10 and 150 µg (Oxoid). The API20E bionumber was 2 004 004. Both isolates were negative by PCR for the 267 bp ureC amplicon, consistent with *P. damselae* subsp. *piscicida* identification.

Mortalities in farmed yellowtail kingfish during outbreaks in Australia have been relatively low compared to those reported in Japan and Europe, with per cage mortality rates not exceeding 10% and generally around ~1–2% across an entire farm lease. It is also worth noting that outbreaks, and associated isolations of *P. damselae* subsp. *piscicida*, have not occurred every season in Australia, even when leases have remained stocked throughout with no fallow period.

| Long-read assembly method | Chr. 1 | Chr. 2 | Plas. 1 | Plas. 2 pPHDP70 | Plas. 3 | Plas. 4 | Plas. 5 pPHDP10 |
|---------------------------|--------|--------|---------|----------------|--------|--------|----------------|
| (a) QMA0505               |        |        |         |                |        |        |                |
| PacBio 1 nM (Flye)        | 3230005| 1136179| 136327  | 81408          | 37525  | 23188  | –              |
| PacBio 12 nM (Flye)       | 3230071| 1136170| 136327  | 81412          | 36448  | 23189  | –              |
| PacBio 15 nM (Flye)       | 3230074| 1136181| 136328  | 81412          | 36447  | 23189  | –              |
| PacBio 1 nM (Hgap3)       | 3230051| 1135725| 136328  | 81412          | 75215  | –      | –              |
| Nanopore (not curated, Flye) | 3230449| 1136362| 136317  | 81403          | 36456  | 23188  | 7991           |
| Nanopore (curated, Flye)  | 3230241| 1136236| 136326  | 81411          | 36456  | 23190  | 7991           |
| (b) QMA0506               |        |        |         |                |        |        |                |
| PacBio 1 nM (Flye)        | 3171776| 1133618| 136327  | 81411          | 38781  | 23189  | –              |
| PacBio 12 nM (Flye)       | 3171786| 1133152| 136328  | 81412          | 38782  | 23189  | –              |
| PacBio 15 nM (Flye)       | 3171764| 1133550| 136328  | 81412          | 39858  | 23190  | –              |
| PacBio 1 nM (Hgap3)       | 3198996| 1135525| 161014  | 109463         | –      | –      | –              |
| Nanopore (Flye, not curated) | 3230247| 1134456| 136333  | 81431          | 36447  | 23192  | 7987           |
| Nanopore (Flye, curated)  | 3230122| 1134735| 136321  | 81418          | 36447  | 23189  | 7991           |

Genome assemblies

Oxford Nanopore reads permitted assembly and closure of draft genomes of QMA0505 and QMA0506 using Flye, with a coverage of at least 90× and 26×, respectively. Additionally, short Illumina reads used to polish the drafts provided an additional 107× and 361× coverage, respectively. For each strain, both chromosomes and plasmids were fully assembled and closed (Table 2a, b). Once polished, the sequences were annotated and manually curated, resulting in complete, high-quality genomes. For QMA0505, 589 and 240 operons were predicted in chromosome 1 and chromosome 2, respectively, with the largest on chromosome 1 (~12 kbp) encoding a type II secretion system between IC627_14670 and IC627_14735, and the largest on chromosome 2 (~15 kbp) encoding a phosphotransferase system (PTS) predicted to be a mannose/fructose/sorbose/N-acetylgalactosamine transport system from IC627_17835 to IC627_17895.
Validation and curation of Nanopore chromosome assemblies

In QMA0505, the alignment of polished PacBio and polished Nanopore assemblies revealed 410 SNPs outside the rRNA and tRNA coding sequences, corresponding to 0.013% in the chromosome 1 assembly. Of these, 45 SNPs could not be resolved due to insufficient Illumina read coverage and 57 were ambiguous. In chromosome 1, polished-PacBio assembly bases were consistent with Illumina data in 239 of the cases (214 deletions, 24 insertions and 1 mismatch compared to the Nanopore assembly), while for Nanopore assemblies it occurred in 69 SNPs (14 deletions, 43 insertions, 12 mismatches). In chromosome 2 of QMA0505, 359 SNPs were found (0.0326%), with 10.2% of unidentified bases and 17.5% of bases lacking Illumina coverage. PacBio base calling was consistent with Illumina data in 40.1% of the SNPs (with 141 deletions and 3 mismatches), while the Nanopore consensus was consistent with Illumina reads in 32.2% of the cases. For chromosome 1 of QMA0506, the number of SNPs outside rRNA and tRNA coding sequences was 882, but only 184 (20.9%) were resolved with Illumina reads. Of these, Illumina reads were consistent in 123 deletions, 3 mismatches and 7 insertions with PacBio assemblies, and in 4 insertions, 3 deletions and 44 mismatches with Nanopore. The remaining 698 SNPs comprised 501 ambiguous bases and 197 were not covered by Illumina reads. For chromosome 2 of QMA0506, 372 SNPs were detected between the PacBio and Nanopore assembly alignments, with 277 uncertain bases and 5 without Illumina coverage. Polished PacBio assemblies were consistent with Illumina base calling in 14.5% of the cases (48 deletions, 4 insertions and 2 mismatches), while for Nanopore assemblies it occurred in 9.7% of the SNPs (2 deletions, 4 insertions, 30 mismatches). The regions that were not resolved by manual curation with Illumina were corrected using PCR and Sanger sequencing.

Curation of the Australian plasmids

MAFFT alignment of QMA0505 and QMA0506 pPHDP10 plasmids allowed the detection of 12 putative SNPs. However, read-mapping of Illumina reads onto the polished Nanopore assemblies and manually calling consensus resulted in identical final sequences for the plasmid from both isolates. For the other plasmids, 46, 966, 18 and 22 putative SNPs were identified between plasmids 1, 2, 3 and 4, respectively. Overall, ~95% of the SNPs were resolved, with the majority being located in homopolymeric regions.

Phylogenetic reconstruction

The phylogenetic analyses of non-recombinant sites of the core genome common to all isolates revealed that Australian P. damselae subsp. piscicida strains are distinct from, but more closely related to, the Japanese and American strains, while the European isolates cluster in a separate clade (Fig. 1). The four closely related strains within the Australia/Asia/USA clade, QMA0505, QMA0506, 91-197 and OT-51443, contain 145 and 72 non-recombinant SNPs in chromosomes 1 and 2, respectively, with the highest proportion of recombination occurring in chromosome 2.
Insertion sequence elements (ISEs), large rearrangements and pseudogenes in *P. damselae* subsp. *piscicida* chromosomes

Both chromosomes belonging to the Australian isolates are larger than the almost complete genome of the Japanese OT-51443 (3 132 149 and 1 010 592 bp) and the fully closed American 91-197 (3 172 118 and 1 054 589 bp). The comparison between the genomes reveals that insertion sequences greatly influence the architecture both of the chromosomes and plasmids, resulting in large-scale rearrangements and functional gene content (Fig. 2a, b).

In 91-197 and OT-51443, various types of ISE account for 1.463 and 1.485% of the coding sequences in chromosome 1, and 14.372 and 14.416% of the coding sequences in chromosome 2, respectively (Table 3). The Australian strains show a similar difference between the two chromosomes, but there is more substantial proliferation of ISEs. In chromosome 1 and 2, respectively, of QMA0505, 9.219 and 22.772% of coding sequences are contained in putative ISEs, representing 6.229 and 17.623% of the chromosome size. In QMA0506, ISEs are 9.245 and 23.019% of the coding sequences, and 6.228 and 17.549% of the chromosomes size. Interestingly, the IS1-like element *ISPda1* family transposases that are the most abundant ISE class in both Australian strains and almost equally represented on both chromosomes were not identified in 91-197 and OT-51443 (Table 3).

As a result of the proliferation of ISEs, the four genomes have undergone a process of general pseudogenization. In chromosome 1 of 91-197, 9.371% of genes have lost their function, while in chromosome 2, the frequency increased to 13.503%. However, the Australian isolates show again a more extensive presence of pseudogenes; in chromosome 1 of QMA0505, 342 out of 3135 (10.909%) genes were inactivated, and in chromosome 2, 246 of 1212 (20.297%) were inactivated.

With respect to the large-scale genetic rearrangements mediated by ISEs, inversion of 22 kbp on chromosome 1 (Fig. 2a, region i) and 15 kbp on chromosome 2 (Fig. 2b, region iv) flanked by ISEs were identified in all four isolates. A ~34 kbp region present in chromosome 1 of both Australian and USA strains is absent in the Japanese strain (IC627_07795 to IC627_07640) (Fig. 2a, region ii). Here, genes encode a set of essential proteins, including a type VI secretion system protein VgrG (IC627_07640), tellurite-resistance protein TerB (IC627_07675), zinc transport protein ZntB (IC627_07680), miniconductance mechanosensitive channel (IC627_07745) and YdcF family protein (IC627_07795). A second, ~60 kbp region of chromosome 1, is unique to the two Australian strains (Fig. 2a, region iii). Located between two tRNA-(ms[2]io[6]A)-hydroxylases (IC627_09455 and IC627_09735), 56 putative coding sequences share highest BLAST identity (94.07%) with *Providencia stuartii* strain AR_0026 (CP026704.1) but only 8% coverage (Table S2). Finally, the largest predicted operon in chromosome 2, the PTS, is also flanked by insertion sequences (IC627_17835 to IC627_17895) (Fig. 2b, region v).

*P. damselae* subsp. *piscicida* plasmids and their insertion sequences

In both Australian strains, the plasmid pPHDP10 comprises 7991 bp with a G+C content of 39.8mol% and contains seven coding sequences: a DNA invertase *hin*, one hypothetical protein, a chorismate mutase, *aip56* and three insertion sequences. The Italian MT1415 strain reference plasmid (DQ069059.1) is 9631 bp and the Spanish L091106-03H is 9736 bp, exceeding the Australian plasmid by more than 1.6 kb and 1.7 kb, respectively. A second distinguishing feature of the Australian and the European pPHDP10 is the inversion that has occurred in the region of *aip56* and the chorismate mutase. The insertion sequence content in the pPHDP10 plasmid is variable amongst strains. In the Australian strains, the oppositely oriented insertion sequences are downstream of the *aip56*/chorismate mutase region, while the Spanish strain contains only one insertion element further downstream. In contrast, the Italian strain MT1415 contains multiple, mostly fragmented, insertion elements on both sides of...
Table 3. Features table of chromosomes 1 and 2 of the Australian strains (QMA0505 and QMA0506), 91-197 and OT-51443

| Feature                             | QMA0505 Chr. 1 | QMA0505 Chr. 2 | QMA0506 Chr. 1 | QMA0506 Chr. 2 | 91-197 Chr. 1 | 91-197 Chr. 2 | OT-51443 Chr. 1 | OT-51443 Chr. 2 |
|-------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Size (base-pairs)                   | 3230241        | 1136236        | 3230122        | 1134735        | 3172118        | 1054589        | 3132149        | 1010592        |
| G+C content (mol%)                  | 41.4           | 39.3           | 41.4           | 39.3           | 41.6           | 39.3           | 41.6           | 39.3           |
| ISEs                                |                |                |                |                |                |                |                |                |
| IS1 family transposase              | 53             | 30             | 55             | 31             | 2              | 152            | 2              | 144            |
| IS1-like element ISPda1 family transposase | 179          | 166            | 177            | 165            | 0              | 0              | 0              | 0              |
| IS3 family transposase              | 13             | 22             | 13             | 21             | 0              | 0              | 0              | 0              |
| IS30 family transposase             | 1              | 0              | 1              | 0              | 0              | 0              | 0              | 0              |
| IS6 family transposase              | 1              | 0              | 1              | 0              | 0              | 0              | 0              | 0              |
| IS9I family transposase             | 27             | 39             | 25             | 37             | 0              | 0              | 0              | 0              |
| IS9I-like element ISPda2 family transposase | 7            | 14             | 9              | 17             | 0              | 0              | 0              | 0              |
| ISNCY family transposase            | 1              | 0              | 1              | 0              | 0              | 0              | 0              | 0              |
| Transposase                         | 5              | 1              | 5              | 1              | 8              | 10             | 8              | 10             |
| Transposase zinc-binding domain-containing protein | 2          | 4              | 2              | 4              | 0              | 0              | 0              | 0              |
| Putative transposase                | 0              | 0              | 0              | 0              | 36             | 53             | 35             | 52             |
| Transposase DDE domain protein      | 0              | 0              | 0              | 0              | 0              | 0              | 1              | 0              |
| Transposon Tn7 transposition protein TnsA | 0          | 0              | 0              | 0              | 1              | 0              | 1              | 0              |
| ISE total length (base-pairs)       | 201226         | 200241         | 201183         | 199136         | 35800          | 78022          | 35638          | 78130          |
| % IS/CDS                            | 9.219          | 22.772         | 9.245          | 23.019         | 1.463          | 14.372         | 1.485          | 14.416         |
| % IS/size                           | 6.229          | 17.623         | 6.228          | 17.549         | 1.129          | 7.398          | 1.138          | 7.731          |
| Pseudogenes                         |                |                |                |                |                |                |                |                |
| No. of pseudogenes                  | 342            | 246            | 326            | 212            | 301            | 202            | NA             | NA             |
| %Pseudogenes/CDS                    | 10.909         | 20.297         | 10.429         | 17.681         | 9.371          | 13.503         | NA             | NA             |

CDS, Coding sequence; IS, insertion sequence; NA, Not annotated
the region. Finally, MT1415 also has a DUF4158 domain-containing protein instead of a complete insertion sequence, and this insertion sequence is absent in L091106-03H (Fig. 3).

In addition to pPHDP10, four other plasmids with highly similar features, but differing in ISEs, were found in both Australian strains (Table 2b). The plasmids bear some similarities with large plasmids found in some overseas isolates.

Both QMA0505 and QMA0506 have the pPHDP70 plasmid, but the Australian strains carry a form that is larger than those found in the Spanish DI21 and the L091106-03H strains. The plasmid is 81,411 bp in QMA0505 and 81,418 bp in QMA0506, while in DI21 it is 68,686 bp and in L091106-03H it is 68,595 bp (Fig. 4). There are also several major rearrangements. Firstly, the pPHDP70 plasmids from Spanish isolates lack a ~5 kbp region in which a fimbrial/pilus periplasmic chaperone and a hypothetical protein are present in the Australian strains (IC628_22550 to IC628_22530 in QMA0506 and IC627_22570 to IC627_22595 in

Fig. 4. Comparative analysis of pPHDP70 plasmids from *P. damsela* subsp. *piscicida* strains DI21, L091106-03H, QMA0505 and QMA0506.
QMA0505). This 5 kbp region has a 47% coverage and 99.59% identity by BLAST with IS91-like element ISPda2 family transposase from *Vibrio toranzonii* (WP_161680561.1). The plasmids also have an inversion between IC627_22690 and IC627_22665, where a ParA family protein (IC627_22695), a ParB/RepB/SpoOJ family partition protein (IC627_22690), a helix-turn-helix domain containing protein (IC627_22685) and the transposases flanking them (IC627_22680 and IC627_22705) are present. A ~3 kbp region containing only a RhsD family protein is present in L091106-03H, while in the other strains this region also includes insertion sequences upstream and downstream of the gene (IC627_22710, IC627_22720, IC628_22600 and IC628_22610). In the Australian strains, a further rearrangement at position 61783 (IC627_22725) to position 69434 (IC627_22760) includes an additional insertion sequence, hypothetical protein and a conjugal transfer protein TraF. Finally, a DNA replication terminus site-binding protein Tus and a DNA-binding protein coding sequence that are both similar to those found in *Vibrio paraohaemolyticus* are present in the Australian strains (99% coverage and 99.31% identity, and 99% coverage and 98.73% identity, respectively).

The largest plasmids in QMA0505 and QMA0506 are 136326 and 136321 bp, respectively. Among the available *P. damselae* subsp. *piscicida* genomes, only the Japanese strain OT-51443 carries a plasmid of a similar size, 145279 bp. All three plasmids encode type III and IV secretion systems, a recombinase family protein, and chaperone proteins Sycd/LcrH family and VscY (Fig. S1).

A plasmid of ~36 kb is highly similar in both QMA0505 and QMA0506, with 17 SNPs out of 18 SNPs in coding regions being as insertion sequences (Fig. S2). The most similar plasmid reported to date is a ~40 kb plasmid found in the Japanese strain OT-51443. In all three plasmids, a type II toxin–antitoxin system HigB family toxin, a type III secretion protein and type IV secretion system proteins are encoded. Moreover, a Hok/Gef family protein and the partition proteins belonging to the ParAB family are present. There is also an approximately 23 kb plasmid that is similar to one found in the Japanese strain OT-51443. The latter is ~25 kb and the difference in size is accounted for by the presence two additional ISEs (Fig. S3).

**Virulence repertoire of Australian *P. damselae* subsp. *piscicida* strains**

Both Australian strains carry the pHDP10 plasmid containing the *aip56* gene (NC_013775.1), encoding the best-characterized virulence factor in *P. damselae* subsp. *piscicida*, as well as plasmid pHDP70 bearing the piscibactin gene cluster. PDP_0080 and p55, potential virulence factors identified to date in overseas strains, were also located in both Australian isolates. While the PDP_0080 gene (HQ599846.1) was annotated on chromosome 1 as a type II secretion system pilot lipoprotein GspS-beta (IC627_08880 and IC628_08855), the p55 genes in QMA0505 (IC627_21025) and in QMA0506 (IC628_18250) were annotated on chromosome 2 as SH3 domain-containing proteins. Furthermore, chromosomes 1 of both isolates contain a transfer-messenger RNA gene *ssrA* (IC627_12340 and IC628_12300) preceded by the SsrA-binding protein SmrB gene.

**DISCUSSION**

**High-quality and cost-effective genome sequences of Australian *P. damselae* subsp. *piscicida***

The combination of long and short reads, obtained from Oxford Nanopore MinION and Illumina technologies, respectively, were at least as accurate as PacBio assemblies polished with the same Illumina data (Table 2a, b), and even more efficient in resolving plasmid sequences. Although PacBio reads gave more accurate sequences at a single nucleotide level, we were unable to complete the assembly of the plasmids present in the Australian strains from these reads, although this may reflect the efficiency of the Blue Pippin size selection prior to the sequencing run (Table 2a). Comparing costs and resources needed for PacBio sequencing, Nanopore has significant time and cost advantages in addition to the ability to complete all steps in a small laboratory, with the only disadvantage being slightly less accurate resolution of homopolymers.

**Information from *P. damselae* subsp. *piscicida* genomes supports a vector-borne *P. damselae* subsp. *piscicida* life-cycle hypothesis**

*P. damselae* subsp. *piscicida* infections in seabass in Egypt coincided with *Caligus elongatus* (sea lice) infestations and the latter agent was hypothesized to serve as a disease transmission vector for *P. damselae* subsp. *piscicida* [34]. Caligid sea lice are also reported on yellowtail in Australia [35]. Indeed, genomes of *P. damselae* subsp. *piscicida* support the idea of an arthropod disease vector. The absence of the urease gene, a feature distinguishing *P. damselae* subsp. *piscicida* from sister subspecies *P. damselae* subsp. *damselae*, is essential for infection of flea vectors by *Yersinia pestis* [36, 37]. The cry8A gene encoding pesticidal protein from *Bacillus thuringiensis*, a subpathogen characterized by continuous mobilome-mediated expansion of invertebrate host range [38], is found in *P. damselae* subsp. *piscicida* chromosome 1 (IC627_12360). Moreover, AIP56, the virulence factor unique to *P. damselae* subsp. *piscicida*, has been intensively studied, in terms of its mechanism of action [39, 40], cellular pathways [41] and other auxiliary molecules involved in host intoxication [42]. However, little is known about its origin. The C-terminal portion of the toxin is similar to a hypothetical protein found in the bacteriophage APSE-2 [5]. This virus infects *Hamiltonella defensa*, a bacterial endosymbiont of some aphids that protects them from parasitoids [43], but APSE is also reported in *Arsenophonus*, another endosymbiont of arthropods but with a wider host range compared with *H. defensa* [44]. It is worth noting that yellowtail
kingfish farmed in Australia have occasional infestations with caligid sea lice and that biting isopods are also observed from time to time.

Support for vector-borne transmission is indirectly provided in the phylogenetic reconstruction of non-recombinant sites that clearly groups Australian strains together with the Japanese and American (Atlantic) strains (Fig. 1). No major surface-water exchanges between the Northern and Southern hemispheres can explain extensive gene flow between Japan, USA and Australia [45], and yellowtail (Seriola quinqueradiata in the Northern hemisphere and Seriola lalandi in Australia), a major host of P. damselae subsp. piscicida, are different species belonging to different populations [46]. They do not undergo exchanges nor migrations, probably due to the hostile environment they encounter close to the equator. Besides, P. damselae subsp. piscicida has a very limited ability to survive outside its host [47]. Also, transmission by vector can sustain both a high-level of horizontal gene transfer and the extremely high virulence that are found in P. damselae subsp. piscicida. Taken together, these support the need for further experimental evaluation to test the hypothesis of a vector-borne lifestyle for P. damselae subsp. piscicida.

**MGEs drive the independent adaptation process in P. damselae subsp. piscicida**

MGEs play a fundamental role in the P. damselae subsp. piscicida genome. Australian P. damselae subsp. piscicida strains carry five plasmids, requiring a substantial allocation of resources for their replication and maintenance. Among them, pPHDP10 and pPHDP70 are key components of the P. damselae subsp. piscicida genome and define its pathogenicity by encoding AIP56 and piscibactin, respectively. The latter is located on a pathogenicity island that closely resembles the Yersinia high-pathogenicity island (HPI) [13]. Pathogenicity islands and plasmids are well known for their ability to severely alter the phenotype and, as a result, the virulence of several bacteria. Some of them are essential to the emergence of novel or highly pathogenic strains and species that stably maintain ‘foreign’ genetic elements in their genomes, such as the type III secretion system in Salmonella pathogenicity island 1 (SPI-1) [48]. Bacteriophages also play a substantial role in the evolution and adaptation of pathogens. The presence of a unique ~60 kbp region between two tRNA-(ms[2]io[6]A)-hydroxylases in QMA0505 may indicate that a phage integration event has taken place. Importantly, their presence has been shown to alter the host phenotype to the point of transforming it to a highly lethal pathogen. For example, in Vibrio cholerae, the two key virulence factors have been acquired by horizontal gene transfer and are encoded on the integrated filamentous phage CTXφ [49, 50]. Interestingly, similarly to AIP56, cholera toxin (CT) is an A-B toxin that is indispensable for Vibrio cholerae virulence. Also, the presence of the Shiga toxin prophage in Shigella dysenteriae determines the onset of severe complications, such as haemolytic uraemic syndrome in humans. Some phages modify Shigella flexneri Y by altering the O-antigen [51], changing the serotype and compromising acquired type-specific immunity. The genomic plasticity of P. damselae subsp. piscicida is highlighted by the considerable number of transposases that frequently flank clusters of related genes. For example, on chromosome 1 of QMA0505, the PTS genes and the flagellar machinery are both enclosed within two insertions sequences. The abundance of insertion sequences, however, also balances the acquisition of novel genes by causing a generalized loss-of-function in other coding sequences, a process that has been elucidated previously in P. damselae subsp. piscicida [52, 53]. The Australian strains clearly follow the trend of genome reduction, but there are differences in term of locations or types of insertion sequences compared with isolates from the USA, Japan and Europe, indicative of independent and localized evolution. The same process of expansion of ISEs and subsequent inactivation of multiple genes is found in many specialized pathogens, including the human pathogens, Yersinia pestis, Salmonella enterica subsp. enterica serovar Typhi, Mycobacterium tuberculosis and Shigella flexneri [54]. A single event appears responsible for the shift from humans to chickens when a subtype of the human Staphylococcus aureus sequence type (ST) 5 lineage in Poland ‘jumped’ to the new host and then spread worldwide via the global poultry value chain [55]. The reductive genome evolution and gene loss that are probably ongoing in P. damselae subsp. piscicida represent a major feature of the host-specialization process in other fish pathogens. The Gram-positive Streptococcus agalactiae includes types such as ST7 and ST283 that infect both human and fish hosts, but other types which have substantially reduced genomes, for example ST260 and ST261, can infect only fish [56, 57]. Similar differences are found between the Gram-negative Edwardsiella tarda and Edwardsiella ictaluri, with the first infecting a variety of animals including humans and the second being restricted to fish hosts, again with a smaller genome [58].

**Potential additional plasmid-borne virulence factors**

The Australian isolates carry the main identified and potential virulence factors of P. damselae subsp. piscicida described to date: AIP56, PDP_0080, p55, SOD and Irp1. Here, we propose two additional virulence factors that may play a fundamental role in P. damselae subsp. piscicida pathogenicity. The pPHDP10 plasmids in Australian strains are considerably smaller than those found in strains from the USA, Europe and Japan. However, the plasmid retains the chorismate mutase gene. This enzyme is part of the shikimate pathway, catalysing an equilibrium reaction that interconverts chorismate and prephenate in the production of phenylalanine and tyrosine [59–61]. In pathogenic yersinias, Yersinia pestis, Yersinia pseudotuberculosis and Yersinia enterocolitica biogroup 1B, the gene irp9 can convert chorismate into salicylate, from which the siderophore yersiniabactin is then synthesized [62]. The pPHDP70 plasmid has a HPI similar to the HPI of Yersinia, where a cluster of irp genes encodes the siderophore piscibactin [13]. Furthermore, pPHDP70, along with pPHDP10, is widely present in P.
**Conclusions**

*P. damselae* subsp. *piscicida* is a highly host-adapted fish pathogen infecting a diversity of species in warm and temperate marine water in the Northern hemisphere, and the high-quality genomes of Southern hemisphere isolates are provided here, to our knowledge, for the first time. The Australian isolates are distinct from Northern hemisphere strains according to non-recombinant core-genome SNP phylogeny, but are more closely related to Japanese and American isolates than their European counterparts. Virulence of *P. damselae* subsp. *piscicida* centres around a cohort of plasmids with rather high conservation, particularly pHDP10 and pHDP70. We were able to resolve the complete cohort of five plasmids in Australian isolates by Nanopore sequencing, coupled with correction using Illumina reads. Both pHDP10 and pHDP70 plasmids are present in Australian strains, they carry the critical AIP56 and Irp virulence factors, but differ from overseas Australian isolates by Nanopore sequencing, coupled with correction using Illumina reads. Both pHDP10 and pHDP70 plasmids are present in Australian strains, they carry the critical AIP56 and Irp virulence factors, but differ from overseas strains in type and orientation of insertion sequences. Evolution of chromosomes and other plasmids are also largely driven by transposable elements. We suggest that, as in other primary pathogens, the ssrA–smpB system may be critical to *P. damselae* subsp. *piscicida* virulence and intracellular survival. Also, we hypothesize that chorismate mutase, universally encoded on pHDP10 immediately upstream of *aip56*, may supply of precursors for siderophore piscibactin biosynthesis, the genes for which are located on pHDP70.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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