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ABSTRACT

Phylogenetic analyses were done for the *Shewanella* strains isolated from Baltic Sea (38 strains), US DOE Hanford Uranium bioremediation site [Hanford Reach of the Columbia River (HRCR), 11 strains], Pacific Ocean and Hawaiian sediments (8 strains), and strains from other resources (16 strains) with three out group strains, *Rhodospseudomonas palustris*, *Clostridium cellulolyticum*, and *Thermoanaerobacter ethanolicus* X514, using DNA relatedness derived from WCGA-based DNA-DNA hybridizations, sequence similarities of 16S rRNA gene and *gyrB* gene, and sequence similarities of 6 loci of *Shewanella* genome selected from a shared gene list of the *Shewanella* strains with whole genome sequenced based on the average nucleotide identity of them (ANI). The phylogenetic trees based on 16S rRNA and *gyrB* gene sequences, and DNA relatedness derived from WCGA hybridizations of the tested *Shewanella* strains share exactly the same sub-clusters with very few exceptions, in which the strains were basically grouped by species. However, the phylogenetic analysis based on DNA relatedness derived from WCGA hybridizations dramatically increased the differentiation resolution at species and strains level within *Shewanella* genus. When the tree based on DNA relatedness derived from WCGA hybridizations was compared to the tree based on the combined sequences of the selected functional genes (6 loci), we found that the resolutions of both methods are similar, but the clustering of the tree based on DNA relatedness derived from WMGA hybridizations was clearer. These results indicate that WCGA-based DNA-DNA hybridization is an idea alternative of conventional DNA-DNA hybridization methods and it is superior to the phylogenetics methods based on sequence similarities of single genes. Detailed analysis is being performed for the re-classification of the strains examined.

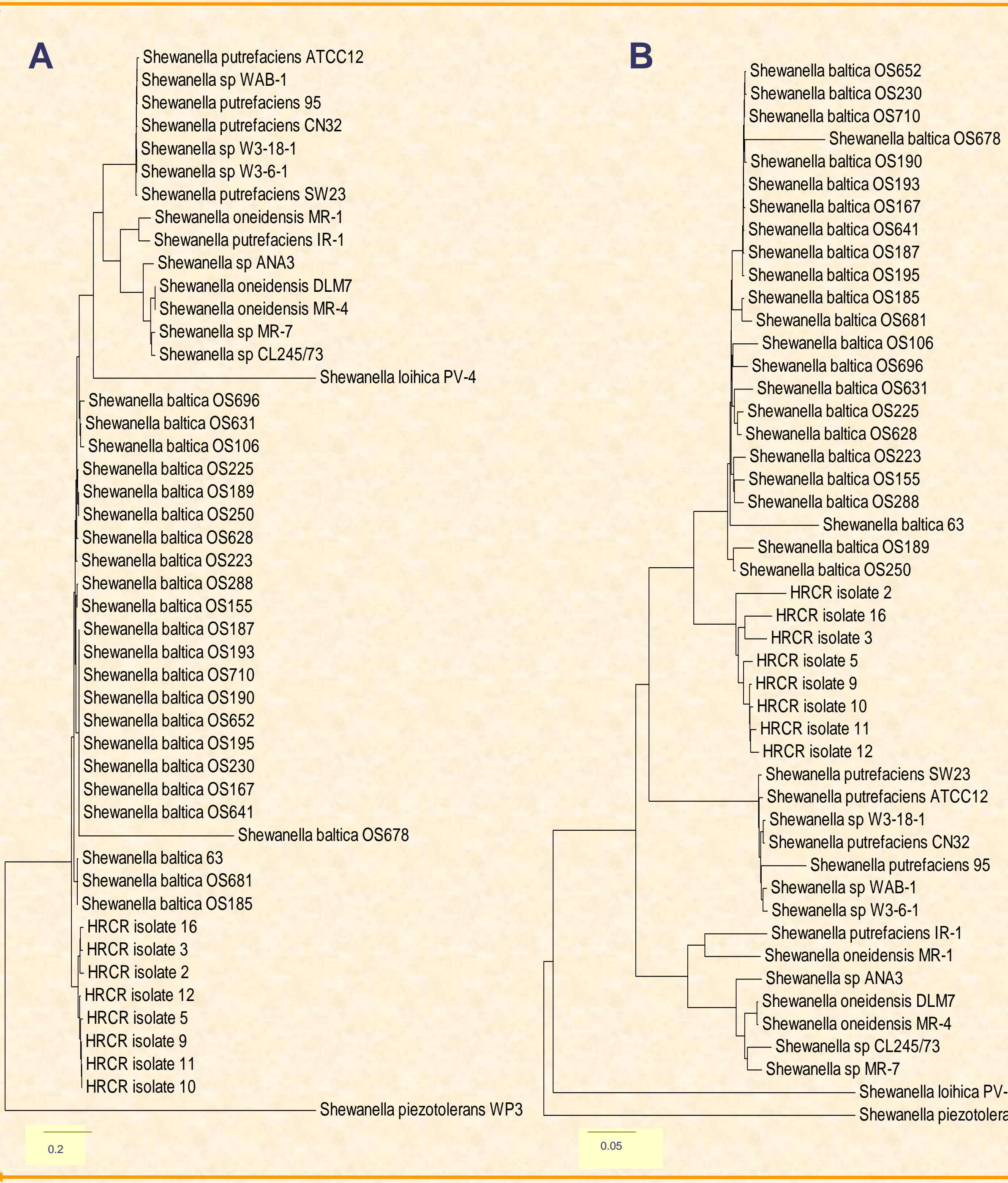
Keywords: Phylogenetic analysis, *Shewanella*, whole genome Microarray

METHODS AND STRAINS

1. Strains tested (table 1);
2. DNA isolation. The genomic DNAs were isolated from pure cultures using phenolchloroform method.
3. 16S rRNA and *gyrB* amplification and phylogenetic tree construction. 16S rRNA and *gyrB* were amplified from genomic DNAs, Sequenced at Oklahoma Medical Research Center. Phylogenetic tree were constructed using MEGA software with NJ method.
4. Microarray Construction, Labeling, and Hybridization. Genomic DNAs were printed on glass slides at concentrations of 200 ng/μL. Genomic DNA from collected *Shewanella* spp., as well as several strains not belonging to *Shewanella* (*Thermoanaerobacter ethanolicus* X514, *Rhodospseudomonas palustris*, and *Paracoccus denitrificans*) as outside groups were printed on the arrays. Whole genomic DNA of target strains was fluorescently labeled using the random priming method. Microarray hybridization was carried out on Tecan and performed as described by Wu, L et al (2004).
5. Microarray Scanning and Data Analysis. A ScanArray 5000 Microarray Analysis System was used for scanning microarrays at a resolution of 5 μm with 75% laser power and 65% PMT gain. Scanned image displays were analyzed using ImaGene version 6.0. A grid of individual circles defining the location of each DNA spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. Mean signal intensity was determined for each spot. For decreasing the variations DNA amount from printing, the corresponding signals of Syto 61 of the same arrays stained after completely washed were used for normalization.
6. Data analysis. A distant matrix of DNA relatedness was constructed according to mean signal intensity among tested strains, and clustered using MEGA software. Phylogenetic analyses were done for sequence similarities of 16S rRNA *gyrB* gene, and multiple loci using the same methods.

No.	Bacteria Strains	Source of Isolation	No.	Bacteria Strains	Source of Isolation
1	<i>Shewanella</i> sp WAB-1	Wabash River, IN	39	<i>Shewanella baltica</i> OS117	Baltic Sea, Gottedand Deep
2	<i>Shewanella</i> sp W3-6-1	marine sediments, Pacific Ocean (897m, 9-10 cm core), Washington State	40	<i>Shewanella baltica</i> OS109	Baltic Sea, Gottedand Deep
3	<i>Shewanella</i> sp W3-18-1	marine sediments, Pacific Ocean (830m, 5-6 cm core), Washington State	41	<i>Shewanella baltica</i> OS107	Baltic Sea, Gottedand Deep
4	<i>Shewanella frigidimarina</i> SW38	North Sea, UK	42	<i>Shewanella baltica</i> OS106	Baltic Sea, Gottedand Deep
5	<i>Shewanella putrefaciens</i> SW2.3	temperate marine estuary, UK	43	<i>Shewanella algae</i> OK-1	ventosolium producing red alga
6	<i>Shewanella</i> sp SP-7	clinical isolates, from Seattle WA	44	<i>Shewanella</i> sp MR-7	Black Sea, 60 m depth
7	<i>Shewanella</i> sp SP-32	clinical isolates, from Astoria	45	<i>Shewanella oneidensis</i> MR-4	Black Sea, 5 m depth
8	<i>Shewanella</i> sp SP-3	clinical isolate, unknown source	46	<i>Shewanella</i> sp MR-30	freshwater sediments, Green Bay, WI
9	<i>Shewanella</i> sp SP-22	clinical isolates, from Seattle WA	47	<i>Shewanella oneidensis</i> MR-1	Lake Onondaga sediments
10	<i>Shewanella</i> sp SP-10	clinical isolates, from Seattle WA	48	<i>Shewanella</i> sp LMP-1	chemodrine, Lower Mystic Pond, MA
11	<i>Shewanella amazonensis</i> SB28	low salinity mud flat sediment at a depth of 1 m, Cabo Cassipone, Armaiz, Brazil, 1996	49	<i>Shewanella putrefaciens</i> IR-1	Rice Paddy, Korea
12	<i>Shewanella</i> sp PV-4	Naha Vents, Hawaii	50	<i>Shewanella oneidensis</i> DLM7	Lower Green Bay sediments, Lake Michigan
13	<i>Shewanella baltica</i> OS710	Baltic Sea, Gottedand Deep	51	<i>Shewanella putrefaciens</i> CN-32	subsurface rock at Carru Negro, NM, 1994, Yale U
14	<i>Shewanella baltica</i> OS696	Baltic Sea, Gottedand Deep	52	<i>Shewanella</i> sp CL245/73	human cerebrospinal fluid
15	<i>Shewanella baltica</i> OS681	Baltic Sea, Gottedand Deep	53	<i>Shewanella</i> sp CE-1	Melt Pool, near Cape Evans, Ross Island, Antarctica
16	<i>Shewanella baltica</i> OS678	Baltic Sea, Gottedand Deep	54	<i>Shewanella</i> sp BC-1	Karst Stream water, Blue Clay, IN
17	<i>Shewanella baltica</i> OS652	Baltic Sea, Gottedand Deep	55	<i>Shewanella putrefaciens</i> ATCC 8012	surface tainted butter
18	<i>Shewanella baltica</i> OS650	Baltic Sea, Gottedand Deep	56	<i>Shewanella putrefaciens</i> ATCC 19857	machine oil
19	<i>Shewanella baltica</i> OS645	Baltic Sea, Gottedand Deep	57	<i>Shewanella putrefaciens</i> ATCC 12099	machine oil
20	<i>Shewanella baltica</i> OS641	Baltic Sea, Gottedand Deep	58	<i>Shewanella putrefaciens</i> ATCC 12099	arsenic-treated wood in Finnish estuary, Woods Hole, MA
21	<i>Shewanella baltica</i> OS631	Baltic Sea, Gottedand Deep	59	<i>Shewanella putrefaciens</i> 200	oil pipeline, Alberta, Canada
22	<i>Shewanella baltica</i> OS628	Baltic Sea, Gottedand Deep	60	<i>Shewanella putrefaciens</i> 95	surface tainted butter
23	<i>Shewanella baltica</i> OS625	Baltic Sea, Gottedand Deep	61	<i>Shewanella baltica</i> 63	oil tank
24	<i>Shewanella baltica</i> OS622	Baltic Sea, Gottedand Deep	62	<i>Shewanella</i> SP W5-18	
25	<i>Shewanella baltica</i> OS616	Baltic Sea, Gottedand Deep	63	<i>Shewanella</i> sp 1	Hanford Reach of the Columbia River
26	<i>Shewanella baltica</i> OS615	Baltic Sea, Gottedand Deep	64	<i>Shewanella</i> sp 2	Hanford Reach of the Columbia River
27	<i>Shewanella baltica</i> OS610	Baltic Sea, Gottedand Deep	65	<i>Shewanella</i> sp 3	Hanford Reach of the Columbia River
28	<i>Shewanella baltica</i> OS605	Baltic Sea, Gottedand Deep	66	<i>Shewanella</i> sp 4	Hanford Reach of the Columbia River
29	<i>Shewanella baltica</i> OS602	Baltic Sea, Gottedand Deep	67	<i>Shewanella</i> sp 5	Hanford Reach of the Columbia River
30	<i>Shewanella baltica</i> OS595	Baltic Sea, Gottedand Deep	68	<i>Shewanella</i> sp 9	Hanford Reach of the Columbia River
31	<i>Shewanella baltica</i> OS593	Baltic Sea, Gottedand Deep	69	<i>Shewanella</i> sp 10	Hanford Reach of the Columbia River
32	<i>Shewanella baltica</i> OS590	Baltic Sea, Gottedand Deep	70	<i>Shewanella</i> sp 11	Hanford Reach of the Columbia River
33	<i>Shewanella baltica</i> OS589	Baltic Sea, Gottedand Deep	71	<i>Shewanella</i> sp 12	Hanford Reach of the Columbia River
34	<i>Shewanella baltica</i> OS587	Baltic Sea, Gottedand Deep	72	<i>Shewanella</i> sp 16	Hanford Reach of the Columbia River
35	<i>Shewanella baltica</i> OS585	Baltic Sea, Gottedand Deep	73	<i>Shewanella</i> sp 19	Hanford Reach of the Columbia River
36	<i>Shewanella</i> sp 184	surface tainted butter	74	<i>Rhodospseudomonas palustris</i>	
37	<i>Shewanella baltica</i> OS167	Baltic Sea, Gottedand Deep	75	<i>Thermoanaerobacter ethanolicus</i> X514	
38	<i>Shewanella baltica</i> OS155	Baltic Sea, Gottedand Deep	76	<i>Clostridium cellulolyticum</i>	

RESULTS: Multiple Locus Sequence Typing (MLST)



RESULTS: Phylogenetic Analyses based on DNA Relatedness, Sequence similarities of 16S RNA and *gyrB* genes

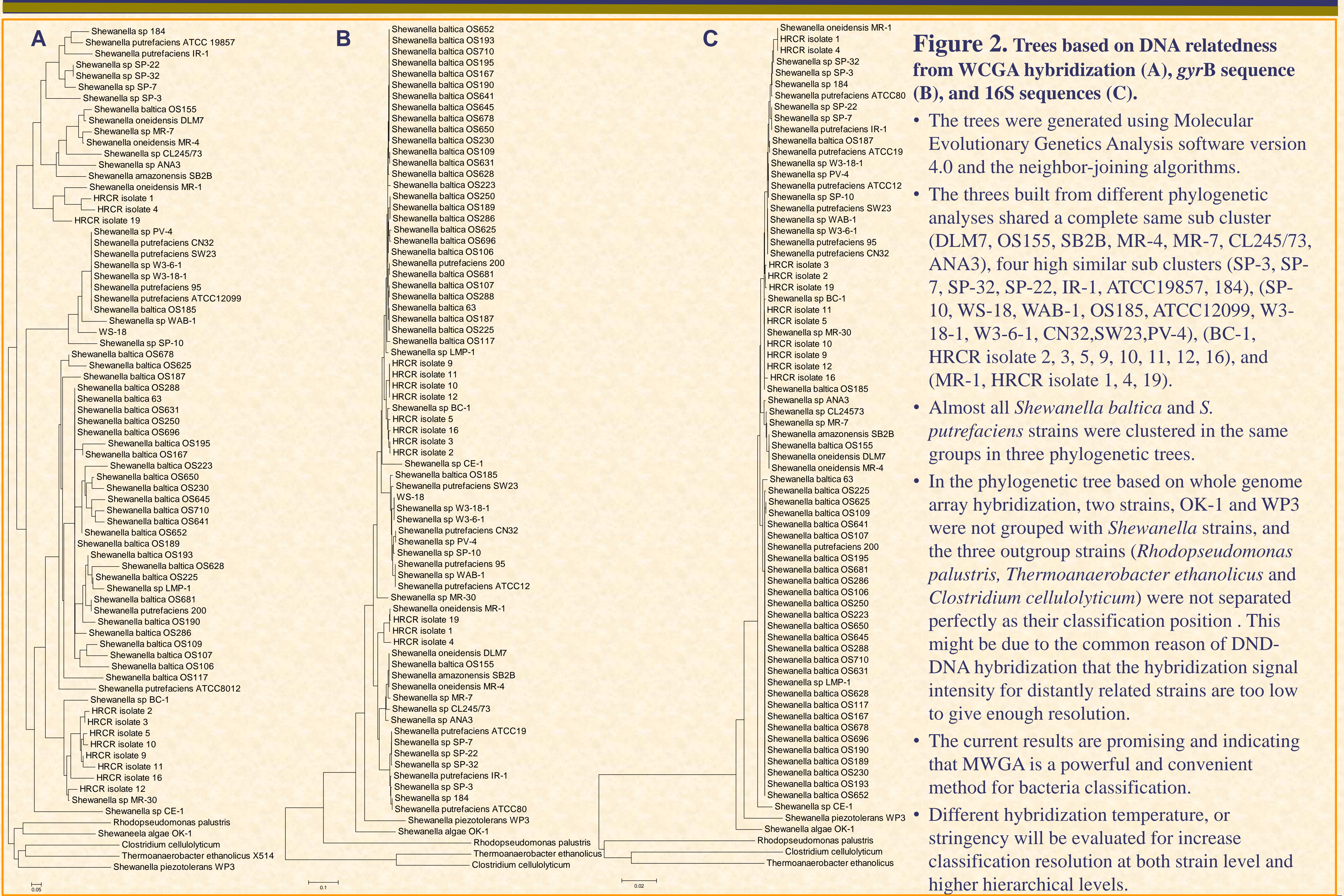


Figure 1. Trees based on MLST

- Genes selected: Three genes coding permease, phosphoglycolate phosphatase, and thymidylate kinase; one gene coding OsmC/Ohr family protein; an unknown periplasmic protein, and another unknown protein.
- Sequences of these genes were collected for about 20 sequenced *shewanella* strains.
- For those strains the whole genome sequences not available, these genes were PCR amplified and sequenced.
- Phylogenetic trees were generated based on sequences of each single functional gene and the combined sequences of all selected functional genes using MEGA 4.0.
- The trees based on sequences of a single gene coding an unknown protein (Fig. 1 A) and the combined sequences of all selected functional genes (Fig. 1 B) are shown.
- The differentiation resolution of phylogenetic analysis based on a single functional gene is very low and the classification is not consistent with other methods; however, while based on the combined sequences it is higher and the clustering of the tree is more clear and consistent with other methods.
- When the tree based on DNA relatedness derived from WCGA hybridizations was compared to the tree based on the combined sequences of the selected functional genes, we found that the resolutions of both methods were similar, but the clustering of the tree based on DAN relatedness is clearer.

CONCLUSIONS

- The accuracy of the classification by WCGA hybridizations was confirmed by the phylogenetic analyses using 16S RNA and *gyrB* genes.
- The differentiation resolution of the WCGA hybridizations method is higher than those using sequence similarities of 16S RNA and *gyrB* genes, and the multiple loci shared by different genomes.
- Using WCGA hybridizations, the 11 HRCR strains can be clustered into two groups: one group contains strains 2, 3, 5, 9, 10, 11, 12, and 16, which are closely related to *Shewanella* sp. BC-1; another group contains strains 1, 4, and 19, which are similar to *Shewanella oneidensis* MR-1. This classification is consistent with that based on *gyrB* gene sequence similarities, and is also consistent with that based on the 16S RNA gene similarities, with one exception that strain 19 is not with the group similar to MR-1, but with the big group.
- Using WCGA hybridization method, most of the Baltic Sea strains are classified together, with only two exceptions that strain OS185 is classified with *Shewanella putrefaciens* strains, and strain OS155 is classified with *Shewanella oneidensis* strains. The classifications of these strains are similar based on 16S RNA and *gyrB* gene sequence similarities, but with a few different exceptions. WCGA hybridization can cluster the major group of Baltic Sea strains further into several sub-clusters, which is different from the other two methods.

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