Taxonogenomics reveal multiple novel genomospecies associated with clinical isolates of *Stenotrophomonas maltophilia*

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**Abstract**

*Stenotrophomonas maltophilia* has evolved as one of the leading multidrug-resistant pathogens responsible for a variety of nosocomial infections especially in highly debilitated patients. As information on the genomic and intraspecies diversity of this clinically important pathogen is limited, we sequenced the whole genome of 27 clinical isolates from hospitalized patients. Phylogenomic analysis along with the genomes of type strains suggested that the clinical isolates are distributed over the *Stenotrophomonas maltophilia* complex (Smc) within the genus *Stenotrophomonas*. Further genome-based taxonomy coupled with the genomes of type strains of the genus *Stenotrophomonas* allowed us to identify five cryptic genomospecies, which are associated with the clinical isolates of *S. maltophilia* and are potentially novel species. These isolates share a very small core genome that implies a high level of genetic diversity within the isolates. Recombination analysis of core genomes revealed that the impact of recombination is more than mutation in the diversification of clinical *S. maltophilia* isolates. Distribution analysis of well-characterized antibiotic-resistance and efflux pump genes of *S. maltophilia* across multiple novel genomospecies provided insights into its antibiotic-resistant ability. This study supports the existence of multiple cryptic species within the Smc besides *S. maltophilia*, which are associated with human infections, and highlights the importance of genome-based approaches to delineate bacterial species. This data will aid in improving clinical diagnosis and for understanding species-specific clinical manifestations of infection due to *Stenotrophomonas* species.

**DATA SUMMARY**

1. The draft genome assembly of 27 clinical isolates of *S. maltophilia* under this study have been deposited in GenBank and individual accession numbers are provided in Table 1.

2. Phylogenetic tree file, i.e. Newick file (.nwk), generated from maximum-likelihood reconstruction based on concatenation of protein sequence from 23 phylogenomic reference genes of 27 *S. maltophilia* clinical isolates and type strains of the genus *Stenotrophomonas* are deposited in Figshare; DOI:10.6084/m9.figshare.5356132 (https://figshare.com/s/2efe1b9e515343e5017).

3. Phylogenetic tree file, i.e. Newick file (.nwk), for a robust phylogenetic tree based on the alignment of protein sequences from 400 core genes of 27 *S. maltophilia* clinical isolates under study along with the type strains of members of the *Stenotrophomonas maltophilia* complex are deposited in Figshare; DOI:10.6084/m9.figshare.5356156 (https://figshare.com/s/2db426f19b14a6706e43).

4. Data file (.xlsx) used to generate the heat map of average nucleotide identity (ANI) values of *S. maltophilia* clinical isolates with the type strains of species belonging to the genus *Stenotrophomonas* is deposited in Figshare; https://figshare.com/s/dc32d3b7be5f18012fbb.

5. Data file (.xlsx) used to generate the heat map of digital DNA–DNA hybridization (dDDH) values of *S. maltophilia* clinical isolates with the type strains of species belonging to the genus *Stenotrophomonas* is deposited in Figshare; https://figshare.com/s/8c9d8b9e76661984d92.

6. Data file (.csv) used to generate the heatmap of presence and absence of antimicrobial resistance genes is deposited under this study have been deposited in GenBank and supplementary table and three supplementary figures are available with the online version of this article.
The taxonomic status of the genus Stenotrophomonas currently comprises 13 validated species according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN; http://www.bacterio.net), which are versatile and have the ability to adapt to diverse environmental niches [1, 2]. Stenotrophomonas maltophilia is an important and predominant species of the genus Stenotrophomonas with a wide range of activities, including plant growth promotion, breakdown of man-made pollutants and production of secondary metabolites, and it has an important role in multi-drug-resistant infections to humans and animals [2–4]. S. maltophilia is a multi-drug-resistant opportunistic pathogen responsible for causing infections in hospitalized patients as well as cystic fibrosis and cancer patients [5–8]. According to a recent World Health Organization report, S. maltophilia is one of the leading multi-drug-resistant bacteria in healthcare settings worldwide [9].

The taxonomic status of S. maltophilia within the genus is complicated because several previously proposed species, namely S. africana, Pseudomonas geniculata, Pseudomonas hibiscicola and Pseudomonas beteli, are considered as synonyms of S. maltophilia [10]. S. maltophilia and its synonym species along with the validly described Stenotrophomonas pavanii belong to the Stenotrophomonas maltophilia complex (Smc) [11, 12]. Whole-genome sequencing of the type strains of validly described and misclassified species belonging to the genus Stenotrophomonas revealed that synonyms of S. maltophilia, i.e. S. africana, P. geniculata, P. hibiscicola and P. beteli, represent distinct species as per modern genome-based taxonomic criteria [13]. In addition to this taxonomic complication, clinical and environmental isolates of S. maltophilia exhibit high levels of phenotypic and genotypic diversity [14]. Various molecular typing methods such as amplified fragment length polymorphism (AFLP) [1], rep-PCR [15], gyrB [10] and multi-locus sequence typing and analysis [16–18] have shown that there is a high level of genetic diversity amongst S. maltophilia isolates. Although these approaches have provided insights into the phylogeny and genetic diversity among S. maltophilia isolates, their limited resolution at the strain level means they are not useful for studies of intraspecies diversity. Genomic studies of clinical and environmental S. maltophilia isolates also suggested a high level of genomic diversity among them [19–23], but systematic studies focusing on phylogenomics and taxogenomics are lacking. Thus, there is a need to understand the intraspecies diversity of S. maltophilia clinical isolates by using genome-based approaches, which is important to identify novel species associated with human infections.

Sequencing of a clinical strain, K279a, of S. maltophilia revealed that the presence of numerous drug resistance determinants and efflux pumps into its genome [24]. S. maltophilia is resistant to a broad array of antibiotics due to intrinsic resistance mechanisms, which are common to all S. maltophilia isolates. Such resistance mechanisms include low membrane permeability, the presence of efflux pumps and antibiotic-modifying enzymes [7, 25]. The intrinsic resistance includes chromosomal but not horizontally acquired genes, which are present in all strains of bacterial species prior to antibiotic exposure. Moreover, apart from the intrinsic resistance mechanisms, acquired mechanisms have also been reported in S. maltophilia, involving acquisition of resistance genes through horizontal gene transfer and mutations [26, 27].

In the present study, whole genome sequencing of 27 clinical isolates identified as S. maltophilia isolated from hospitalized patients at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, was carried out. To study phylogenetic placements of sequenced clinical isolates within the genus Stenotrophomonas and to discover novel genomospecies, we used type strain-based phylogenomics and modern taxonomic criteria. Based on this, we concluded that multiple novel genomospecies are present amongst these clinical isolates of S. maltophilia. We also studied the gene content of Smc members along with novel genomospecies and found a small core genome size, which again supported the diverse nature of these clinical isolates. Our finding of potential novel species of Stenotrophomonas associated with human infections may open up a new path for further studies on the epidemiology, disease spectrum, virulence and resistance traits of infections.

**Impact Statement**

*Stenotrophomonas maltophilia* is a rapidly emerging multi-drug-resistant opportunistic pathogen responsible for nosocomial infections and a serious threat to healthcare settings worldwide. The genus *Stenotrophomonas* is taxonomically challenging due to several reclassifications and misclassifications associated with it. Genotypic methods suggest a high level of genetic diversity among *S. maltophilia* isolates. Thus, there is a need to assess the intra-species diversity of *S. maltophilia* among clinical isolates and to delineate them to the correct species. Type strains of the genus *Stenotrophomonas* are now available in public databases. Thus, we assessed intra-species diversity among clinical isolates of *S. maltophilia* by genome sequencing and integrated them with the genomes of type strains of the genus *Stenotrophomonas* using modern taxonomic methods. This allowed us to delineate clinical isolates within the genus and discover potential novel species of the genus responsible for clinical infections. We also studied the contribution of point mutations, homologous recombination and horizontal gene transfer in the diversification of clinical *S. maltophilia* isolates. Our finding of potential novel species of *Stenotrophomonas* associated with human infections may open up a new path for further studies on the epidemiology, disease spectrum, virulence and resistance traits of infections.
isolates. To elucidate the role of homologous recombination and mutations in the diversification of the Smc, we performed recombination analysis, which suggested that the impact of homologous recombination includes more than mutations in diversification. We also assessed the distributions of drug resistance and efflux pump genes across novel genospecies. Our finding of potential novel species associated with the clinical isolates of *S. maltophilia* may be important for clinicians in understanding the epidemiology and management of the disease caused by this multi-drug-resistant pathogen.

**METHODS**

**Bacterial isolates and culture conditions**

Twenty-seven isolates identified as *S. maltophilia* from hospitalized patients at a tertiary care hospital, PGIMER, were included in this study (Table 1). They were isolated from different clinical specimens, i.e. blood (*n* = 18), respiratory (*n* = 7), pus (*n* = 1) and cerebrospinal fluid (*n* = 1). The isolates were grown either on nutrient agar or in nutrient broth at 37°C from frozen stocks. Ethics approval and each patient’s written consent was not required as it was a part of routine clinical testing.

**DNA isolation, Illumina library construction and sequencing**

Approximately 15 ml of culture was grown in nutrient broth at 37°C with constant shaking at 200 r.p.m. DNA isolation was carried out by using a ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) as per the manufacturer’s instructions. DNA was quantified by using a Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific). Illumina sequencing libraries were prepared by using an Illumina Nextera XT sample preparation kit (Illumina) with dual indexing adapters from Illumina by strictly following the manufacturer’s guidelines. Illumina libraries were quantified by using a KAPA Library Quantification kit for Illumina (KAPA Biosystems). Sequencing libraries were pooled and sequenced using an in-house Illumina Miseq (Illumina) platform with 2×250 bp paired-end runs.

**Genome assembly and annotation**

The illumina reads were *de novo* assembled into the high-quality draft genome by using CLC Genomics Workbench 6.5.1 (CLC Bio-Qiagen) with default parameters except a minimum contig length set to 500 bp. The quality of the assembled genome in terms of completeness and contamination was accessed using CheckM v1.0.7 with default settings [28]. The assembled genomes were submitted to the NCBI GenBank database and accession numbers are given in Table 1. The genomes were annotated using the NCBI-Prokaryotic genome annotation pipeline [29].

**Phylogenetic analysis**

The 16S rRNA gene was extracted from the sequenced genome by using the RNAmmer 1.2 server [30] available at http://www.cbs.dtu.dk/services/RNAmmer/. Protein sequences for 23 essential bacterial phylogenetic reference genes (dnaG, rplA, rplB, rplC, rplD, rplE, rplF, rplK, rplL, rplM, rplN, rplP, rpsL, rpmA, rpoB, rpsB, rpsC, rpsE, rpsJ, rpsK, rpsM, rpsS, tsf) were extracted from the genome by using the AmphoraNet pipeline [31] available at http://pitgroup.org/amphoranet/. The extracted sequences were aligned by using CLUSTALW and a maximum-likelihood (ML) phylogenetic tree was reconstructed by using the General Time Reversible model, and Gamma distributed and Invariant sites (G+I) with 1000 bootstrap replications using MEGA version 6.06 [32]. The phylogenetic tree based on the whole genome was reconstructed by using PhyloPhlan [33], which uses ubiquitous and phylogenetically informative proteins conserved among the bacteria. Orthologues of these proteins in the genome were detected using USEARCH v5.2.32 [34] followed by the generation of multiple sequence alignments of these proteins using MUSCLE v3.8.31. A final concatenated dataset containing 4231 aligned amino acid positions was generated, and phylogenetic tree reconstruction was performed using FastTree version 2.1. [35]. The resulting phylogenetic tree was visualized by using iTOL v4 (https://itol.embl.de/) [36].

**Genome similarity assessment**

For genome similarity assessment we used average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), which have emerged as modern genome-based taxonomic methods [37]. ANI was calculated by using iSpecies 1.2.1 [38] and dDDH was calculated by using the web tool Genome to Genome Distance Calculator, GGDC 2.1 (http://ggdc.dsmz.de/distcalc2.php). We used Formula 2 alone for calculation of dDDH as it determines dDDH independent of the genome length and is recommended for use with draft genomes [39]. Heat maps of ANI and dDDH values were constructed using GENE-E software (https://software.broadinstitute.org/GENE-E/).

**Pan-genome analysis**

Pan and core genome analysis were performed using the pan-genome analysis pipeline (PGAP pipeline version 1.2.1) with the MultiParanoid (MP) method [40]. A minimum score value of 40 and e-value of 1e-10 were used as a cut-off for BLAST. PanGP version 1.0.1 [41] was used to analyse the pan-genome profile of clinical isolates of *S. maltophilia* and six reference genomes of members of the Smc. The power-law regression (*y*<sub>pan</sub>=*A*<sub>pan</sub>*x*<sup>β</sup>*B*<sub>pan</sub>+*C*<sub>pan</sub>) was used to model the pan-genomes generated from all permutations, where *y*<sub>pan</sub> is the total number of gene families in the pan-genome, *x* is the number of genomes considered, and *A*<sub>pan</sub>, *B*<sub>pan</sub> and *C*<sub>pan</sub> are fitting parameters. When 0<(*B*<sub>pan</sub>−1), the pan-genome should be considered open because it is an unstrained function over the number of genomes. When *B*<sub>pan</sub> <0, the pan-genome is considered closed because it approaches a constant as more genomes are considered. The number of core genes after addition of each new genome was plotted as a function of the number of genomes added sequentially, in a similar manner to the pan-genome plot. The exponential curve fit model, *y*<sub>core</sub>=*A*<sub>core</sub>*e<sup>*B*<sub>core</sub>*x</sup>
| Isolate ID  | Source     | Year  | Genome size (bp) | No. of contigs | Fold coverage | N50 (bp) | % GC  | No. of CDS* | Total bp in reads | Completeness (CheckM) | Contamination (CheckM) | NCBI accession |
|------------|------------|-------|------------------|----------------|---------------|----------|-------|-------------|-------------------|----------------------|------------------------|-----------------|
| SM20065    | Blood      | 2012  | 4303718          | 137            | 266           | 57893    | 66.5  | 3969       | 1200729721        | 99.01                | 0.00                   | LXXA000000000 |
| SM3226     | Blood      | 2012  | 4485531          | 181            | 157           | 44537    | 66.6  | 3910       | 706934712         | 99.89                | 0.18                   | LXXB000000000 |
| SM325416   | Blood      | 2013  | 4499756          | 166            | 234           | 67358    | 66.6  | 3967       | 1054601188        | 99.15                | 0.03                   | LXXC000000000 |
| SM7180     | Respiratory | 2012  | 4559054          | 164            | 239           | 62326    | 66.5  | 3980       | 1092251589        | 99.66                | 0.00                   | LXXD000000000 |
| SM7882     | Respiratory | 2012  | 4404542          | 143            | 407           | 62986    | 66.5  | 3897       | 1793580861        | 97.93                | 0.69                   | LXXE000000000 |
| SM480      | Respiratory | 2013  | 4294147          | 143            | 210           | 81284    | 66.6  | 3741       | 905075243         | 95.63                | 0.34                   | LXXF000000000 |
| SM11522    | Blood      | 2012  | 4772386          | 163            | 238           | 66742    | 66.2  | 4227       | 1138488981        | 95.62                | 0.69                   | LXXG000000000 |
| SM2546     | Respiratory | 2013  | 4622345          | 145            | 267           | 64336    | 64.5  | 4099       | 1238064180        | 98.62                | 0.39                   | LXXH000000000 |
| SM4416     | Blood      | 2012  | 4348678          | 254            | 133           | 42949    | 66.8  | 3806       | 582189524         | 99.26                | 0.05                   | LXXI000000000 |
| SM100      | Blood      | 2010  | 4670638          | 224            | 65            | 38440    | 66.4  | 4185       | 307302019         | 98.74                | 0.43                   | LXXJ000000000 |
| SM19467    | Blood      | 2012  | 4590360          | 162            | 201           | 54275    | 66.5  | 4084       | 926278460         | 99.10                | 0.34                   | LXXK000000000 |
| SM30540    | Blood      | 2013  | 4544171          | 147            | 197           | 54368    | 66.6  | 4042       | 898867912         | 99.31                | 0.00                   | LXXL000000000 |
| SM5815     | Blood      | 2010  | 4927374          | 247            | 125           | 48474    | 66.4  | 4432       | 618830138         | 99.74                | 2.12                   | LXXM000000000 |
| SM17711    | Blood      | 2012  | 4334100          | 143            | 195           | 69796    | 66.9  | 3803       | 846083847         | 98.62                | 0.00                   | LXXN000000000 |
| SM24179    | Blood      | 2012  | 4281782          | 140            | 252           | 76600    | 66.9  | 3754       | 1082737078        | 95.66                | 0.00                   | LXXO000000000 |
| SM6957     | Blood      | 2013  | 4300278          | 128            | 245           | 74488    | 66.7  | 3814       | 1054847302        | 98.26                | 0.00                   | LXXP000000000 |
| SM1911     | pus        | 2010  | 4279279          | 230            | 67            | 47434    | 66.7  | 3738       | 289442792         | 98.03                | 0.11                   | LXXQ000000000 |
| SM31360    | Blood      | 2012  | 4316491          | 136            | 240           | 62147    | 66.6  | 3814       | 1038281752        | 98.14                | 0.17                   | LXXR000000000 |
| SM760      | Respiratory | 2010  | 4227019          | 253            | 109           | 38897    | 66.7  | 3681       | 461064721         | 97.75                | 0.11                   | LXXY000000000 |
| SM1006     | Blood      | 2013  | 4308146          | 119            | 339           | 76966    | 66.7  | 3799       | 1464096438        | 98.83                | 0.00                   | LXXS000000000 |
| SM3112     | Respiratory | 2012  | 4272442          | 150            | 207           | 69629    | 66.6  | 3771       | 887873333         | 99.27                | 0.39                   | LXXT000000000 |
| SM16975    | Blood      | 2012  | 4582512          | 119            | 256           | 83180    | 66.4  | 4093       | 1173248392        | 97.76                | 0.39                   | LXXW000000000 |
| SM10507    | CSF        | 2012  | 4783681          | 167            | 140           | 71880    | 66.4  | 4296       | 671034607         | 99.80                | 1.03                   | LXXU000000000 |
| SM16360    | Blood      | 2012  | 4712691          | 116            | 258           | 96011    | 66.6  | 4163       | 1219487828        | 98.62                | 1.49                   | LXXV000000000 |
| SM1389     | Blood      | 2010  | 4350271          | 122            | 314           | 64824    | 66.5  | 3862       | 1369078280        | 98.03                | 0.00                   | LXXW000000000 |
| SM38795    | Blood      | 2013  | 4227221          | 136            | 245           | 79994    | 66.6  | 3786       | 1039225345        | 96.16                | 0.34                   | LXXX000000000 |
| SM3123     | Respiratory | 2010  | 4018348          | 114            | 215           | 138936   | 66.9  | 3491       | 865543650         | 95.55                | 0.33                   | LXXY000000000 |

*CDS, coding DNA sequences.
†CSF, cerebrospinal fluid.
Homologous recombination analysis

The genomes of clinical isolates of *S. maltophilia* along with the five type strains belonging to the Smc were aligned using Mauve version 20150226 build 10 (c) [42]. Core genome alignment generated from Mauve was further used to reconstruct the phylogenetic tree using PhyML 3.1 [43]. The core genome alignment and PhyML tree were further used to calculate the relative rate of recombination to mutation events using ClonalFrameML [44] with 100 bootstrap replications. The PhyML and ClonalFrameML phylogenetic tree was visualized by using iTOL v4 (https://itol.embl.de/) [36].

RESULTS

Whole genome sequencing of *S. maltophilia* clinical isolates

Whole genome sequencing was carried out for *S. maltophilia* isolated during 2010–2013 from clinical specimens of different patients (Table 1). The genome features and assembly statistics are detailed in Table 1. High-quality draft genomes were obtained with coverage ranging from 65× to 407× fold. There is no direct significant correlation found between assembly quality and coverage, suggesting that other factors, such as library quality or percentage of repetitive DNA in each genome, may influence the assembly quality. The estimated genome completeness for this genome dataset ranges from 95.55 to 99.89 % and estimated contamination ranges from 0 to 2.12 % (Table 1). The number of predicted coding DNA sequences (CDSs) ranged from 3491 to 4432 and GC content of the assembled genomes is around 66 mol% (Table 1).

Phylogenetic placement of sequenced clinical *S. maltophilia* isolates within the genus *Stenotrophomonas*

The phylogenetic placement of sequenced clinical *S. maltophilia* was assessed by reconstructing a phylogenetic tree along with the type strains of species belonging to the genus (Table S1, available in the online version of this article). A phylogenetic tree was reconstructed based on 16S rRNA gene sequences, which plays an important role in microbial identification and taxonomy with 97 % cut-off for distinct species. 16S rRNA gene sequences of the clinical isolates from this study showed >97 % similarity with the type strains of all species of the Smc (Fig. S1). Due to the limited resolution provided by 16S rRNA-based phylogeny, a phylogenomic tree was obtained further using translated protein sequences of 23 conserved housekeeping genes. The analysis showed the placement of *S. maltophilia* clinical isolates in the Smc with high bootstrap values (Fig. 1). While both analyses suggested the distribution of clinical isolates over the Smc lineage, certain discrepancies in branching among the phylogenetic tree based on 16S rRNA and 23 phylogenomic marker genes were observed, indicating the need for a highly robust tree for taxonomic classification.

To address these discrepancies, we reconstructed a phylogenetic tree based on protein sequences of 400 core genes of the Smc including type strains of members of the Smc along with *S. maltophilia* clinical isolates under study (Fig. 2). The phylogenetic tree showed that the 27 clinical isolates of *S. maltophilia* were distributed over five major monophyletic groups. Eleven isolates grouped together with *S. maltophilia* MTCC 434 T while both *P. hibisicola* ATCC 19867 T and *S. pavanii* DSM 25135 T were grouped with two isolates under study. Isolate SM3123 formed a monophyletic clade with *P. beteli* LMG 978 T. The type strains of *P. geniculata* and *S. africana* did not group with any of the clinical isolates under study.

Genome similarity assessment and discovery of novel genomospecies

A robust phylogenetic tree of members of the Smc clearly revealed the existence of multiple distinct lineages within the Smc. We calculated ANI and dDDH values with the type strains of valid and misclassified species of the genus *Stenotrophomonas* for the assessment of overall genome similarity and to identify potential novel species. The heat map of ANI and dDDH values of clinical isolates of *S. maltophilia* with the type strains of the genus *Stenotrophomonas* is shown in Fig. 3. Based on the cut-off values for species delineation using ANI (96 %) and dDDH (70 %) [46], there are six distinct groups in *S. maltophilia* isolates that should be considered as separate bacterial species, and referred to below as genomospecies (Fig. 3). Genomospecies 1 (G1) consisting of 11 isolates that are grouped with reference strain *S. maltophilia* MTCC 434 T represents the core *S. maltophilia* group. Genomospecies 2 (G2), genomospecies 3 (G3) and genomospecies 4 (G4) comprised two, nine and two isolates, respectively, which did not group with any *Stenotrophomonas* species type strain (Fig. 3). Genomospecies 5 (G5) included two isolates that grouped with *P. hibisicola* ATCC 19867 T. Isolate SM3123 was a singlet as it did not group with any type strain within the genus *Stenotrophomonas* and is represented as genomospecies 6 (G6). The genome similarity results for the 27 Smc clinical isolates revealed their distribution over six genomospecies, among which G1 belongs to *S. maltophilia* and the remaining
genomospecies (G2–G6) are potentially novel species (Table 2).

Pan-genome analysis
To obtain insight into the core genome, genomospecies-specific genes and strain-specific gene content, we performed pan-genome analysis of S. maltophilia genomospecies along with the type strains of Smc species. This analysis provided a measure of the intra-genomospecies variation in gene content. The orthologous CDSs shared among Smc members is 1917, which is ~21.23% of the pan-genome size (9031 CDSs) (Fig. 4). The genomospecies-specific core genomes ranged from 2840 to 4464 CDSs, representing ~31 to ~49% of the pan-genome size (Fig. 4). Core genome size is smaller than the group-wise core genomes. Among the group-wise core genome genomospecies, G2 (2840 CDSs) and G1 (2861 CDSs) have smaller core genomes due to a large number of genomes included in the analysis (Fig. 4). The strain-specific genes ranged from two to 253 CDSs, a widely variable genomic fraction (Fig. 4). Genomospecies G1 also had a small number of strain-specific genes, which is again in concordance with the fact that large numbers of the genomes were included in the analysis (Fig. 4).

The pan-genome plot (Fig. 5) clearly shows that even after the addition of all CDSs from 33 genomes, the plot is yet to reach a plateau and further addition of genomes will increase the pan-genome size. The power law regression model shows that the pan-genome of the Smc is ‘open’, as the γ-parameter value (Bpan) is 0.45, and sequencing of isolates from the Smc is required to identify all genes of this complex. The core genome size decreases dramatically with the inclusion of each new genome, the curve almost approaches a plateau and further addition of new genomes may result in decreased core genome size (Fig. 5). Similar behaviour is observed in the plot of strain-specific CDSs against the number of genomes, the number of strain-specific CDSs gradually decreasing with the addition of new genomes (Fig. S2).

Role of homologous recombination and mutations in diversification of clinical S. maltophilia isolates
To investigate the role of homologous recombination and mutations in the diversification of clinical isolates of S. maltophilia, we used the application ClonalFrameML. The average relative rate of recombination (R) to mutation (θ) of the Smc was estimated to be R/θ=0.376066, mean DNA import length was δ=211 bp, and the mean divergence of imported DNA was ρ=0.059. This suggests the occurrence of ~2.659 mutational events for each recombination event. The relative impact of recombination to mutation (r/m) is ~4.74 across the overall phylogeny of the Smc. To investigate the effect of recombination on the phylogenetic tree topology we used ClonalFrameML to reconstruct a more accurate phylogeny by removing the genomic divergence generated by recombination. Branch lengths of the ClonalFrameML tree were not consistent with the ML phylogeny, indicating the impact of recombination on the diversification of these isolates (Fig. S3).

Resistome analysis
The resistome of a well-studied strain, S. maltophilia K279a, has been characterized and data on its drug resistance profile are available [24, 26]. We assessed the distribution of known antibiotic-resistant and efflux pump genes across various genomospecies of S. maltophilia.
clinical isolates (Fig. 6). The two chromosomally encoded β-lactamases \( \text{bla}L1 \) (Zn\(^{2+}\)-dependent metalloenzyme) and \( \text{bla}L2 \) (serine β-lactamases), plus \( \text{ampC} \), which are characteristics of \( S. \) maltophilia [7], are present in all isolates except SM 3123, which belongs to genomespecies G6 and does not harbour any of these β-lactamases. Resistance to the aminoglycosides group of antibiotics is mediated by aminoglycoside-modifying enzymes such as aminoglycoside 6'-N-acetyltransferase (aac (6')-Iz) [47], aminoglycoside 2'-N-acetyltransferase (aac (2')-Iz) [24], aminoglycoside phosphotransferase (aph (3')-IIc) [48] and streptomycin 3 phosphotransferase [24]. The distribution of aac (6')-Iz and aac (2')-Iz is limited to the Smc as they are present in six and 11 isolates, respectively, the majority of which belong to genomespecies G1. The streptomycin 3'-phosphotransferase and aminoglycoside phosphotransferase are present in all the isolates along with other members of the Smc but absent from isolates SM 11522, SM 38795, SM 5815, and SM 3123. The chloramphenicol acetyltransferase gene, \( \text{cat} \), mediates resistance to chloramphenicol, which is exclusively present in \( S. \) maltophilia MTCC 434\(^4 \) and SM 11522. All strains except SM 3123 carry the chromosomal \( \text{Smqnr} \) gene, which is responsible for resistance to quinolones. The gene \( \text{spgM} \), involved in lipopolysaccharide biosynthesis and moderately involved in resistance to gentamicin, nalidixic acid, cefazidime, piperacillin-tazobactam, polymyxin B, polymyxin E and vancomycin [49], is also present in all the isolates under study. The \( \text{sul} \) gene, which is responsible for resistance to the trimethoprim/sulfamethoxazole class of antibiotics [27], is not present in any of these isolates. There are five families of efflux pumps reported to be present in \( S. \) maltophilia: the resistance-nodulation-cell-division (RND) family, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, ATP-binding cassette (ABC) family, and multidrug and toxic compound extrusion (MATE) family [25, 26], which are present in all the isolates under study with a few exceptions (Fig. 6). The well-characterized RND-type efflux pumps in the \( S. \) maltophilia genome are \( \text{smeABC} \), \( \text{smeDEF} \), \( \text{smeJK} \), \( \text{smeOP} \), \( \text{smeVWX} \) and \( \text{smeYZ} \). Apart from \( \text{smeABC} \), the remaining RND-type efflux pumps are present in all the isolates under study. \( \text{smeABC} \) is absent from SM 325416, SM 38795, SM 3123 and \( P. \) hibiscicola ATCC 19867. The \( \text{emrAB} \) efflux pump belonging to the MFS family is present in all the isolates under study, and
confers resistance to hydrophobic antibiotics and compounds such as nalidixic acid, thiolactomycin and organomercurials [26]. SMR family pumps are considered responsible for resistance to β-lactams, macrolides, tetracyclines and quaternary ammonium compounds [50]. The sugE and emrE pumps are well-characterized SMR efflux pumps in S. maltophilia and are present in all the isolates. Two efflux pumps belonging to the ABC transporter family, smrA and macAB, were previously characterized from S. maltophilia and are present in all the isolates under study. The smrA pump is known to confer resistance to fluoroquinolones and tetracycline [50] and the macABC efflux pump confers intrinsic resistance to aminoglycosides, macrolides and polymyxins, which are present in all the isolates under study [51]. A unique tripartite fusaric acid efflux pump fuaABC responsible for fusaric acid resistance was reported in S. maltophilia [52], and is reported to be present in all isolates except SM3123. There are two genes, pmpM and norM, encoding MATE efflux pumps [26], which are present in all the isolates and are known to be responsible for resistance to the quinolone family of drugs that includes ciprofloxacin, norfloxacin and ofloxacin.

**DISCUSSION**

*Stenotrophomonas* is a taxonomically challenging genus due to multiple taxonomic revisions in the past. *S. maltophilia* is an emerging opportunistic pathogen with high genetic diversity and is the only species in the genus that is known to be responsible for clinical infections. However, another species, *S. africana*, was isolated from human infections, and was later reclassified as *S. maltophilia* [53, 54]. Whole-genome sequencing of the type strains and historically associated reference strains revealed that *S. africana* represents another species of clinical importance [13]. Thus, there is a need to assess the intra-species diversity among clinical isolates of *S. maltophilia*. Advancements in sequencing

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**Fig. 3.** Heatmap of ANI and dDDH values among 27 clinical isolates with the type strains of members of the genus *Stenotrophomonas* under species delineation thresholds. The left side heat map represents ANI and the right side dDDH values. Colour variation in heat maps shows the variation in identity values as shown by the scale on the bottom. Isolate names highlighted with the same colour belong to the same genomospecies, and those that do not group with any isolate under study are highlighted as black.

**Table 2.** List of genomospecies identified among 27 clinical isolates of *S. maltophilia* along with their species status

| Genomospecies  | Species     | Isolates                                                                 |
|----------------|-------------|---------------------------------------------------------------------------|
| Genomospecies 1 (G1) | *S. maltophilia* | SM20065, SM3226, SM325416, SM7180, SM7882, SM480, SM11522, SM2546, SM4416, SM100, SM19467 |
| Genomospecies 2 (G2) | Novel       | SM30540, SM5815                                                           |
| Genomospecies 3 (G3) | Novel       | SM17711, SM24179, SM6957, SM1911, SM13670, SM760, SM1006, SM3112, SM16975 |
| Genomospecies 4 (G4) | Novel       | SM10507, SM16360                                                         |
| Genomospecies 5 (G5) | *P. hibiscola* | SM1389, SM38795                                                         |
| Genomospecies 6 (G6) | Novel       | SM3123                                                                 |
technologies have enabled us to study the intra-species population structure based on genome sequence information [55]. Therefore, we carried out a whole-genome sequencing of 27 clinical isolates identified as *S. maltophilia* from a hospital located in northern India. Phylogenetic analysis using 16S rRNA and 23 housekeeping genes with the type strains of members of the genus *Stenotrophomonas* revealed that the clinical isolates were distributed exclusively over the Smc. This finding has implication for our understanding of the ecology of clinical *S. maltophilia* isolates within the genus, which is important for the utilization of other non-pathogenic members of the genus *Stenotrophomonas* for biotechnical purposes. Further phylogenomic and taxonomonomic analysis revealed the heterogeneous structure of the Smc. The current nomenclature suggests the presence of only two valid species (*S. maltophilia* and *S. pavanii*) and four misclassified species (*P. hibiscicola*, *P. geniculata*, *P. betele* and *S. africana*) belonging to the Smc. Our analysis suggests that there are six genomospecies among the clinical isolates of *S. maltophilia*; thus, the Smc should include at least ten distinct genomospecies. Genomospecies 1, which belongs to the core *S. maltophilia* group, is a dominant group (11/27: 40.27%) among sequenced isolates followed by Genomospecies 3, which is a putatively novel species (9/27: 33.33%) (Table 2). Two isolates from our study belong to *P. hibiscicola*, suggesting that *P. hibiscicola* is a putative novel species with an ability to cause human infections. This study also highlights the importance of type strain genomes in making accurate species assignments and in the discovery of novel species in the post-genomic era.

The pan-genome analysis suggests that the Smc has an open pan-genome and addition of newly sequenced genomes is required to identify all genes in the Smc. The small core genome size (21.23%) suggests high genetic diversity and genomic heterogeneity among the isolates (Fig. 4). Further recombination analysis suggests that there is selection pressure acting on isolates of *S. maltophilia* for pathoadaptation,

![Fig. 4. Number of orthologous CDSs belonging to the core, genomospecies-specific and strain-specific genes across the Smc. Strain names are given outside the circle. From outside to inside, the first and second circles represent the number of strain-specific CDSs and genomospecies-specific core-genome CDSs, respectively. The third circle at the centre represents the number of core genome CDSs of the Smc.](image-url)
which leads to the introduction of variations through homologous recombination and mutations. The impact of recombination is higher in the diversification of clinical S. maltophilia isolates because a single recombination event causes multiple nucleotide changes in the genome. However, the larger pan-genome, which is nearly five times larger than the core-genome, suggests that variation mediated by non-homologous gene transfer is also playing a role in the diversification of clinical S. maltophilia isolates. The novel genomospecies have a unique gene pool, which is different from S. maltophilia, suggesting that gene gain and loss events are shaping the genomes of clinical S. maltophilia isolates during the course of evolution.

Clinical isolates of S. maltophilia are well known for their high level of intrinsic resistance to most of the commonly used antibacterial agents, including β-lactams (cephalosporin, carbapenems), macrolides, fluoroquinolones, aminoglycosides, chloramphenicol, tetracyclines and polymyxins [7, 9, 25, 56]. In addition, the emergence of resistance against the treatment of choice, trimethoprim-sulfamethoxazole, is increasing [57, 58]. Along with intrinsic drug resistance genes, the multi-drug resistance phenotype is also mediated by intrinsically encoded efflux pumps [26]. The distribution of well-characterized antibiotic resistance and efflux pump genes of S. maltophilia across multiple novel genomospecies has provided insights into its antibiotic resistance capability. Further varying levels of resistant phenotype changes among these isolates can be correlated with point mutations and expression differences in resistant genes [59].

The identification of multiple genomospecies, which represent potential novel species of Stenotrophomonas, associated with human infections can serve as an important asset to clinicians. These clinical isolates of S. maltophilia are found to be considerably different from each other, despite originating from the same hospital. Further studies supplemented with polyphasic approaches are underway to ascertain if these putative genomospecies represent novel species. This information can be helpful for clinicians to manage infections caused by this clinically significant
pathogen. Studies on the epidemiology, disease spectrum resistance and virulence traits for infections caused by putative novel species are required for species-specific diagnosis and treatment.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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