Biofilm forming capabilities and protein secretion systems distinguish ecologically diverse lineages of *Xanthomonas* from rice

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Running title:

Ecological and evolutionary genomics of rice associated *Xanthomonas*

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Abstract

Xanthomonas oryzae is a bacterial pathogen that causes a serious disease of rice (RPX: rice pathogenic *Xanthomonas*). However, there are several reports of *Xanthomonas* strains from healthy rice tissues that are reported to be not pathogenic (NPX: non-pathogenic *Xanthomonas*). In the present study we report additional NPX strains and a detailed genomic investigation of ecologically and geographically diverse *Xanthomonas* isolates from rice plant. Phylogenomic analysis revealed two major lineages corresponding to NPX and RPX species. Interestingly, one of the NPX strain is intermediary to both the lineages suggesting on-going diversification and selection. Genealogical analysis indicated that impact of recombination is more for NPX as compared to RPX which is further supported by exclusive presence of a type IV secretion system (T4SS) in the major lineage of NPX. Pangenome analysis revealed large number of lifestyle specific genes with atypical GC content indicating role of horizontal gene transfer in genome diversification. Acquisition of type III secretion system and its effectome along with a type VI secretion system also seem to have played a major role in the pathogenic lineage. Interestingly, known virulence genes encoding cellobiohydrolase and non-fimbrial adhesin *yapH* are exclusive to pathogenic lineage. Higher biofilm forming capability of the strains from pathogenic lineage also clearly distinguishes them from non-pathogenic lineage. The commonality of loci encoding exopolysacharide, *rpf* signalling molecule, iron uptake, xanthomonadin pigment, etc. suggests their essentiality in adaptation to rice plant. Overall our study has uncovered a distinct evolutionary history of pathogenic and non-pathogenic *Xanthomonas* species associated with rice plant.

Keywords: Non-pathogenic, evolution, type secretion systems, biofilm, virulence-related gene clusters, ecology, *Xanthomonas*.

Importance

Two bacterial pathogens belonging to the genus *Xanthomonas* cause foliar diseases of rice. Although there are reports of rice-associated non-pathogenic *Xanthomonas*, so far these counterparts of the pathogen have not drawn much attention. Our detailed and comprehensive genomic investigation has revealed systematic evolutionary diversification corresponding to diverse lifestyles in *Xanthomonas* strains associated with rice. Our study has also revealed genetic features that can be associated with the adaptation of *Xanthomonas* lineages with two diverse life-styles on the rice plant. In particular distinct set protein secretion systems along with their effectome have played critical role in emergence as a successful pathogen of rice from its non-pathogenic counterparts. Interestingly functions required for attachment the host plant and also formation of biofilm seems to have led to emergence of pathogenic species. This knowledge will open up new avenue for better management of pathogenic strains for sustainable cultivation of a major staple food crop.

Introduction

Xanthomonas is a complex group of bacteria that is primarily known for its pathogenic lifestyle causing infection in 125 monocots and 268 dicots (Leyns, De Cleene et al. 1984, Hayward 1993, Vauterin, Yang et al. 1996, Chan and Goodwin 1999) with remarkable host and tissue specificities. Among the 33 species of *Xanthomonas* (Parte 2018), *Xanthomonas oryzae* is solely known to be associated with rice pathogenicity which has further two pathovars on the basis of rice plant tissue infected by them as *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, infecting vascular and parenchyma tissues, respectively (NIÑO-LIU, Ronald et al. 2006). However, there are some reports of other *Xanthomonas* strains found to be associated with healthy rice seeds (Vauterin, Yang et al. 1996, Fang, Lin et al. 2015, Triplett, Verdier et al. 2015). Rice pathogenic *Xanthomonas* (RPX) are widely studied, however, their non-pathogenic counterparts (NPX) isolated from healthy plants are vastly overlooked. These non-pathogenic bacterial populations may be coevolving with the plant and their systematic study might give insights into the evolution of pathogenic counterparts. With the advent of the genomics era and renewed interest in bacterial ecology and evolution, non-pathogenic bacterial strains are now coming in the limelight.

Extensive research is underway to identify the genetic features fundamentally required for pathogenicity and virulence potential of *Xanthomonas*. This work has aided in the identification of virulence genes and pathogenesis-related clusters (Van Sluys, Monteiro-Vitorello et al. 2002, Toth, Pritchard et al. 2006). Protein secretion is a fundamental determining feature for pathogenic or symbiotic interactions as well as for inter-bacterial competition. There are six types of bacterial secretion systems reported till now, out of which five are present in pathogenic *Xanthomonas oryzae* strains (Ray, Rajeshwari et al. 2002, Büttner and Bonas 2010, Souza, Andrade et al. 2011, Pruitt, Schwessinger et al. 2015). *Xanthomonas* sp. undergoes different stages in the infection process such as colonizing leaf surfaces as epiphytes, invasion of the host, etc. On the other hand, the host plant has immune receptors for monitoring their extracellular and intracellular environment for the presence of bacteria. The *rax* gene cluster encodes a T1SS and a secreted sulphated peptide called RaxX which is recognized by rice receptor kinase XA21(Pruitt, Schwessinger et al. 2015). The ability to adhere to the host surface is important for successful infection. Among non-fimbrial adhesins, *xadA, xadB* help in attachment to the leaf surface and *yapH* aids in colonization of bacteria in xylem vessels (Das, Rangaraj et al. 2009). Amongst the fimbrial adhesins, the type IV pilus also reported to help in attachment to the host(Juhas, Crook et al. 2008). *In planta* experiments indicate that *xadA*, *xadB* and *yapH* mutants show deficiency in the initial stages of infection, namely leaf attachment and entry (Das, Rangaraj et al. 2009).

Xanthomonas strains are known to have two representative T2SS gene clusters *xps* and *xcs*. Out of these, *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola are equipped with one (*xps*) and *X. campestris* pv. campestris, *X. axonopodis* pv. citri have both the systems (Szczesny, Jordan et al. 2010). *Xanthomonas oryzae* uses the *xps* system for secreting hydrolytic enzymes to degrade rice cell walls, a process that appears to be crucial for pathogenesis (Jha et al., 2007). Further, the T3SS is one of the key pathogenicity factors in gram-negative bacterial pathogens and is used to inject effectors into the host plant (Ghosh 2004). Another secretion system known as the T4SS is involved in transporting effectors and nucleoprotein complexes to the extracellular milieu or directly into the cytoplasm of other cells (Juhas, Crook et al. 2008). The T4SS is known to be involved in horizontal gene transfer, thus contributing to the genome plasticity and evolution of the bacteria. T4SS is found to be absent among *X. oryzae* strains, though it is present in *X. albelineans, X. citri, X. campestris* and *Stenotrophomonas maltophilia* (Souza, Andrade et al. 2011).T6SS is a recently discovered secretion system described in human pathogens *Pseudomonas aeruginosa* and *Vibrio cholera* (Mougous, Cuff et al. 2006, Pukatzki, Ma et al. 2006). T6SS proteins are structurally and evolutionary related to phage proteins and contribute towards virulence,

symbiosis, inter-bacterial interactions etc. (Sarris, Skandalis et al. 2010, Schwarz, West et al. 2010, Bernal, Llamas et al. 2018).

In response to ambient conditions, bacteria have evolved regulatory systems to regulate expression of pathogenicity related genes. Availability of nutrients, population density, oxygen levels etc. are some of the factors influencing bacterial growth and colonization. The *rpf* gene cluster is involved in synthesis, detection and signal transduction of diffusible signal factor (DSF) (Tang, Liu et al. 1991). In pathogenic *Xanthomonas* strains the DSF is widely known to regulate pathogenicity (Barber, Tang et al. 1997, Wang, He et al. 2004, He, Zhang et al. 2007, Ryan, Fouhy et al. 2007, He and Zhang 2008). External signals are often recognized and transduced via two-component signal transduction systems (Qian, Han et al. 2008). There are > 50 predicted two component systems in *Xanthomonas* but, RavA/RavR, RpfC/RpfG, RaxH/RaxR, ColR/ColS and response regulator HrpG are known to contribute to the virulence (Büttner and Bonas 2010). HrpG perceives environmental signals via an unknown sensor kinase (Wengelnik, Marie et al. 1996, Wengelnik, Van den Ackerveken et al. 1996, Wengelnik, Rossier et al. 1999). However, its expression is controlled by PhoPQ, a two component system and member of GntR regulatory family (Tsuge, Nakayama et al. 2006, Lee, Jeong et al. 2008, Feng, Song et al. 2009). Further, *hrpG* activates *hrpX*, a regulatory gene which further induces HrpG-induced genes (Wengelnik and Bonas 1996, Tsuge, Terashima et al. 2005, Koebnik, Krüger et al. 2006).

Iron uptake is always one of the limiting factors in bacterial growth and is shown to affect virulence in case of animal and plant pathogenic bacteria (Cody and Gross 1987, Meyer, Neely et al. 1996, Bearden, Fetherston et al. 1997, Franza, Mahé et al. 2005, Lawlor, O'Connor et al. 2007). Bacteria encode multiple iron uptake pathways whereas, in iron-limiting conditions, bacteria secrete siderophores (low-molecular weight iron chelators), facilitating ferric ion acquisition. Iron regulated *xssABCDE* (*Xanthomonas* siderophore synthesis) and *xsuA* (*Xanthomonas* siderophore utilization) genes encode proteins involved in biosynthesis and utilization of *X. oryzae* vibrioferrine-type siderophore (Pandey and Sonti 2010). In addition to ferric ions, ferrous iron uptake is carried out via *feo* (ferrous iron transporter) system. Further, the *fur* (ferric uptake regulator) gene product is shown to have a regulatory role in siderophore production (Subramoni, Pandey et al. 2012). Genome-wide expression and genetic and functional studies of *xibR* (*Xanthomonas* iron binding regulator) mutant have revealed that it regulates iron homeostasis in response to changing iron availability in environment (Pandey, Patnana et al. 2016). Further, all species of *Xanthomonas* are widely known to produce copious amounts of extracellular polysaccharide

(EPS) that is known as xanthan gum. In *Xanthomonas* the EPS is encoded by a 16kb *gum* gene cluster, whose mutants have been largely studied for effects on xanthan gum biosynthesis and virulence (Chou, Chou et al. 1997, Katzen, Ferreiro et al. 1998, Dharmapuri and Sonti 1999). Xanthomonadin is a yellow, membrane-bound halogenated, aryl-polyene pigment that is characteristic to *Xanthomonas* group of bacteria (Stephens and Starr 1963, Goel, Rajagopal et al. 2002). This pigment is encoded by the *pig* cluster and provides protection against photooxidative damage (Goel, Rajagopal et al. 2001, Cao, Wang et al. 2018).

A comprehensive genomic investigation of pathogenic *Xanthomonas* with non-pathogenic *Xanthomonas* strains from the same host can help in identifying the functions acquired, lost or modified during adaptation to a particular lifestyle by a bacterial strain. As the genome of a successful pathogen is shaped by genetic, ecological and evolutionary factors, this comparative study may give a clearer picture of the evolution of bacterial pathogenesis on rice in particular and plants in general. Such a study can also help in developing newer methods of disease control and putative genomic markers for proper diagnosis/identification.

Methods

Isolation of rice associated *Xanthomonas*

Healthy rice seeds of basmati variety (Pusa basmati 1121) were collected from farmer's field in Fazilka, Punjab, India. For bacterial isolation, 5g of seeds were partially crushed (almost 80%) in 0.85 % NaCl (normal saline) using sterile mortar and pestle. This heterogeneous mixture was then suspended in 50 ml of the solution (10%; cottyn et al, 2001). Solution was then incubated for 2hrs at 28°C and serial dilutions up to 10^{-6} were plated on Nutrient Agar (NA), Peptone Sucrose Agar (PSA) and Glucose Yeast extract Calcium carbonate Agar (GYCA). Plates were incubated at 28°C upto 6 days. Bacterial colonies were screened for *Xanthomonas* strains based on 16SrRNA sequences. After isolation, cultures were grown and maintained on PSA medium.

In planta pathogenicity tests

5ml of cultures grown upto 0.8 OD were pellet down and dissolved in phosphate buffer saline (PBS). These were used to inoculate 30 days old plants of Pusa basmati 1121. Inoculations were done by cutting edges of leaves with culture dipped scissors.

Genome sequencing, assembly and annotation

Genomic DNA extraction was carried out using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Qualitative assessment of DNA was performed using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis. Quantitative test was performed using Qubit 2.0 fluorometer (Life Technologies). Nextera XT sample preparation kits (Illumina, Inc., San Diego, CA, USA) were used to prepare Illumina paired-end sequencing libraries (250 x 2 read length) with dual indexing adapters. In-house sequencing of the Illumina libraries was carried out on Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). Adapter trimming was performed automatically by MiSeq control software (MCS), and remaining adapters were detected by NCBI server and was removed by manual trimming. Sequencing reads were *de novo* assembled into high quality draft genome on CLC Genomics Workbench v7.5 (CLC bio, Aarhus, Denmark) using default settings. Genome annotation was performed by NCBI PGAP pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok).

Phylogenomic and taxonogenomic analysis

Phylogenomic analysis based on more than 400 putative conserved genes was constructed using PhyloPhlAn (Segata, Börnigen et al. 2013) from whole genome proteome data. Ortholog searching, multiple sequence alignment and phylogenetic construction were done using Usearch v5.2.32 (Edgar 2010), MUSCLE v3.8.31 (Edgar 2004) and FastTree v2.1 (Price, Dehal et al. 2009) respectively. All strains under study were used as an input to PhyloPhlAn. *Stenotrophomonas maltophilia* was used as an outgroup. Taxonogenomic analysis of all the strains carried out using OrthoANI values calculated by using USEARCH (Edgar 2010) for taxonomic status of bacterial species.

Virulence related gene clusters

Various genes clusters related to virulence (supplementary table 1) examined in the present study were retrieved from NCBI. The tBLASTn searches were performed using retrieved sequences as query. Cut-off for similarity was set to be 40% and coverage was 50%. Cluster figures were generated using Easyfig v2.2.2(Sullivan, Petty et al. 2011)and heat maps for the blast results were generated using GENE-Ev3.0.215 (https://www.broadinstitute.org/).

Lineage-wise gene content analysis

Lineage-wise core genes were fetched from gene profiling done by roary pan genome pipeline (Page, Cummins et al. 2015). Since, strains we were dealing with were from three

different species (*X. oryzae*, *X. maliensis* and *Xanthomonas* sp.), pan genome analysis was performed using identity cut-off of 60%. Further, GC content of genes were calculated and genes with atypical GC content were identified (GC content not in range of 64.5 +-2%). Functional analysis of the lineage specific core genes was performed using EggNOG, PROKKA and GenBank annotation was also considered.

Biofilm assay

Cultures were grown in NB (Nutrient broth) media at 28℃ at 180 rpm until OD at 600nm reaches 0.5. Cultures were then diluted 1:10 with NB media containing 2% glucose. Then, 1ml of diluted culture was dispensed into borosilicate glass tubes. Test tubes were then incubated at 28℃ for 7 days. After 7 days, media was discarded and non-adherent cells were removed by washing three times with sterile water. Quantification of biofilm formation was assessed by crystal violet (CV) staining. Briefly, biofilms were fixed at 60℃ for 20 min, and stained with 1.5ml of 0.1% CV for 45 min. The dye was discarded, and the plate was rinsed in standing sterile water and then allowed to dry for 30 min at RT. Stained biofilms were dissolved in 1.5ml of ethanol: acetone (80:20) and finally OD was read at 595nm using spectrophotometer.

Recombination and mutation analysis

Impact of recombination and mutations was evaluated among the strains under study using ClonalFrameML analysis. Core genome alignment was performed by MAUVE v2.3.1(Darling, Mau et al. 2004) and it was further used to generate maximum likelihood tree by PhyML 3.1(Guindon, Dufayard et al. 2010).These alignment and tree were further subjected to ClonalFrameML analysis (Didelot and Wilson 2015) to calculate frequency and impact of recombination and mutation.

Results

Strain isolation and pathogenicity tests

We were able to isolate three strains (PPL1, PPL2 and PPL3) belonging to genus *Xanthomonas* from healthy rice seeds. Further, their pathogenicity was checked by leaf clip inoculation studies. Pathogenic strain *Xanthomonas oryzae* pv. oryzae BXO1 was used as a positive control and PBS (phosphate buffered saline) was used as control for wounding. At 14 days post inoculation (dpi), leaves infected with BXO1 showed lesion of 13 cm length on an average of 10 leaves taken into account, however, PPL1, PPL2, PPL3 showed 0.61 cm,

0.29 cm and 0.8 cm lesions were respectively. Negative control (PBS) was having wound symptoms that measured 0.43 cm of (figure 1).

Additionally, five other rice associated *Xanthomonas* strains (LMG27592 (Africa), LMG12459, LMG12460, LMG12461 (China) and LMG12462 (Antunes, Machado et al.) were shortlisted for analysis due to the reports on their non-pathogenic behavior towards rice (Vauterin, Yang et al. 1996). These were procured from the Belgian Co-ordinated Collections of Micro-organisms/ Laboratory of Microbiology Gent Bacteria Collection (BCCM/LMG).

In-house whole genome sequencing and assembly

Whole genome sequencing of eight NPX isolates was carried out on in-house Illumina MiSeq platform. The high quality de novo assembly of the Illumina reads resulted in genomes with coverage ranging from 78x to 203x and with N50 values of 32.9 kb to 77.6 kb (table 1). The genome size for all the NPX strains was found to be approximately 5 Mb, which is similar to the pathogenic *Xanthomonas* genus, suggesting no large-scale reductive evolution in these non pathogenic strains. Further, genome sequences of four additional NPX strains available on NCBI GenBank database were retrieved. These strains are reported to be isolated from surface-sterilized rice seeds (SHU166, SHU199, SHU308 (USA) and *X. sacchari* R1 (China). However, pathogenicity status of these isolates is not reported yet.

Phylogenomic analysis of the RPX and NPX isolates

Phylogenomic relationship among RPX and NPX isolates was evaluated at the whole genome level (figure 2). Analysis of more than 400 conserved genes resulted in a highly resolved phylogenomic tree. Based on a previous study from our group, there are five distinct lineages of Asian *Xanthomonas oryzae* pv. oryzae strains (Midha, Bansal et al. 2017). We have selected one representative strain from each of the lineages (BXO1, IXO222, XOOP, XOOK and IXO599 from L-I, L-II, L-III, L-IV and L-V respectively for the analysis. Besides, we also included representative strains of different geographic locations (BXO1 from India; AXO1947, CIX44 from Africa; PXO86 from the Philippines; X8-1A from US; YM15 from China and CFBP2286 from Malaysia). In addition, we have also included one *X. oryzae* pv. oryzicola strain (BLS256). Further, since one of the strains among the NPX (*X. sacchari* R1) was classified in *X. sacchari*, type strains of *X. sacchari* and its closest relative *X. albilineans* were also included in the analysis (López, Lopez-Soriano et al. 2018). For phylogenetic construction, *Stenotrophomonas maltophilia* ATCC13637 (T) was taken as an outgroup.

Phylogenetic analysis clearly depicts that all the NPX isolates formed a distinct major lineage I (ML-I) while the RPX are forming another major lineage II (ML-II). Interestingly, one of the NPX isolates, *X. maliensis*, did not cluster in ML-I and was an outlier of ML-II which consists of RPX (figure 2).

Taxonogenomic status of rice seed associated strains

To assess the taxonomy of the NPX, we looked for the 16S rRNA identity of the isolates. All the isolates (except *X. maliensis)* had identity value of 99.59% with *X. sacchari* CFBP4641 (T) and 98.50% with *X. albilineans* CFBP2523 (T). Further, to explicitly assess taxonomic status of ML-I and *X. maliensis* strains, we used OrthoANI values, to define species status (figure 3). The type strains of *X. sacchari* and *X. albilineans* were also included in this analysis. OrthoANI uses species delineation cut off of 96% similarity (Richter and Rosselló-Móra 2009). All ML-I isolates were having ANI values of around 93% with *X. sacchari* CFBP4641 (T) and around 83% with *X. albilineans* CFBP2523 (T), hence, they comprise a putative novel species *Xanthomonas sp.* Further, *X. maliensis*is already reported as a distinct species (Triplett, Verdier et al. 2015). Thus it can be concluded that, all of the rice associated strains were not belonging to *X. oryzae*. They belong to three different species i.e. *X. oryzae* (ML-II), *X. maliensis* and putative novel species *Xanthomonas* sp. (ML-I)

Recombination and mutation impact on lifestyle of bacteria

Recombination and mutations are major driving forces of bacterial evolution. To measure their impact on the major lineage strains (ML-I and ML-II), we carried out ClonalFrameML analysis. Interestingly, the output suggests that recombinational events are not very frequent amongst these clades of *Xanthomonas* strains. There is occurrence of around 5 and 12 mutational events for each recombinational event in ML-I and ML-II respectively. However, even though there is low occurrence, recombination has more impact (r/m = 1.233) towards the evolution of strains in ML-I. But, this scenario is not seen in the pathogenic strains, where the impact of recombination is only half that of mutation (r/m = 0.54) (table 2).

Distinct gene content among RPX and NPX

To evaluate the gene-content wise relatedness of strains under study, we focused on their core and pan-genome content (figure 4). Here, core was least (1039) and pan was maximum (17,221) for all strains (including *Stenotrophomonas maltophilia*). Whereas, for all *Xanthomonas* strains (excluding *S. maltophilia*) under consideration, core increased (1291) and pan decreased (14231). Core and pan values were (1314, 13385) for ML-I and ML-II strains taken together. Interestingly, core values increased and pan values decreased

dramatically, when ML-I and ML-II strains were considered separately (2224, 6766; 2686, 9415). Interestingly, when *X. maliensis* was included in ML-I, the core decreased and pan increased (1626, 8357), whereas inclusion of *X. maliensis* did not have such an effect on ML-II (2391, 10601). Gene content wise analysis also emphasized that although a non-pathogenic isolate, gene content wise *X. maliensis* is more related to ML-II (pathogenic lineage).

Whether differences in lifestyles of the bacterial isolates are also reflected in the gene content? To address this, we carried out pangenome analysis in two sets. Set one was to find genes unique to RPX and NPX strains and set two was for genes unique to ML-I and ML-II (i.e. excluding *X. maliensis*). Set one will give us distinct gene content present in all pathogenic (Unique RPX, n = 195) or non-pathogenic (Unique NPX, n = 133) isolates. However, *X. maliensis* is phylogenomically more closely related to pathogenic ones, so it may lead to biasness in the analysis, hence, gene content exclusively present in ML-I (Unique ML-I, n = 686) (i.e. non-pathogenic isolates, but excluding *X. maliensis*) and unique to ML-II (Unique ML-II, n = 583), i.e. present in pathogenic isolates were also considered. Interestingly, as soon as *X. maliensis*is excluded from the analysis, number of unique genes increased by approximately three-fold.

Typical GC content for *Xanthomonas* sp. is within 64.5%+-2.5% range. Among these, the unique RPX and unique ML-II, 47% and 42% of genes respectively, had atypical GC content respectively, whereas, in case of unique NPX and unique ML-I it was 87% and 86% respectively. Interestingly, genes with atypical GC content among unique NPX or ML-I, all were having GC in higher range (>67%) whereas amongst the unique RPX or ML-II atypical GC genes had GC in both high and low GC ranges (<62% and >67%). Further, functional analysis of these genes was carried out. Among 25 COG classes, our data was represented in 17 classes while approx. 50% were having unknown function or hypothetical proteins (Figure 5). Overall, genes related to metabolism (such as carbohydrate, lipid, inorganic ion and secondary metabolites metabolism biosynthesis and transport) and information storage and processing such as transcription were more in non-pathogenic as compared to pathogenic. While, genes unique to the pathogenic pool were more of cell wall/membrane/envelope biogenesis, motility related or intracellular trafficking, secretion and vesicular transport related genes. Whereas, genes related to amino acid and nucleotide transport and metabolism; replication, recombination and repair etc. were comparable among both the pathogenic and non-pathogenic strains.

Further inspection into the pangenome results were carried out by looking for the annotation of the genes unique to different sets of strains described above. Interestingly, unique RPX and

ML-II genes for strains following pathogenic lifestyles showed presence of genes related to various types of secretion systems, PhoPQ-activated pathogenicity-related protein, etc. Strikingly, pan-genome analysis depicted that biofilm biosynthesis cluster (*pgaABCD*) is absent from NPX except for *X. maliensis* but is present in RPX. Further, this was also confirmed experimentally by biofilm analysis (figure 6). The strains from pathogenic lineage display higher biofilm abilities than strains from non-pathogenic lineage, except *X. maliensis*.

Pathogenicity gene(s) and gene cluster(s) in rice associated *Xanthomonas*

Xanthomonas is a model phytopathogen and numerous genetic studies have identified major pathogenicity related gene(s) and gene clusters (Büttner and Bonas 2010). We proceeded to examine the status of various well known *Xanthomonas* pathogenicity genes and gene clusters (supplementary table 1) amongst the three *Xanthomonas* species associated with rice. First, we focused on all the protein secretion systems, effectors produced by them and their regulators which could be playing a critical role in their interaction with the host (figure 7(a), (b)). Interestingly, T1SS *raxX*, *raxST*, *raxA* showed variable or complete absence in non-pathogenic isolates, however, they were present in the pathogenic isolates. A single T2SS i.e. *xps*, is present in pathogenic and non-pathogenic strains, whereas *X. maliensis* has both of the secretion systems (*xps* and *xcs*).Type II system secreted effectors or cell wall degrading enzymes which are well studied for *Xanthomonas* were examined in this study were cellulases (*cbsA*, *clsA*, *cel8A*, *cel9A*); xylanases *(xynA*, *xyn10A*, *xyn5A*, *xyn51A*,*xynB*, *xyn10B*, *xyn10C*); pectate lyases (*pel10A*, *pel1A*, *pel1B*, *pel1C*, *pel3A*, *pel4A*, *pel9A*); lipase (*lipA*) and *aguA* and *pglA* (Dow, Scofield et al. 1987, Ray, Rajeshwari et al. 2000, Rajeshwari, Jha et al. 2005, Potnis, Krasileva et al. 2011). We could observe diversity in the repertoire of cell wall degrading enzymes among RPX and NPX isolates. Among the cellulases, *cbsA* is present only in pathogenic (except for *X. oryzae* pv. oryzicola CFBP2286, where it has a frameshift mutation) strains and was completely lacking in all the NPX isolates. Interestingly, *cel8A* and *cel9A* were in all rice non-pathogenic and only present in non-pathogenic strains (except for absence of *cel8A* in *X. maliensis*) and *clsA* is present in all isolates. However, xylanases and pectate lyases showed variations in their repertoire among pathogenic and non-pathogenic isolates. Among xylanases *xynA*, *xyn10A*, *xynB* and *xyn10B* are present in pathogenic and *X. maliensis*; while *xyn10C* is present only in *X. maliensis*. The xylanases *xyn5A* and *xyn51A* are present in all the isolates (except for absence of *xyn5A* inCFBP2286, CIX44 and YM15). Further, *aguA* and *lipA* genes are present in all the isolates and *pglA* is present in ML-II and *X. maliensis*. In case of pectate lyases, *pel9A* is present in pathogenic and *X. maliensis* except for PXO86 and *pel1B* was present in all NPX strains except for *X. sacchari* R1. While, *pel1A*

and *pel4A* are present in all the strains (except for absence of *pel1A* in X8-1A) and *pel3A*, *pel10A* and *pel1C* are not present in any of the strains under study. T3SS and its effectors are present in pathogenic strains (ML-II) and absent from all non-pathogenic strains (ML-I and *X. maliensis*). While T4SS is only present in ML-I isolates. Among adhesins (type V effectors), *yapH* is only present in pathogenic isolates, however, *xadA*, *xadB* and *pilQ* are present among all the isolates.

We also scanned for the presence of genes encoding virulence regulators (figure 7 (c)). The *rpf* cluster, is present in both groups of bacteria, although non-pathogenic isolates were having a diversified *rpf* cluster as compared to pathogenic isolates. Similarly, we looked for other regulators of virulence, most of them were present in all the isolates under study. However, *hrpG*, *hrpX* global regulators are present in ML-II and *X. maliensis* and absent in ML-I strains. Other two component systems viz., RavA/RavR, RpfC/RpfG, RaxH/RaxR and ColR/ColS are present in all the isolates.

Iron uptake (ferric and ferrous) in response to changing iron availability in environment is mediated via various two component systems, siderophores and *xibR*. Here, all strains showed presence of genes responsible for this, with diversified genes in ML-I lineage, except for *xssA*, which is present only in ML-II and *X. maliensis*. Further, genes responsible for EPS production (*gum* cluster), yellow pigment production (xanthomonadin pigment) and flagellar glycosylation (*gigX* cluster) are present in all the isolates.

Discussion

Distinct evolutionary trajectories in rice associated *Xanthomonas*

Genome based studies are allowing researchers to obtain detailed and comprehensive insights into bacterial evolution. Even though they are of diverse geographic origin, it can be clearly concluded from the present whole genome based analysis that large numbers of rice associated *Xanthomonas* strains (both RPX and NPX) formed two distinct major lineages (ML-I and ML-II) associated with diverse lifestyles. Interestingly, according to the genome based taxonomic and phylogenetic analysis, NPX (except *X. maliensis)* belongs to a putative novel species, whose formal proposal is under progress. Further, *X. sacchari* R1 which was previously classified into *X. sacchari*, is now shown to be misclassified. It is very interesting that NPX strains from diverse geographic areas are clustering together. This indicates that the NPX strains are not random associations with rice and suggest that they represent genuine adaptations of *Xanthomonas* to a non-pathogenic lifestyle on this host.

In addition to these two rice associated species, *X. maliensis* is more related to pathogenic lineage (ML-II) eventhough its reported to haves non-pathogenic lifestyle (Triplett, Verdier et al. 2015). This points out that this species has emerged independently to that of the non-pathogenic *Xanthomonas* sp. in close association with rice plant. The phylogenetic tree suggests that the NPX and RPX lineages diverged a long time ago and belong to two distinct species as seen by ANI values among them. Phylogenetic clustering of RPX and NPX isolates also suggests a distinct rate and pattern of divergence taking place in these two sets of isolates and this is further emphasised by higher recombination rates in NPX than in RPX isolates.

Genome based studies not only allow us to understand bacterial diversity at the sequence level but also make it possible to compare the gene content of pathogenic and non-pathogenic isolates. This can provide insights into evolution of different lifestyles of the bacteria from a common ancestor. Pangenome analysis supports distinct evolutionary trajectories led by pathogenic and non-pathogenic isolates by revealing gene-content differences among them. As a pathogenic group, their genomes are expected to be dynamic as seen by relatively higher pan-genome size of rice pathogenic species. Core-pan gene analysis also confirmed that *X. maliensis*is more related to ML-II and not to ML-I. Strikingly, a number of unique genes are shared by *X. maliensis* and RPX, which are not present in other non-pathogenic isolates (ML-I), reaffirming the closer relationship of *X. maliensis* with RPX. Interestingly, pangenome for both the major lineages were open, while, atypical GC content clearly indicated independent acquisition of unique genes in pathogenic and non-pathogenic bacteria from different sources. At the same time, large numbers of unique genes with unknown functions in both the groups highlight the need for more functional genomics and genetic studies for a detailed understanding of both the pathogenic and non-pathogenic lineages.

Interestingly, pangenome allowed us to identify gene cluster for biofilm biosynthesis (*pgaABCD*) in all the strains of the pathogenic lineage, suggesting importance of its function on evolution and emergence in pathogenic lineage of *Xanthomonas* in rice. Absence of this gene cluster in all non-pathogenic strains except *X. maliensis* reiterate that acquisition of the gene cluster has played pivotal role in success of pathogenic species. Hence comparative genomic strategies have potential to identify novel genomic loci and functions associated with pathogenic life-style and also the potential to target this function in management of pathogenic lineage.

Xanthomonas is a model pathogenic bacterial pathogen to understand molecular aspects of host-pathogen interactions, virulence and pathogenicity (Lu, Patil et al. 2008, Büttner and

Bonas 2010, Jacques, Arlat et al. 2016). Apart from pan-genome analysis, the present study has provided novel insights into the status and evolution of well characterized pathogenicity associated genes/gene clusters in two evolutionarily distinct and ecologically related isolates having diverse host-microbe interactions (pathogenic and non-pathogenic) (Price, Dehal et al.) (figure 8). For example, the hallmark of pathogenicity of *Xanthomonas* is T3SS and its effectors which are well known to be present in RPX strains (White, Potnis et al. 2009) are interestingly not present in any of the non-pathogenic strains. Hence, T3SS and its effectors seem to have been acquired by the ancestor of the pathogenic lineage (ML-II) after the divergence of pathogenic and non-pathogenic lineages and have played a primary role in their emergence and pathogenicity.

Interestingly, HrpG, HrpX regulons known to be critical for pathogenicity of *Xanthomonas* are present in all RPX strains and even in *X. maliensis* as well, but are not present in ML-I. It is consistent with previous reports of their importance for pathogenicity and it was acquired by common ancestor of pathogenic and *X. maliensis*, i.e. after diversion of ML-I during the evolution. Similarly presence of T6SS in RPX but absence in ML1 suggests its importance in success of pathogenic *Xanthomonas* lineage. Conversely, presence of T4SS in ML1 suggests is importance in emergence and success of major lineage of NPX strains. This is in accordance with the higher rate of recombination in ML-I as compared to ML-II, as T4SS is involved in bacterial conjugation and acquiring nucleoprotein complexes by the bacteria. In this case, it seems to be acquired by ancestor of ML-I after divergence of ML-II and *X. maliensis*.

On the other hand, among the two type II secretion systems (*xps* and *xcs*), RPX and all the NPX isolates were having *xps*. Presence of an additional non-canonical T2SS *xps* cluster in *X. maliensis* but absence in all other strains indicate on-going dynamics and selection within these species ~~(Szczesny, Jordan et al. 2010)~~. Further, cell wall degrading enzymes (secreted by T2SS) are also present in all the strains under study, though with differential repertoires. Conservation of T2SS and most of the effectors where we further see redundancy suggests that they were present in ancestor of both the lineages and were acquired for plant adaptation. The exceptions are *cbsA* and *cel9A* genes encoding cellobiosidase and cellulase and are exclusively present in pathogenic and non-pathogenic strains respectively suggesting their specific acquisition by RPX and NPX lineages. In an earlier study, cellobiosidase gene is reported to be a major virulence factor (Jha, Rajeshwari et al. 2007).

Apart from protein secretion systems, adhesion to the host is the most crucial step for bacteria irrespective of the lifestyle followed. However, redundancy is reported in this case as well as,

mutations in majority of these genes do not completely abolish virulence (Das, Rangaraj et al. 2009). However, in the present study, a distinct gene profile for adhesions was observed with RPX isolates showed more adhesins than NPX isolates suggesting their importance in origin and evolution of the pathogenic lifestyle. Interestingly, mutation in *yapH* encoding gene abolished virulence of *Xanthomonas* due to deficiency in leaf attachment and entry (Das, Rangaraj et al. 2009). Exclusive presence of *yapH* in RPX suggests its importance in emergence of pathogenic strains of *Xanthomonas* in rice. Conservation of EPS biosynthetic pathway, siderophore biosynthetic gene clusters, iron uptake genes and regulatory genes (*xibR*) and other well-known genes like *rpf* cluster, several two component systems for mediating environmental signals and *gigX* cluster for flagellar glycosylation (Yu, Chen et al. 2018) are present in all the rice associated *Xanthomonas* point to their primary role in plant adaptation per se. At the same time, this study also allowed us to track genes that are implicated in virulence but are under reductive evolution in NPX. For example, the type I secretion system related *raxX* cluster that encodes a peptide recognized by Xa21 rice resistance gene is present in all RPX strains but seem to be under reductive evolution in non-pathogenic strains. It indicates that strategies once suitable for adaptation which have been hijacked or exploited by pathogenic counterparts but are being lost from the non-pathogenic counterparts. Hence such plant adaptation genes which are now evolved to be virulence genes can be important candidates for genetic studies of pathogenic isolates of rice.

It is interesting to note that *X. maliensis* is a non-pathogenic strain lacking T3SS and its effectors, but on the other hand, it is having T6SS, biofilm formation cluster (*pgaABCD)* and *raxX, raxST, rasA* genes of T1SS and does not have T4SS all of which are features associated with the pathogenic strains. Furthermore, it is found to be phylogenomically more related to pathogenic than to non-pathogenic isolates. Hence, this might be representing a second wave of evolution of non-pathogenic isolates, which remain non-pathogenic but have some of the genes which are associated with the pathogenic lineage. What do these intermediate strains represent? Do they represent variations of a non-pathogenic lifestyle that benefit from acquisition of genes that are normally present in pathogenic strains? Alternatively, could they represent non-pathogenic strains that have set off on the path towards pathogenicity? Further studies on these kinds of intermediate strains as well as the non-pathogenic strains can lead to a better understanding of the mechanisms of adaptation of the genus *Xanthomonas* on plants.

Conclusion:

Although the genus *Xanthomonas* has evolved as highly successful plant pathogens, this study highlights the scope and possibilities for detailed comparatives studies on both

pathogenic and non-pathogenic *Xanthomonas* strains that are associated with a particular plant. A small core genome shared amongst all rice associated *Xanthomonas* strains and non-uniformity of various horizontally acquired gene clusters suggest a non-recent divergence of these lineages and occurrence of various events of gene gain and gene loss amongst these rice associated bacteria. Interestingly the study has revealed many well characterized gene clusters that are common to both life-styles which are indicative of a role for these functions in plant adaptation. Moreover, the study has revealed many novel and interesting gene(s) that are unique to each of the groups. These can be promising material for researchers engaged in genetic and molecular studies to understand *Xanthomonas* biology in particular and pathogenesis in general.

Author Contributions

SM performed strain isolation, identification and genome sequencing was performed by SM, SK and KB. KB performed phylogenomic and comparative analysis. KB and SK have performed virulence gene cluster analysis. AK performed biofilm assay. KB have drafted manuscript with inputs from SK, SM and RVS and PBP. PBP conceived the study and participated in its design with KB, SM and RVS. All the authors have read the manuscripts and approved the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Legends

Figure 1: *In planta* infected leaves of rice (Pusa Basmati 1121) (a) leaves showing symptoms after 14 dpi (b) lesion length (cm) for positive (BXO1), negative (PBS) control and PPL1, PPL2 and PPL3 strains.

Figure 2:ML tree based on >400 conserved genes of all RPX (in red) and NPX (in green) strains constructed using PhylPhlAn. *Stenotrophomonas maltophilia* ATCC13637 (T) was used as outgroup and other type strains are designated as (T).

Figure 3: OrthoANI of the RPX and NPX isolates including type strains of *Xanthomonas sacchari* CFBP4641 (T) and *Xanthomonas albilineans* CFBP2523 (T).

Figure 4: Core and pan genome for different combinations of strains indicated along x-axis.

Figure 5: COG functional classification of unique genes to NPX, RPX, ML-I and ML-II.

Figure 6: Biofilm assay: Ability of strains to form biofilms in borosilicate glass tubes determined using crystal violet staining. Here, ML-I, *X. maliensis* and ML-II are represented in green, yellow and red boxes.

Figure 7: Distribution of pathogenicity related gene(s) and gene cluster(s) among ML-II (in red box), ML-I (in green box) and *X. maliensis* (in yellow box). Different colors are corresponding to different clusters and the intensity of the color indicates level of identity with the query as depicted by the scale.

Figure 8: Pattern of acquisition or loss of pathogenicity related clusters among RPX (in red box) and NPX (in green box).

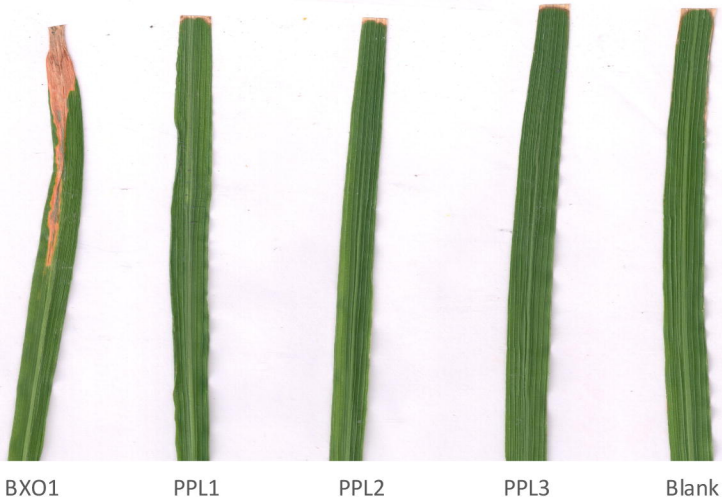
Tables:

Table 1: Genome statistics of genomes sequenced in the present study

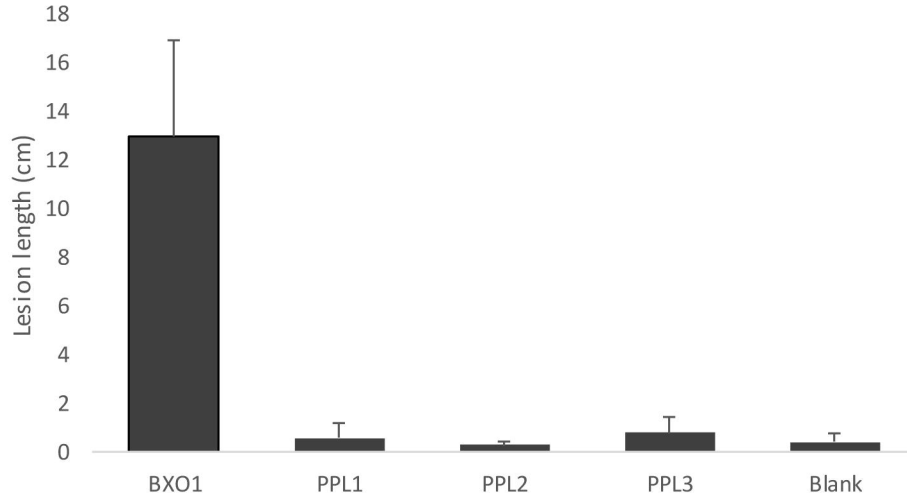
S. N.	Strain Name	Completeness/ Contamination	Fold (X)	N50 (kb)	Contigs	Genome Size	GC (%)	CDS	rRNA + tRNA	Isolation source	Accession No.
1	*X. sacchari* PPL1	96.31/0.22	78	32.9	332	4.8	69	4149	3+51	Rice seeds	NQYO
2	*X. sacchari* PPL2	95.79/0.07	109	48.1	200	4.9	68.8	4105	2+50	Rice seeds	NQYP
3	*X. sacchari* PPL3	98.56/0.55	80	40.3	231	4.8	68.8	4106	3+52	Rice seeds	NMPO
4	*X. maliensis* LMG27592	96.74/0.30	155	77.5	141	5.2	66.1	4369	3+53	Rice seeds	NSET
5	*Xanthomona*sp. LMG12459	98.17/0.86	184	59.3	143	4.8	68.8	4055	3+51	Rice seeds	NSGT
6	*Xanthomona*sp. LMG12460	96.58/0.00	180	50.7	178	4.7	69	3934	4+51	Rice seeds	NKUH
7	*Xanthomona*sp. LMG12461	98.76/0.64	202	71.8	134	4.8	68.9	4060	3+51	Rice seeds	NQYT
8	*Xanthomona*sp. LMG12462	97.64/0.52	176	76	136	4.8	68.9	4062	3+52	Rice seeds	NMPP

Table 2: ClonalFrameML output parameters for ML-I and ML-II.

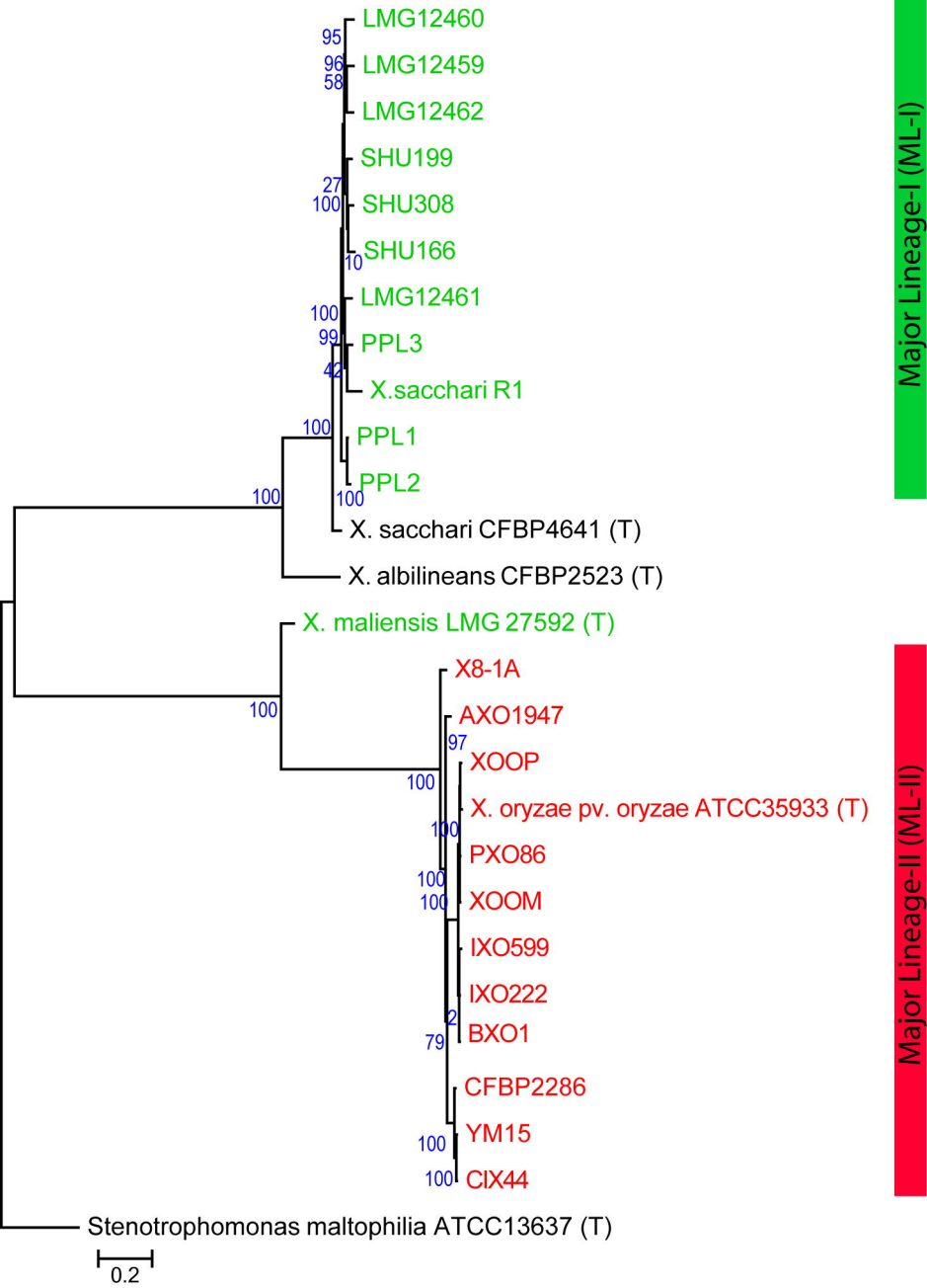
	Ratio of rates of recombination v/s mutation (R/theta)	**Relative impact of recombination v/s mutation(r/m)**
ML-I	0.195	1.233
ML-II	0.083	0.54

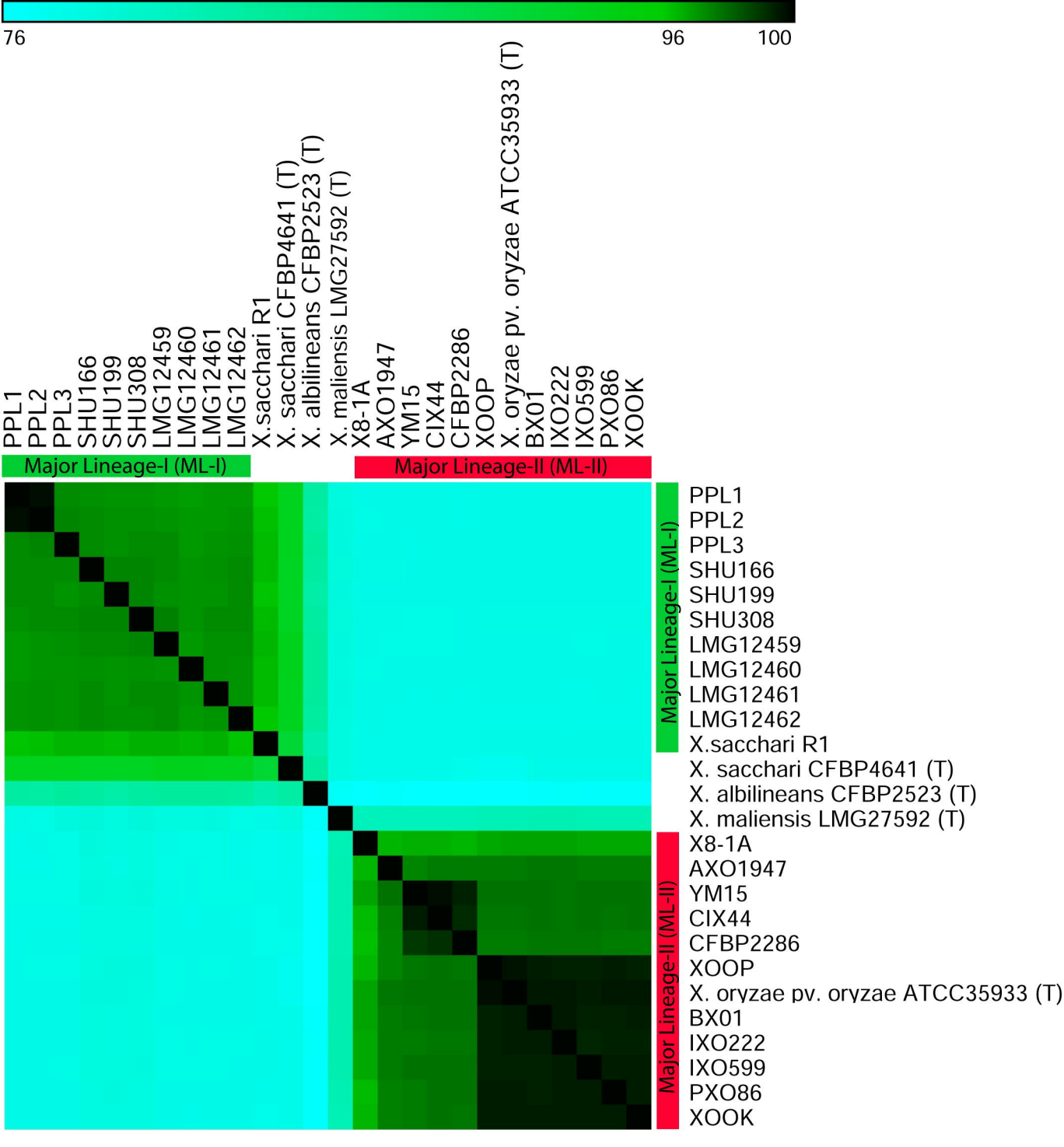

BXO1
PPL1
PPL2
PPL3
Blank

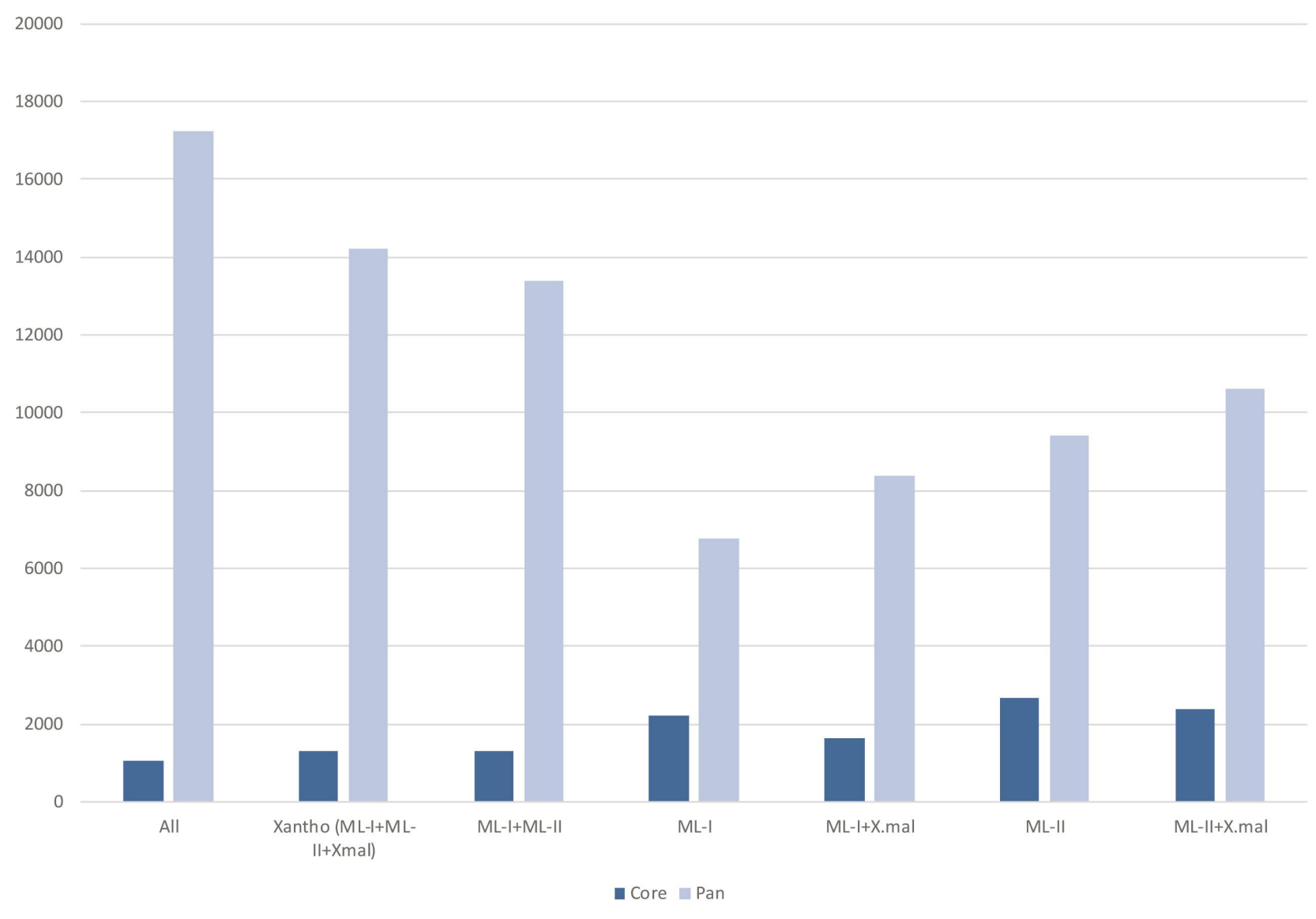
(A)

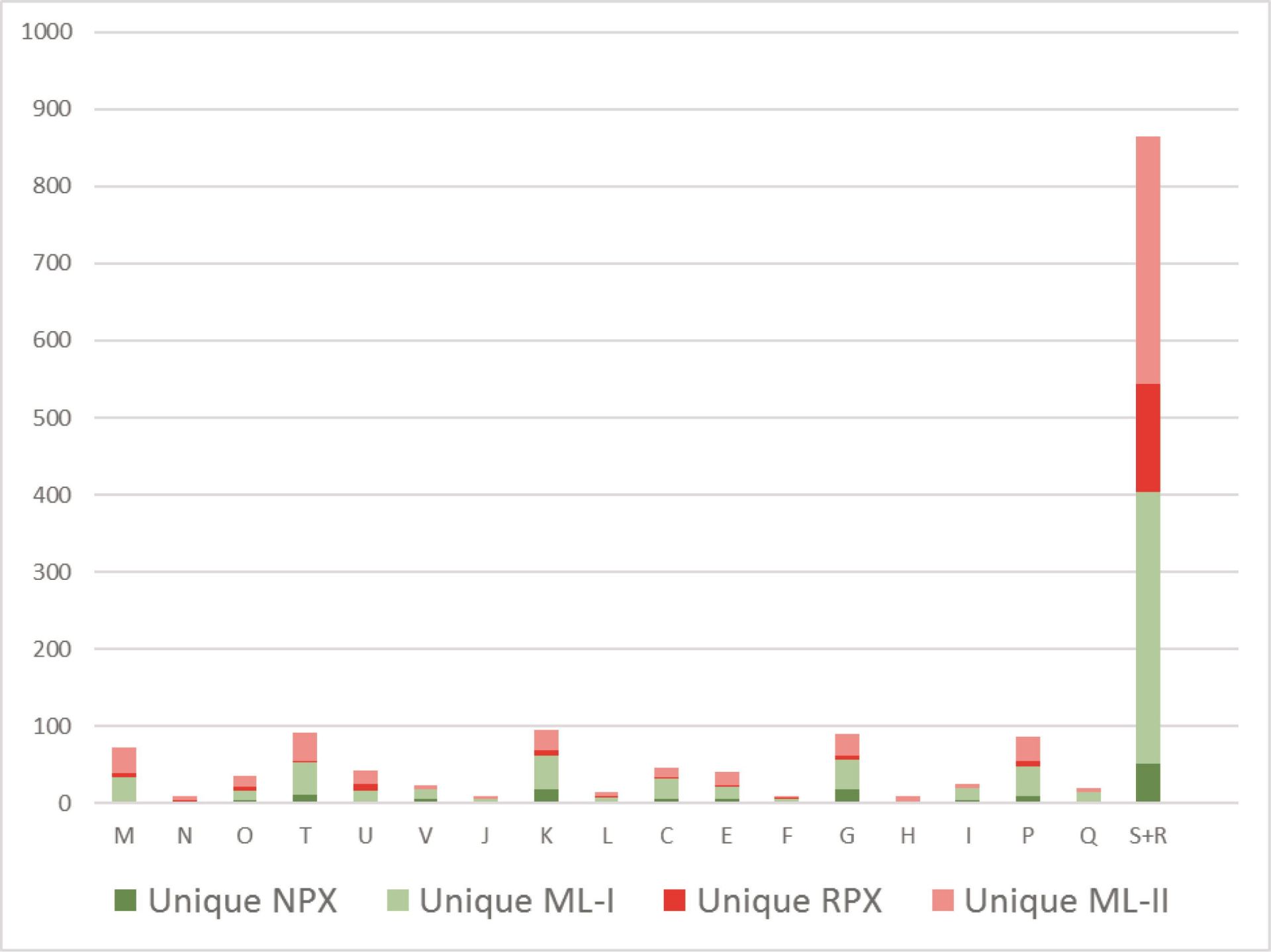

Lesion length (cm)
0
2
4
6
8
10
12
14
16
18
BXO1
PPL1
PPL2
PPL3
Blank

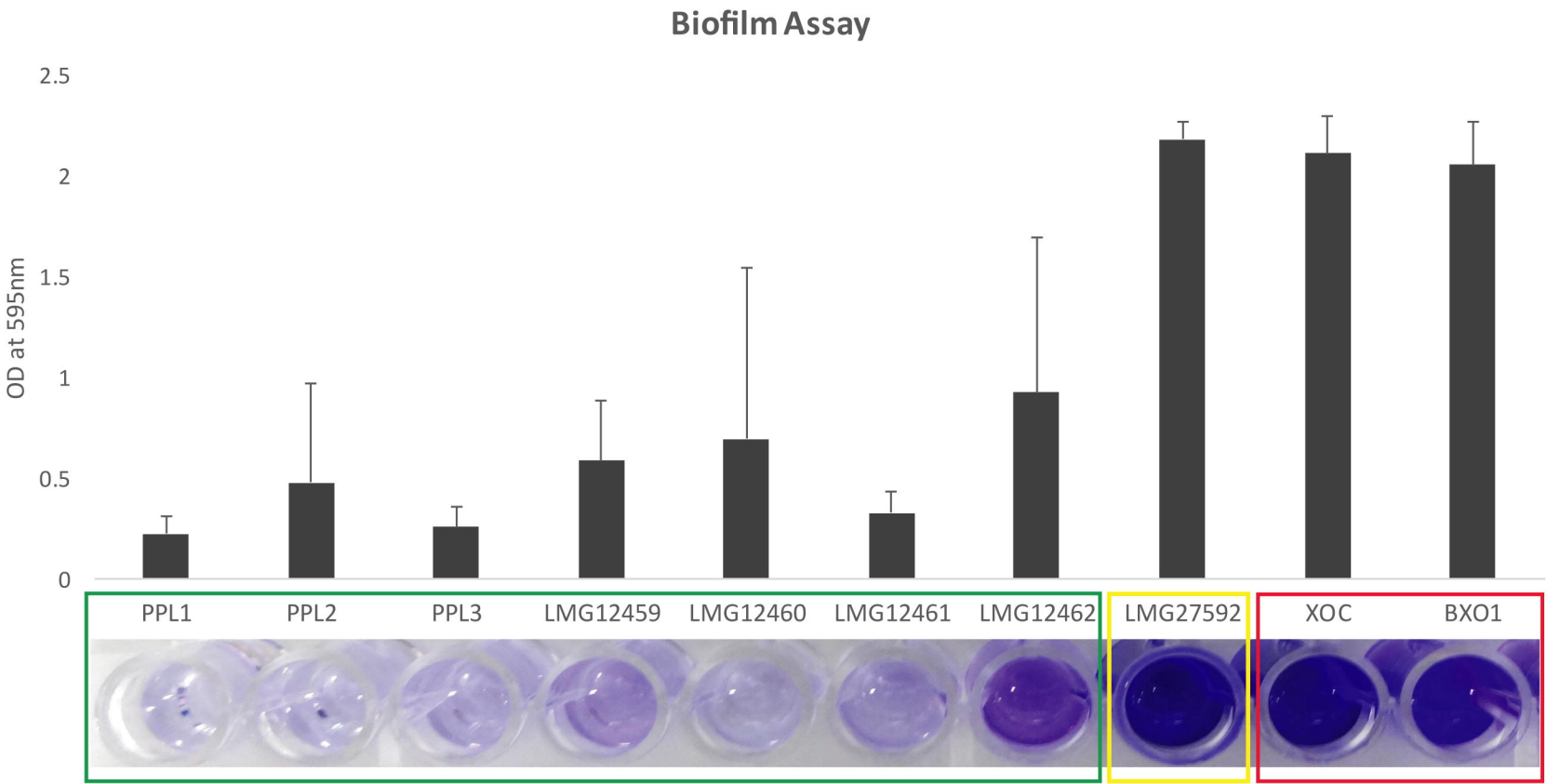
(B)

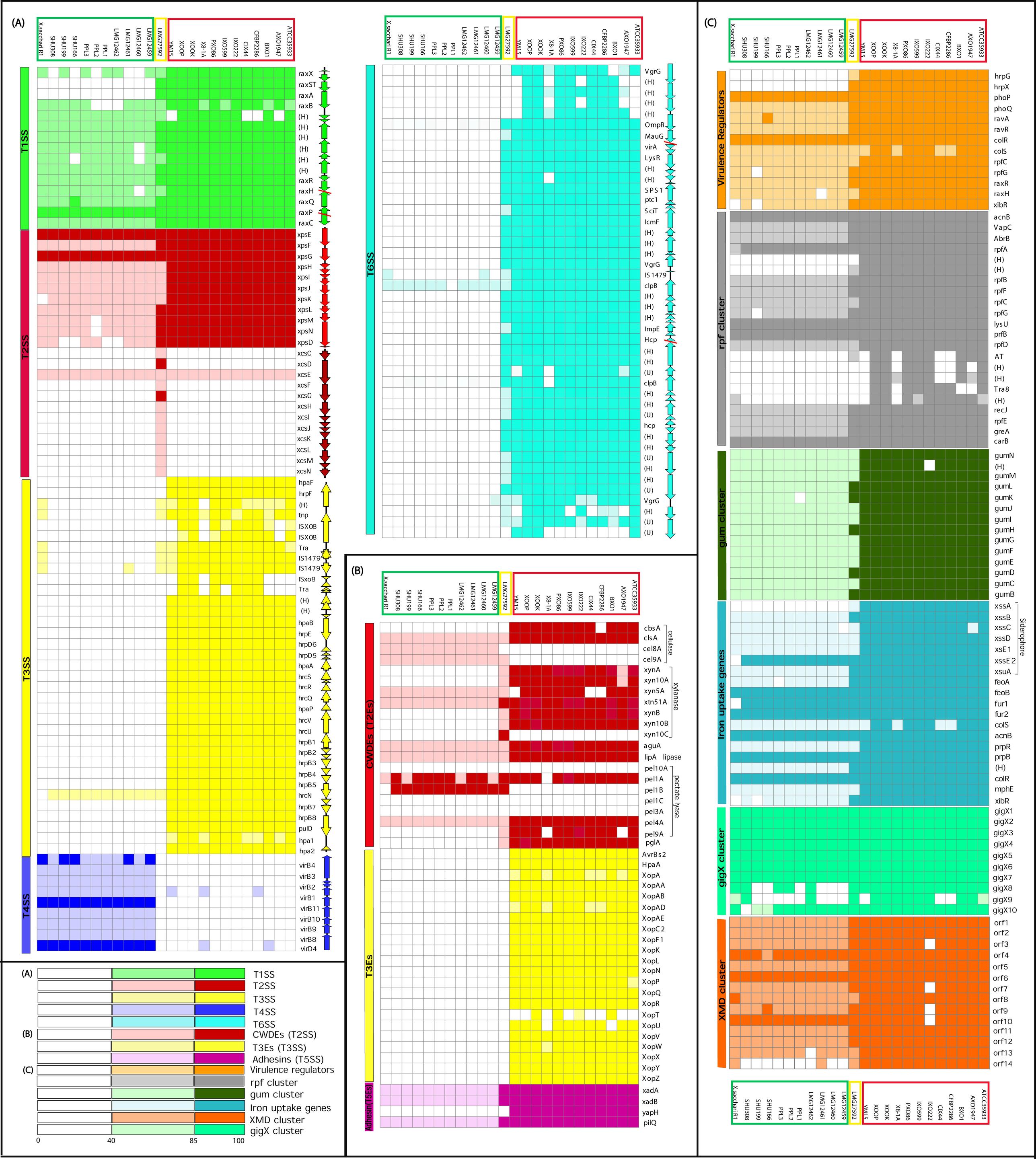

LMG12460
LMG12459
LMG12462
SHU199
SHU308
SHU166
LMG12461
PPL3
X.sacchari R1
PPL1
PPL2
X. sacchari CFBP4641 (T)
X. albilineans CFBP2523 (T)
X. maliensis LMG 27592 (T)
X8-1A
AXO1947
XOOP
X. oryzae pv. oryzae ATCC35933 (T)
PXO86
XOOM
IXO599
IXO222
BXO1
CFBP2286
YM15
CIX44
Stenotrophomonas maltophilia ATCC13637 (T)
0.2
Major Lineage-I (ML-I)
Major Lineage-II (ML-II)

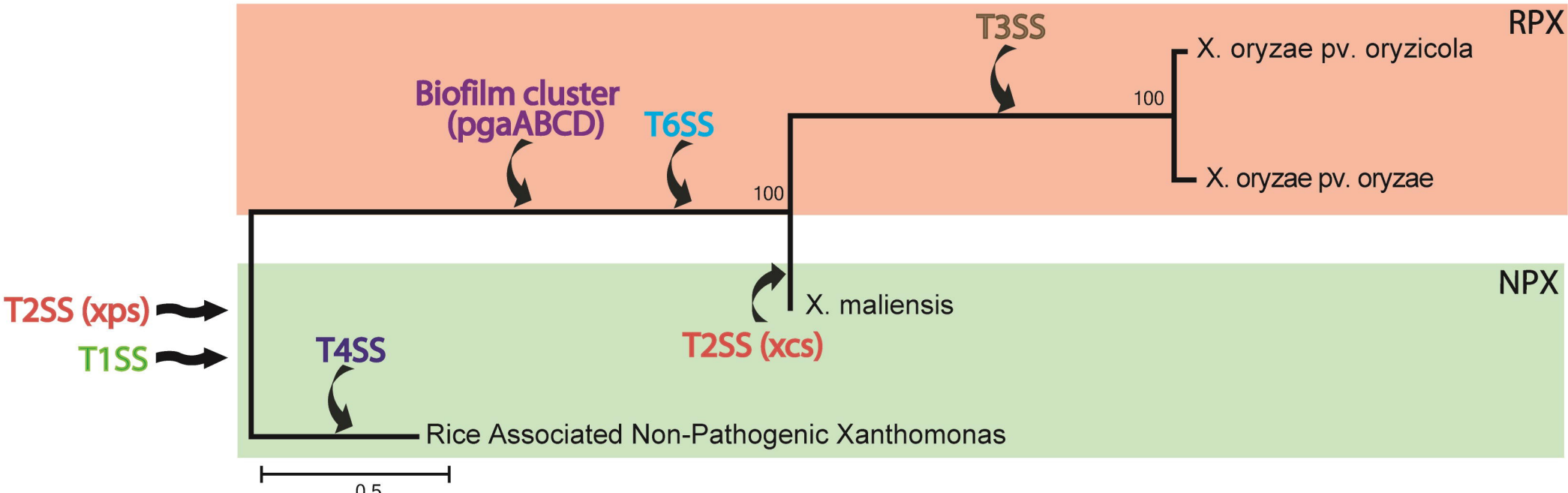

76
96
100
PPL1
PPL2
PPL3
SHU166
SHU199
SHU308
LMG12459
LMG12460
LMG12461
LMG12462
X.sacchari R1
X. sacchari CFBP4641 (T)
X. albilineans CFBP2523 (T)
X. maliensis LMG27592 (T)
X8-1A
AXO1947
YM15
CIX44
CFBP2286
XOOP
X. oryzae pv. oryzae ATCC35933 (T)
BX01
IXO222
IXO599
PXO86
XOOK
Major Lineage-I (ML-I)
Major Lineage-II (ML-II)


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
All
Xantho (ML-I+ML-II+Xmal)
ML-I+ML-II
ML-I
ML-I+X.mal
ML-II
ML-II+X.mal
Core
Pan


1000
900
800
700
600
500
400
300
200
100
0
M
N
O
T
U
V
J
K
L
C
E
F
G
H
I
P
Q
S+R
Unique NPX
Unique ML-I
Unique RPX
Unique ML-II


Biofilm Assay
OD at 595nm
2.5
2
1.5
1
0.5
0
PPL1
PPL2
PPL3
LMG12459
LMG12460
LMG12461
LMG12462
LMG27592
XOC
BXO1


(A)
T1SS
T2SS
T3SS
T4SS
T6SS
(B)
CWDEs (T2Es)
T3Es
Adhesin(T5Es)
cellulase
xylanase
lipase
pectate lyase
(C)
Virulence Regulators
rpf cluster
gum cluster
Iron uptake genes
Siderophore
XMD cluster
gigX cluster
X.sacchari R1
SHU308
SHU199
SHU166
PPL3
PPL2
PPL1
LMG12462
LMG12461
LMG12460
LMG12459
LMG27592
YM15
XOOP
XOOK
X8-1A
PXO86
IXO599
IXO222
CIX44
CFBP2286
BXO1
AXO1947
ATCC35933
T1SS
T2SS
T3SS
T4SS
T6SS
CWDEs (T2SS)
T3Es (T3SS)
Adhesins (T5SS)
Virulence regulators
rpf cluster
gum cluster
Iron uptake genes
XMD cluster
gigX cluster
0
40
85
100


RPX
NPX
T3SS
X. oryzae pv. oryzicola
100
Biofilm cluster
(pgaABCD)
T6SS
X. oryzae pv. oryzae
100
T2SS (xps)
T1SS
X. maliensis
T2SS (xcs)
T4SS
Rice Associated Non-Pathogenic Xanthomonas
0.5