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METHODS

Multilocus Sequence Analysis, a Rapid and Accurate Tool for Taxonomic Classification, Evolutionary Relationship Determination, and Population Biology Studies of the Genus *Shewanella*

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ABSTRACT The genus *Shewanella* comprises a group of marine-dwelling species with worldwide distribution. Several species are regarded as causative agents of food spoilage and opportunistic pathogens of human diseases. In this study, a standard multilocus sequence analysis (MLSA) based on six protein-coding genes (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) was established as a rapid and accurate identification tool in 59 *Shewanella* type strains. This method yielded sufficient resolving power in regard to enough informative sites, adequate sequence divergences, and distinct interspecies branches. The stability of phylogenetic topology was supported by high bootstrap values and concordance with different methods. The reliability of the MLSA scheme was further validated by identical phylogenies and high correlations of genomes. The MLSA approach provided a robust system to exhibit evolutionary relationships in the *Shewanella* genus. The split network tree proposed twelve distinct monophyletic clades with identical G+C contents and high genetic similarities. A total of 86 tested strains were investigated to explore the population biology of the *Shewanella* genus in China. The most prevalent *Shewanella* species was *Shewanella algae*, followed by *Shewanella xiamenensis*, *Shewanella chilikensis*, *Shewanella indica*, *Shewanella seohaensis*, and *Shewanella carassii*. The strains frequently isolated from clinical and food samples highlighted the importance of increasing the surveillance of *Shewanella* species. Based on the combined genetic, genomic, and phenotypic analyses, *Shewanella upenei* should be considered a synonym of *S. algae*, and *Shewanella pacifica* should be reclassified as a synonym of *Shewanella japonica*.

IMPORTANCE The MLSA scheme based on six housekeeping genes (HKGs) (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) is well established as a reliable tool for taxonomic, evolutionary, and population diversity analyses of the genus *Shewanella* in this study. The standard MLSA method allows researchers to make rapid, economical, and precise identification of *Shewanella* strains. The robust phylogenetic network of MLSA provides profound insight into the evolutionary structure of the genus *Shewanella*. The population genetics of *Shewanella* species determined by the MLSA approach plays a pivotal role in clinical diagnosis and routine monitoring. Further stud-

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ies on remaining species and genomic analysis will enhance a more comprehensive understanding of the microbial systematics, phylogenetic relationships, and ecological status of the genus *Shewanella*.

KEYWORDS *Shewanella*, evolutionary relationship, identification, multilocus sequence analysis, population biology, taxonomic classification

The genus *Shewanella*, first described by MacDonell and Colwell, belongs to the family *Shewanellaceae* (1). The members of this genus are Gram-negative, facultatively anaerobic, oxidase-positive, and motile bacteria (2–4). At the time of writing, there are 60 to 70 recognized species in the genus of *Shewanella* (http://www.bacterio.net/shewanella.html) (5–7). The majority of *Shewanella* species inhabit a wide range of environments, including free-living *Shewanella* species in oceans (8–11). Multiple *Shewanella* species are frequently yielded from consumable products as spoilage bacteria and from clinical specimens as opportunistic pathogens (12–14). In addition, the genus *Shewanella* plays a critical role in bioremediation (15), and certain strains have been used in bioelectrical systems (16, 17).

To date, polyphasic approaches are performed to assign the phylogenetic placement and taxonomic classification of *Shewanella* species. Commercial biochemical systems, such as Vitek and API, are available for species identification in clinical laboratories. However, only two species, namely, *Shewanella algae* and *Shewanella putrefaciens*, have been recorded in the database (12, 13). Phylogenetic analysis based on the 16S rRNA gene as a molecular marker was utilized to yield an evolutionary relationship for taxa (18). The disadvantage of the application of the 16S rRNA gene was the low resolving power to discriminate closely related species due to their high sequence similarities (19). Recently, a more rapidly evolving housekeeping gene (HKG) of *gyrB* was selected as an alternative phylogenetic indicator for *Shewanella* species classification (7, 20–22). Nevertheless, the quality of sequences submitted in public databases is poor (22–24). The genome-wide parameters, consisting of *in silico* DNA-DNA hybridization (*is*DDH) (25) and average nucleotide identity (ANI) (26), take the place of the wet-lab DNA-DNA hybridization (DDH) to unravel bacterial systematics. However, the process of genome sequencing is expensive and time-consuming; meanwhile, limited genomes of *Shewanella* type strains are available in public databases. These conditions make this approach impractical in clinical and daily investigations for rapid and efficient identification.

The effective multilocus sequence analysis (MLSA) scheme has been used in the phylogenetic and taxonomic analyses of several bacterial taxa (19, 27). Nevertheless, rare information is delineated among the genus *Shewanella*. Hence, in this study, we established a reliable MLSA method to classify *Shewanella* species by assessing the nucleotide sequences and phylogenies of six individual and concatenated HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) in almost 60 *Shewanella* type strains. The phylogenetic framework of concatenated sequences provided a significant understanding of the evolutionary relationship in the genus *Shewanella* on the basis of multiple distinct taxonomic clades. The MLSA scheme was further utilized to determine the population biology of 86 tested strains collected in China.

RESULTS

Individual gene analysis. In this study, fragments of 16S rRNA gene and six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) were amplified successfully for all *Shewanella* strains. Sequence diversity and phylogenetic analysis of fifty-nine type strains (see Table S2 in the supplemental material) were performed to assess the interspecies taxonomy among the genus *Shewanella*. The results of sequence diversity for the 16S rRNA gene are shown in Table 1. The high occurrences of >98.65% interspecies similarity in the 16S rRNA gene implied the low resolution to distinguish *Shewanella* species. The low bootstrap values indicated the unstable topology in the phylogenetic tree, and close evolutionary branches were discovered (Fig. S1). Among the six HKG analyses, greater

TABLE 1 Nucleotide sequence diversity of 59 *Shewanella* type strains

Locus	Length (bp)	Parsimony informative sites		Nucleotide diversity (Pi)	Similarity (%)		K_a/K_s
		No.	%		Range	Mean	
16S rRNA	1,434	148	10.3	0.043	89.8–100	95.0	NA[a]
gyrA	498	229	46.0	0.223	68.3–100	77.7	0.117
gyrB	1,110–1,119	492	44.0	0.194	73.2–99.9	80.8	0.089
infB	663	289	43.6	0.193	73.5–100	80.7	0.105
recN	633–636	457	71.9	0.360	52.2–99.8	64.0	0.275
rpoA	615	221	35.9	0.125	79.7–100	87.5	0.052
topA	657–660	358	54.2	0.264	65.9–100	73.5	0.168
MLSA	4,176–4,191	2,046	48.8	0.223	71.1–99.9	77.7	0.143

[a]NA, not applicable.

values of parsimony informative sites and nucleotide diversity were obtained (Table 1). In addition, the phylogenetic trees of all HKGs demonstrated more distinct branches and greater bootstrap values than the phylogenetic tree of the 16S rRNA gene (Fig. S1). However, this was not sufficient to differentiate all members of the genus *Shewanella*. Lower bootstrap values for the outer branches and discordance in the partial topology of six HKGs were still observed.

MLSA. The concatenated sequences of protein-coding genes for 59 *Shewanella* type strains comprised 2,046 (48.8%) parsimony informative sites, with a nucleotide diversity value of 0.223 (Table 1). The analysis of sequences indicated that the multilocus sequence analysis (MLSA) scheme possessed an appropriate resolution and balanced the divergent evolutionary rates of six HKGs. The neighbor-joining phylogenetic tree based on concatenated alignment showed independent branches for interspecies, except for two sets of species, *S. algae-S. haliotis-S. upenei* (28) and *S. japonica-S. pacifica* (Fig. 1). These five species were likely to be misclassified, and more approaches were needed to perform the identification. The branches to discriminate *Shewanella* species were supported by high bootstrap values, except for *S. algicola-S. inventionis* and *S. carassii*. Bootstrap results indicated that the taxonomic groups involving those three species shared close evolutionary relationships. The phylogenetic tree of concatenated sequences was also reconstructed by the maximum-likelihood algorithm (Fig. S2). Almost the same topology was obtained, and the only exception was the location of *S. carassii*, which was supported with relatively low bootstrap values as described above.

Comparative analysis between MLSA and genomes. To further validate the reliability of MLSA, a whole-genome-based phylogenetic tree was constructed, and correlation analysis was performed among 28 type strains whose genomes were publicly available. The phylogeny of MLSA yielded a topology similar to that of core genes, and only a slight difference was observed in the position of *S. carassii* (Fig. S3). Similarities between the MLSA and *is*DDH analyses were calculated and are shown in Table S3. The *is*DDH values among distant species were concentrated at 20%. The *is*DDH values were highly correlated with the MLSA similarities ($R^2 = 0.9887$) in closely related *Shewanella* species (Fig. 2). Based on the simulative equation $y = 90.01 \times \exp(0.001112 \times x) - 431.3 \times \exp(-0.1927 \times x)$, the 70% *is*DDH value was equivalent to the 97.3% MLSA similarity, which could serve as a species boundary in the genus *Shewanella*.

Nevertheless, >97.3% concatenated sequence similarities were observed among two sets of species, i.e., *S. algae-S. haliotis-S. upenei* (28) and *S. japonica-S. pacifica*. The corresponding *is*DDH results between those groups of species were in the range of 83.7 to 88.9%, which exceeded the 70% species threshold (Fig. 2). The further pairwise ANI results between type strains of *S. algae-S. haliotis*, *S. algae-S. upenei*, and *S. haliotis-S. upenei* were 98.2, 98.1, and 98.2%, respectively, and the value of that between *S. japonica* and *S. pacifica* was 98.8%. All ANI values were greater than the boundary of 95% for species delineation. The genomic analysis based on *is*DDH and ANI provided

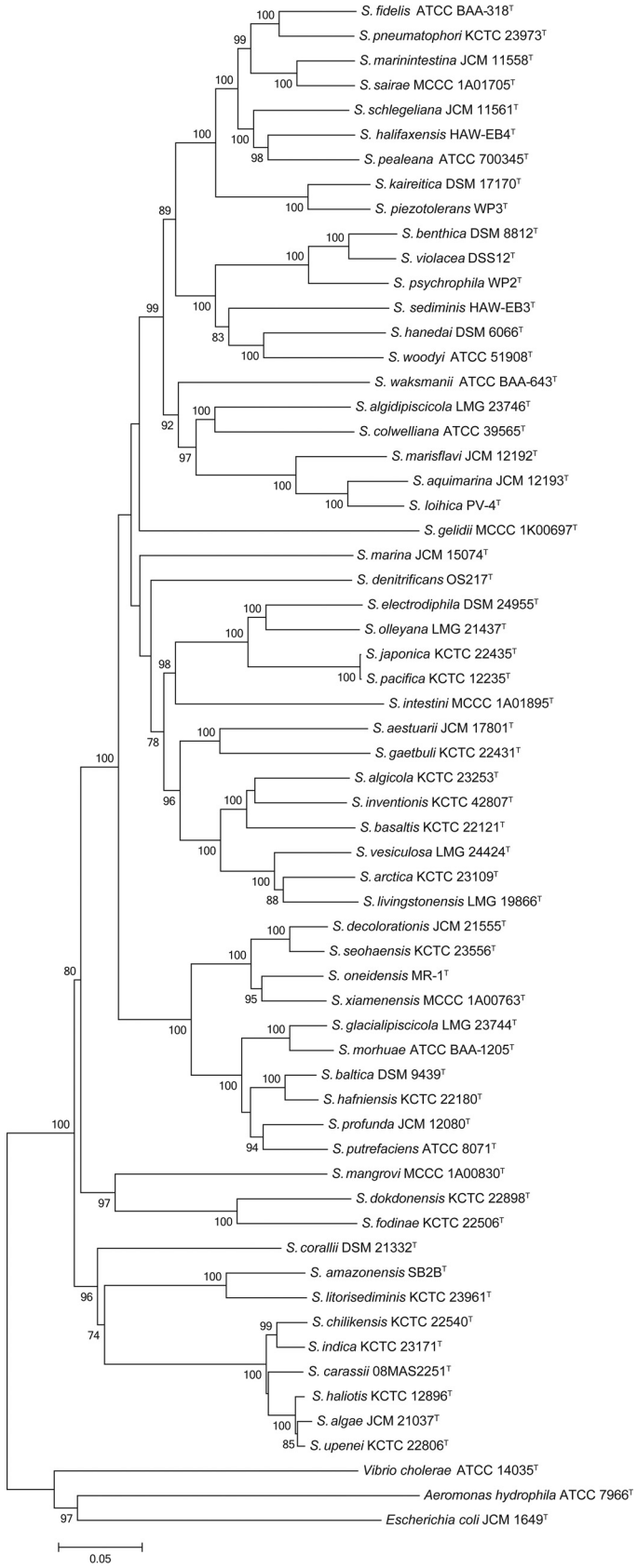

FIG 1 Phylogenetic tree reconstructed by the neighbor-joining method based on six concatenated gene sequences (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA* [4,191 bp]) of 59 *Shewanella* type strains. The robustness

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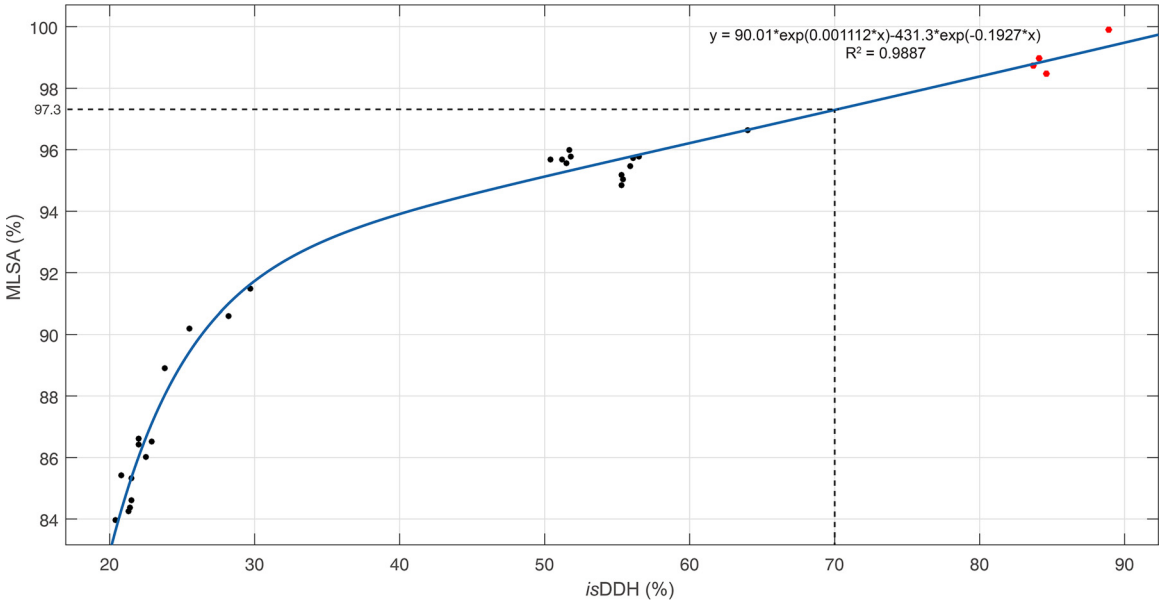

FIG 2 Correlation analysis between similarities of *is*DDH and MLSA for the genus *Shewanella*. The vertical line indicates a 70% *is*DDH threshold, and the horizontal line indicates the corresponding 97.3% MLSA similarity. The four points greater than the species boundary are marked in red.

compelling evidence for correct taxonomic position, indicating that *S. algae*, *S. haliotis*, and *S. upenei* were the same species (28) and that *S. pacifica* belonged to *S. japonica*. Additional phenotypic characteristics were detected among these five strains (Table 2). Minor differences in biochemical results were obtained between *S. algae*, *S. haliotis*, and *S. upenei*. The phenotypic discrepancies between *S. japonica* and *S. pacifica* were discovered for various growth conditions and the assimilation of *N*-acetylglucosamine. These results confirmed the conclusion of a recent report that identified *S. haliotis* as a synonym of *S. algae* according to whole-genome sequencing (28). Considering the genetic, genomic, and phenotypic characteristics, the *S. upenei* reported by Kim et al. (29) should be regarded as a later heterotypic synonym of *S. algae* proposed by Simidu et al. (30); meanwhile, the *S. pacifica* identified by Ivanova et al. (31) should be reclassified as a later heterotypic synonym of *S. japonica* described by Ivanova et al. (32).

Distinct taxonomic clades. Given the results of sequence diversity, topological stability, and concordance with genomes, the MLSA scheme of six protein-coding genes (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) was validated for taxonomic and evolutionary analysis among members of the *Shewanella* genus. The concatenated sequences for 56 species after emendation were subjected to construct the split network tree to explore evolutionary relationships among taxa (Fig. 3). Twelve distinct monophyletic clades were identified, i.e., the *Algae*, *Amazonensis*, *Aquimarina*, *Benthica*, *Colwelliana*, *Fodinae*, *Gaetbuli*, *Hanedai*, *Japonica*, *Livingstonensis*, *Pealeana*, and *Putrefaciens* clades (Table 3). The *Shewanella* species within the same clade shared $<$4 mol% GC variation and $>$84% MLSA concatenated similarity. There are eight orphan *Shewanella* species, namely, *S. corallii*, *S. denitrificans*, *S. gelidii*, *S. intestini*, *S. mangrovi*, *S. marina*, *S. sediminis*, and *S. waksmanii*, which form a distinct branch clearly separated from all taxonomic clades in the phylogenetic network, except for *S. sediminis*. *S. sediminis* harbored a far evolutionary distance similar to both *Hanedai* and *Benthica* clades and was located on the boundary of clade differentiation. Combined with the

FIG 1 Legend (Continued)
of tree topologies was evaluated with 1,000 bootstrap replications, and values of $>$70% are shown at the nodes of the branches. The scale bar indicates substitutions per site. The strains *Aeromonas hydrophila* ATCC 7966^T, *Escherichia coli* JCM 1649^T, and *Vibrio cholerae* ATCC 14035^T served as outgroups.

TABLE 2 Distinctive phenotypic characteristics among five *Shewanella* strains[a]

Characteristic	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
Growth at/in:					
4°C	–	–	–	–	+
35°C	+	+	+	+	–
0% (wt/vol) NaCl	+	+	+	+	–
6% (wt/vol) NaCl	+	+	+	–	+
Ornithine decarboxylase	+	+	+	–	–
Utilization of:					
D-Glucose	+	–	–	+	+
D-Maltose	–	–	–	+	+
N-Acetylglucosamine	+	+	+	+	–
DNA G+C content (mol%)	53.1	52.9	53.1	40.8	40.7

[a]Strains: 1, *S. algae* JCM 21037^T; 2, *S. haliotis* KCTC 12896^T; 3, *S. upenei* KCTC 22806^T; 4, *S. japonica* KMM 3299^T; 5, *S. pacifica* KMM 3597^T. +, Positive; –, negative.

ambiguous relationships between *S. sediminis* and clades *Hanedai* and *Benthica* in a single HKG phylogenetic tree, *S. sediminis* was considered an orphan species. Twelve evolutionary clades were always maintained in phylogenetic trees of individual and concatenated HKGs. There were only slight differences observed, i.e., *S. woodyi-S. hanedai* in *gyrB*, *S. colwelliana-S. algidipiscicola* and *S. gaetbuli-S. aestuarii* in *infB*, and *S. algidipiscicola-S. colwelliana* in *topA*, which were positioned closely but did not group within one clade in phylogenies.

Population genetics of *Shewanella* species in China. Eighty-six *Shewanella* strains isolated from diverse samples were involved in the analysis of sequences and phylogeny to evaluate the intraspecies relationships and investigate the distribution of *Shewanella* species in China. As shown in the concatenated phylogenetic tree (Fig. 4), 86 strains were divided into six compact clusters with high bootstrap support of 100%. Each cluster was represented by a unique *Shewanella* type strain situated in the *Algae*

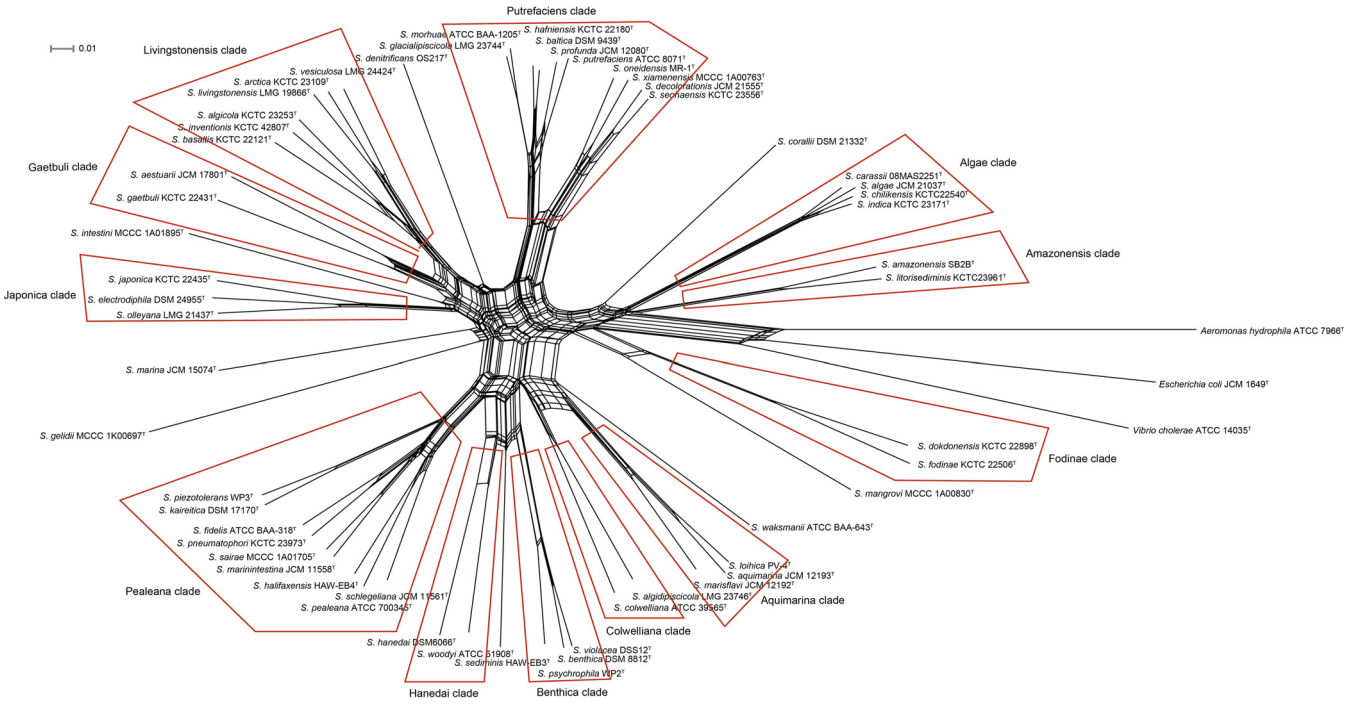

FIG 3 Concatenated split network tree based on six gene loci. The *gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA* gene sequences from 56 validated *Shewanella* species were concatenated and reconstructed using the SplitsTree 4 program. Twelve distinct clades were identified and are indicated in the figure by red outlines.

TABLE 3 G+C content and MLSA concatenated similarity of clades in *Shewanella* species

Clade	Described species	No. of species	G+C content (mol%)[a]	MLSA concatenated similarity (%)
Algae	*S. algae*, *S. carassii*, *S. chilikensis*, and *S. indica*	4	53–54	94.8–96.6
Amazonensis	*S. amazonensis* and *S. litorisediminis*	2	54	91.2
Aquimarina	*S. aquimarina*, *S. loihica*, and *S. marisflavi*	3	50–53	89.0–93.4
Benthica	*S. benthica*, *S. psychrophila*, and *S. violacea*	3	47–49	90.6–94.6
Colwelliana	*S. colwelliana* and *S. algidipiscicola*	2	46–47	85.4
Fodinae	*S. fodinae* and *S. dokdonensis*	2	50–51	87.4
Gaetbuli	*S. gaetbuli* and *S. aestuarii*	2	43	84.3
Hanedai	*S. hanedai* and *S. woodyi*	2	44–46	87.0
Japonica	*S. japonica*, *S. electrodiphila*, and *S. olleyana*	3	43	87.7–89.6
Livingstonensis	*S. livingstonensis*, *S. algicola*, *S. arctica*, *S. basaltis*, *S. inventionis*, and *S. vesiculosa*	6	43–44	85.1–91.8
Pealeana	*S. pealeana*, *S. fidelis*, *S. halifaxensis*, *S. kaireitica*, *S. marinintestina*, *S. piezotolerans*, *S. pneumatophori*, *S. sairae*, and *S. schlegeliana*	9	44–46	84.0–93.5
Putrefaciens	*S. putrefaciens*, *S. baltica*, *S. decolorationis*, *S. glacialipiscicola*, *S. hafniensis*, *S. morhuae*, *S. oneidensis*, *S. profunda*, *S. seohaensis*, and *S. xiamenensis*	10	46–50	84.6–96.3

[a]Calculated based on the concatenated sequences of six HKGs.

and *Putrefaciens* clades. In comparison with the concatenated phylogenetic tree, several unexpected locations were observed in the single HKG tree: strain 08MAS2647 in the *S. algae* cluster fell into the *S. chilikensis* cluster in *gyrA*; strains 08MAS2647, 11MAS2711, 11MAS2745, and 11MAS2746 in the *S. algae* cluster formed a subcluster next to the *S. carassii* cluster in *infB*; and strains in the *S. algae*, *S. carassii*, and *S. chilikensis* clusters exhibited a close affiliation that could not be separated from each other in *recN*. Although some strains could be grouped into clusters properly in individual phylogenetic trees, clusters were supported with low bootstrap values, such as *S. algae* cluster in the 16S rRNA, *gyrA*, and *infB* genes, as well as *S. seohaensis* cluster in the *gyrA* and *gyrB* genes (Fig. S4). Hence, concatenated sequences derived from six HKGs exhibited good performance and robustness in identifying *Shewanella* strains. Since strains were defined as corresponding species in the concatenated phylogenetic tree, ranges of intraspecies and interspecies similarities for genes among the 56 validated *Shewanella* species were measured and are shown in Fig. 5. Overlaps between the intraspecies and interspecies similarities were observed among the genes 16S rRNA, *gyrA*, *infB*, *recN*, *rpoA*, and *topA*. A small interval was detected in the *gyrB* gene, with only 0.1% variance. A notable gap was discovered in concatenated sequences. A minimum intraspecies similarity was found among *S. seohaensis* strains (97.8%), and the maximum interspecies similarity existed between *S. chilikensis* and *S. indica* (96.8%), which differed by 1% variation, corresponding to approximately 40-bp divergences.

A total of 86 *Shewanella* strains collected from China were assessed to define species via the MLSA approach. The most dominant *Shewanella* species was identified as *S. algae* (66.3%), followed by *S. xiamenensis* (11.6%), *S. chilikensis* (9.3%), *S. indica* (8.1%), *S. seohaensis* (3.5%), and *S. carassii* (1.2%). Except for *S. seohaensis*, which was only isolated from the environment, the remaining five species were relevant to clinical patients. It is noteworthy that *S. algae*, *S. xiamenensis*, *S. chilikensis*, and *S. indica* were also discovered in food samples consisting of both marine products and cooked food for sale. Consequently, MLSA as a proper discrimination for *Shewanella* species played a significant role in public health and regular surveillance.

DISCUSSION

In this study, the MLSA scheme, based on six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*), was established to carry out efficient classification, reflect evolutionary relationships, and delineate population biology in the genus *Shewanella*. Totals of 59 recognized type strains and 86 Chinese strains were investigated to explore the interspecies and intraspecies sequence diversity and phylogenetic topology in *Shewanella* species.

Previously, the 16S rRNA gene was applied as a traditional genetic marker among the genus *Shewanella* (7, 33, 34). However, the resolving power of the 16S rRNA gene was restricted, with fewer parsimony informative sites and lower nucleotide diversity

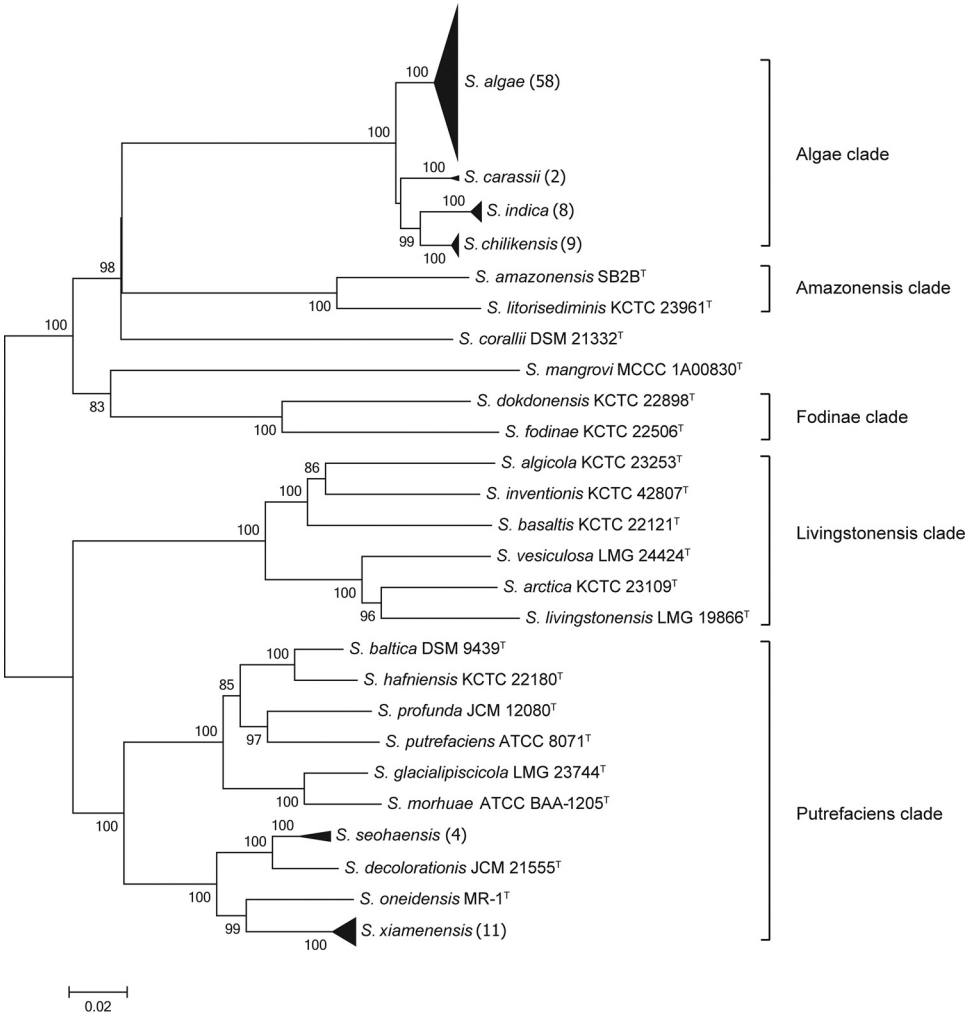

FIG 4 Phylogenetic tree reconstructed by the neighbor-joining method based on six concatenated gene sequences (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*, 4191 bp) of 86 *Shewanella* tested strains and 26 related type strains. The number of tested strains for each compact cluster (black triangle) is shown in parentheses (each of these clusters also contained one type strain). The robustness of tree topologies was evaluated with 1,000 bootstrap replications, and values of >70% are shown at the nodes of the branches. The scale bar indicates the substitutions per site.

values. A narrow range of sequence variation was observed, and multiple pairs of *Shewanella* species shared >99% similarity. The latest proposed threshold of 98.65% for 16S rRNA was insufficient to differentiate species in the genus *Shewanella* (35). In addition, the existence of sequence variation among *rrn* operons would perplex the species definition and evolutionary analysis for taxa (36). Hence, protein-coding genes with a greater genetic resolution were utilized to determine the taxonomic positions of *Shewanella* species.

Comparable analysis was performed among six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*). Misclassification of some tested strains was discerned in the *gyrA* and *infB* genes for the high biological diversity among *S. algae* strains. High interspecies similarities of these HKGs were generated, making it difficult to discern closely related species. The *gyrB* gene has always been used as a basic detection for novel *Shewanella* species identification (7, 20–22). However, the criterion for *gyrB* analysis was not well established, and the boundary between interspecies and intraspecies similarities was inconspicuous. The *recN* gene was the most variable HKG, with the greatest rates of parsimony informative sites and the widest spectrum of interspecies similarity. Although the *recN* gene was unsuccessful in making a distinction in the *Algae* clade, the effective discrimination was proven by high sequence substitution rates in the majority

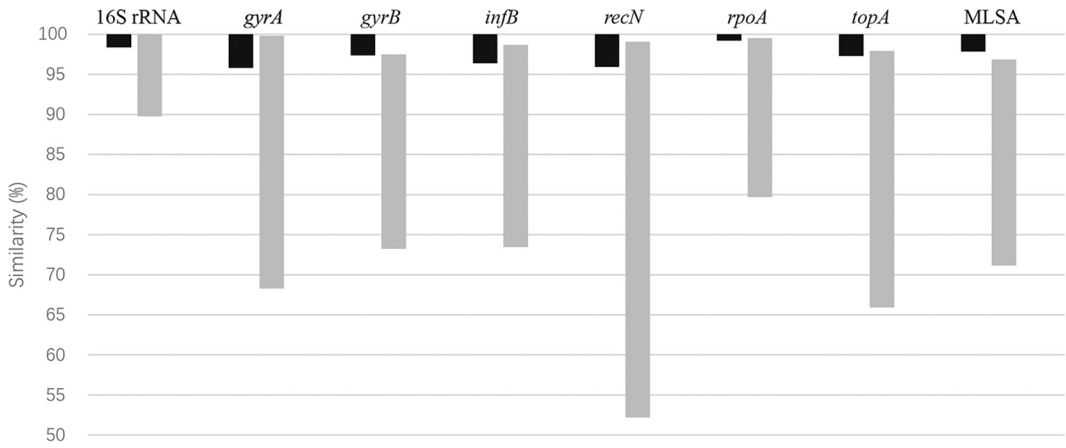

FIG 5 Intraspecies and interspecies similarities of 16S rRNA, six HKGs, and MLSA for 56 validated *Shewanella* species. The ranges of similarity are displayed in black (intraspecies) and gray (interspecies).

of species. The *rpoA* gene was more conserved than other HKGs, with limited variable sites. None of the tested strains were phylogenetically located at unexpected positions, and only a slight overlap was detected between the intraspecies and interspecies ranges. The *topA* gene possessed a high genetic divergence next to the *recN* gene. The unstable taxonomic subtree with a low bootstrap value was discovered in the *Colwelliana* clade. The various evolutionary rates and inconsistencies of phylogenetic topology were discovered in these six loci. Therefore, concatenated sequences with integrated and sufficient information should be taken into account to obtain the exact *Shewanella* species classification.

The concatenation of six HKGs demonstrated enough resolution power to discern *Shewanella* species in regard to variable sites, sequence divergences, and independent branches. A notable gap between the ranges of interspecies and intraspecies similarities was favorable for defining the strains unambiguously at the species level, and 97.3% MLSA similarity was proposed as a species threshold in the genus *Shewanella*. The neighbor-joining phylogenetic tree indicated that all validated species positioned at a distinct branch were clearly separated from closely related taxa. The stability of the phylogenetic tree was proven by bootstrap and topology analysis. The concatenated sequence phylogeny was supported by high bootstrap values among interspecies having a significant advantage over all individual genes. The phylogenetic tree grouped *Shewanella* strains into intraspecies clusters and taxonomic clades with almost 100% bootstrap support. The use of the maximum-likelihood method had a slight impact on the tree topology. The reliability of the MLSA scheme was validated by comparison with genomic sequences. Identical phylogenies were constructed by concatenated sequences of six HKGs and core genes. A high correlation between the similarities of the MLSA and *is*DDH was discovered. Combined with the analysis of the resolution, stability, and reliability for nucleotide sequences and phylogenies, the MLSA approach of six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) showed a significant performance for the precise classification of *Shewanella* species.

Under comprehensive analysis, exceptional cases were only observed among two sets of recognized species, i.e., *S. algae*-*S. haliotis*-*S. upenei* (28) and *S. japonica*-*S. pacifica*. Based on molecular, genomic, and phenotypic analyses, these five species were reclassified correctly, and the taxonomic structure of the *Shewanella* genus was refined. It is noteworthy that previous studies proposing these five novel species depended largely on the individual sequence analysis of 16S rRNA, experimental DDH, and biochemical tests (10, 29–32). The high sequence similarities of 16S rRNA genes between phylogenetic neighbors have already been observed, and the results of wet-lab DDH below 70% were regarded as the gold standard for species classification (37). However, the experimental DDH was hard to reproduce completely by different

laboratories; thus, the digital DDH based on the bacterial genomes was recommended in microbial systematics (25, 26). The phenotypic traits are inclined to be conservative among the *Shewanella* genus, and limited characteristics are suitable to discriminate *Shewanella* species. The deviation of the biochemical results could be attributed to the different manual procedures and bacterial growth statuses. The phenotypic discrepancies in growth conditions and the carbon source utilization observed among *S. japonica* and *S. pacifica* were also reported in the reclassification of *S. affinis* and *S. colwelliana* (38). Therefore, the accurate molecular method of MLSA is considered a promising alternative tool for species identification and is superior to genomic analysis in terms of high efficiency and low cost.

In addition, the MLSA scheme provided a portable and robust system to reflect evolutionary relationships for the genus *Shewanella*. Twelve distinct phylogenetic clades were proposed with identical G+C contents and greater nucleotide similarity in concatenated sequences. The Chinese strains collected from clinical specimens and routine monitoring were located on *Algae* and *Putrefaciens* clades. These results indicated that species in monophyletic clades have a tendency to share a close genetic relationship, tracing back to common ancestry, and occupy similar geographical positions. These clades could be almost retrieved from individual HKG phylogenies, further elucidating the accurate and stable evolutionary structure in the *Shewanella* taxon. Eight orphan species separated from all phylogenetic clades were defined. Attempts to involve the remaining species and identify the novel *Shewanella* species were conducive to exploring taxonomic positions for these species. In summary, the concatenated phylogeny provided significant insight into the evolutionary structure of the *Shewanella* genus.

Furthermore, it has been verified that *Shewanella* species, as marine pathogens, are associated with human diseases (12). Misidentifications to the species level were fairly common in clinical diagnoses due to the poor discernment system (39). In this study, 86 *Shewanella* strains collected from environmental, food, and clinical samples in China were mainly defined as *S. algae*, followed by *S. xiamenensis*, *S. chilikensis*, *S. indica*, *S. seohaensis*, and *S. carassii*, via the MLSA scheme. Five *Shewanella* species were verified to have clinical connections: *S. algae*, *S. carassii*, *S. chilikensis*, *S. indica*, and *S. xiamenensis*. It was likely that some *Shewanella* pathogens identified as *S. algae* in previous studies belonged to *S. carassii*, *S. chilikensis*, and *S. indica* for their high 16S rRNA similarities. Apart from *S. carassii*, four species were also frequently collected from marine products, as well as from cooked food for sale. It was reported that a common mechanism causing *Shewanella* infections was ascribed to the consumption of seafood or raw fish (12). Therefore, more attention is needed to reinforce continuous surveillance for the genus *Shewanella* by the MLSA approach in the processes of clinical diagnosis and food sales.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 145 *Shewanella* strains were involved in this study. Forty-two type strains were collected from the China General Microbiological Culture Collection Center (CGMCC), the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ]), the Japan Collection of Microorganisms (JCM), the Korean Collection for Type Cultures (KCTC), the Belgian Coordinated Collections of Microorganisms (BCCM/LMG Bacteria Collection), and the Marine Culture Collection of China (MCCC). Detailed information of type strains is listed in Table S1. Eighty-six tested strains were isolated from patients ($n = 44$), food ($n = 35$), and the environment ($n = 7$) in four provinces (Anhui, Hainan, Liaoning, and Shandong) of China from 2007 to 2016. The 42 *Shewanella* type strains were incubated at suitable conditions according to the protocols of culture collection. The tested strains were isolated and characterized according to procedure described previously (40). The pure colonies were cultured on Marine Agar 2216 (BD, Difco) at 35°C for 18 h.

DNA extraction, gene selection, and primer design. Genomic DNA from *Shewanella* strains was extracted with a genomic DNA extraction kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The 16S rRNA gene of tested strains was amplified and sequenced with two universal primers (27F and 1492R) described previously (41). Six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) were chosen for the MLSA scheme. The degenerate primers of HKGs for PCR amplification, except the *gyrB* gene (42), were designed from genome sequences of *Shewanella* type strains in the GenBank database

TABLE 4 Primers used in this study

Locus	Primer	Sequence (5′–3′)[a]	Position[b]	Amplicon size (bp)	Annealing temp (°C)	Source or reference
16S rRNA	16S-27F	AGAGTTTGATCCTGGCTCAG	8–27	1,503	52	45
	16S-1492R	GGTTACCTTGTTACGACTT	1492–1510			
gyrA	gyrA-164F	TGAAGAACGATTGGAACAARCCNTAYAARAARTC	164–197	664	56	This study
	gyrA-827R	TTTTCAATCAAACGAGCTTTGTTHACYTGRTAHGG	793–827			
gyrB	UP-1	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	274–314	1,256	58	42
	UP-2r	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT	1486–1529			
infB	infB-1426F	ATGCCACAGACTATTGAAGCDATYCARCAYGC	1426–1457	830	56	This study
	infB-2255R	GCATCAGCACGAACGTTAAARCCNAYMAKRATNGC	2221–2255			
recN	recN-415F	AGTGAGCATCAACTGACCYTRYTNGAYAGYTAYGC	415–449	863	54	This study
	recN-1277R	GGTTGTAAAGGTTGCCCTGGGTTDGTNSWNAC	1246–1277			
rpoA	rpoA-83F	TGGAGCCGCTTGAGCGTGGTTTYGGHCAYAC	83–113	751	56	This study
	rpoA-833R	ATGTAATGAATCGCTTCGGCYTTYARRCAGTT	802–833			
topA	topA-70F	GAATTCATCGTTAAGTCGAGYGTDGGBCAYRT	70–101	860	60	This study
	topA-929R	CGCTGGGCCATCATCATGGTYTTYTTNACNCC	898–929			

[a]The nondegenerate primers in the 5′ region for sequencing are underlined.
[b]Position numbering is based on the complete genome of *Escherichia coli* K-12 (NC_000913.3).

(Table S1) to accommodate a wide taxonomic scope. The nondegenerate primers in the 5′ region for sequencing are underlined in Table 4.

PCR amplification and sequencing. Amplification reactions for six HKGs were performed in a total volume of 25 μl, containing 12.5 μl of 2× EsTaq MasterMix (Cwbiotech, China), 2 μl of each forward and reverse primer (10 μM), 1.5 μl of template DNA (10 to 30 ng/μl), and 7 μl of ultrapure water, using a SensoQuest LabCycler. The PCR mixture was subjected to denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54 to 60°C for 30 s, and extension at 72°C for 60 s, with a final extension step at 72°C for 10 min. More detailed information on annealing temperatures is given in Table 4. PCR amplicons were verified by electrophoresis on 1% agarose. The amplified products were purified and sequenced using the ABI 3730xl platform.

Analysis of nucleotide diversity. The sequences of 16S rRNA, *gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA* genes used for MLSA were trimmed to positions 56 to 1455, 247 to 744, 337 to 1446, 1519 to 2181, 565 to 1200, 139 to 756, and 106 to 768, respectively, corresponding to *Escherichia coli* numbering (43). The evolutionary distances and sequence similarities of the 16S rRNA gene and of individual and concatenated HKGs were calculated using MEGA X v10.05 with Kimura's two-parameter model. The parsimony informative sites and K_a/K_s ratios (K_a, the number of nonsynonymous substitutions per nonsynonymous site; K_s, the number of synonymous substitutions per synonymous site) were analyzed using DnaSP 6.0 (44).

Phylogenetic analysis. The nucleotide sequences were aligned using MEGA X v10.05. The phylogenetic trees of the 16S rRNA gene and the individual and concatenated sequences of six HKGs (*gyrA*, *gyrB*, *infB*, *recN*, *rpoA*, and *topA*) were constructed by neighbor-joining and maximum-likelihood methods with MEGA X v10.05. The model selected was Kimura's two-parameter with the pairwise-deletion option. The parameter used for maximum-likelihood inference was the nearest-neighbor interchange. The robustness of tree topologies was evaluated with 1,000 bootstrap replications, and values of >70% are shown at the nodes of the branches. The split network tree of MLSA was performed by SplitsTree 4.14.4 using the Jukes-Cantor correlation.

Genomic relatedness. Twenty-eight *Shewanella* type strains with complete genomes available in GenBank (Table S1) were utilized to investigate the concordance and correlation between MLSA and genomes. Core genes of genomic sequences identified by OrthoMCL 2.0.9 were concatenated to construct the phylogenetic tree. The *is*DDH results were measured by the Genome-to-Genome Distance Calculator (GGDC) (http://ggdc.dsmz.de/). The ANI values were estimated by using the web-based platform EZBioCloud (http://www.ezbiocloud.net/tools/ani) with the OrthoANIu algorithm. The correlation between the *is*DDH results and MLSA similarities was simulated using MATLAB R2016a (MathWorks, Inc.) with nonlinear interpolation analysis.

Phenotypic characteristics. Further phenotypic tests were performed using the five strains of *Shewanella* species whose *is*DDH values were greater than the species threshold. The type strains of species were examined in parallel under suitable conditions. Physiological and biochemical traits were determined by using commercial strips, including API 20E and API 20NE (bioMérieux, France), in accordance with standard manufacturer's instructions.

Data availability. The nucleotide sequences of the six HKGs have been deposited in the GenBank nucleotide sequence database under the following accession numbers: MH090144 to MH090185 (*gyrA*), MH090186 to MH090202 (*gyrB*), MH090203 to MH090244 (*infB*), MH090245 to MH090286 (*recN*), MH090287 to MH090328 (*rpoA*), and MH090329 to MH090370 (*topA*).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03126-18.

SUPPLEMENTAL FILE 1, PDF file, 3.2 MB.

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