Distribution, abundance, and diversity patterns of the thermoacidophilic “deep-sea hydrothermal vent euryarchaeota 2”

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INTRODUCTION

The diversity of Archaea associated with marine hydrothermal vent habitats is unrivaled in any other ecosystem on Earth (Auguet et al., 2009). Much of this diversity resides within the Euryarchaeota where numerous cultivated and uncultivated lineages appear to be endemic to the deep-sea. One such lineage is the “deep-sea hydrothermal vent euryarchaeota 2” (DHVE2; Takai and Horikoshi, 1999). Previously, our knowledge of the distribution and diversity of the DHVE2 was based primarily on cultivation-independent assessments (Table A1 in Appendix). These studies established that the DHVE2 are widespread in marine hydrothermal environments and can account for up to 15% of the archaeal rRNA gene sequences, suggesting that they are important members of deep-sea hydrothermal ecosystems (Reysenbach et al., 2006). More recently, 16S rRNA gene cloning studies have shown that certain types of deep-sea vent mineral deposits, namely horizontal flanges (shelf-like structures that form in some hydrothermal systems, Figure 1, Tivey, 2007), can harbor an even greater proportion of DHVE2 16S rRNA gene sequences (Nunoura and Takai, 2009).

The first cultured representative of the DHVE2, Aciduliprofundum boonei T469T, was the first obligate thermoacidophile isolated from deep-sea vents despite the low pH of most hydrothermal fluids (pH 2.8–4.5) and predictions of acidic microhabitats within the walls of vent deposits (Tivey, 2004; Reysenbach et al., 2006). Acidic habitats are generated in vent deposits by conductive cooling of end-member fluids or by transport of hydrothermal fluids outward across deposit walls by diffusion (Tivey, 2004). However, when hydrothermal fluids and seawater mix, either in the subsurface or by advection across deposit walls, neutrality of the fluids is quickly reached resulting in most marine hydrothermal vent habitats being circumneutral. Therefore, the pH of end-member fluids and the degree of fluid mixing within an individual deposit are likely important factors in controlling the distribution and abundance of thermoacidophilic DHVE2 both within and between vent fields. Factors that influence the pH of hydrothermal fluids at the vent field scale include the presence of organic sediments, which increases the pH of fluids as at the Guaymas Basin (GB), and inputs of magmatic volatiles as observed in the low pH fluids of the Mariner vent site along the Eastern Lau Spreading Center (ELSC, Tivey, 2007). Furthermore, fluid mixing styles can be influenced by the type of vent deposit as hydrothermal fluids associated with horizontal flanges are conductively cooled with little or no mixing of seawater,
FIGURE 1 | Photograph of deep-sea hydrothermal vent mineral deposits from the Eastern Lau Spreading Center. A series of horizontal flanges are shown in the foreground while two vertical chimneys can be seen in the background. Photo was taken from ROV Jason II (courtesy of Woods Hole Oceanographic Institution).

while the mixing styles of vertical chimney deposits are much more variable (Tivey, 2004). Taken together, these factors suggest that thermoacidophilic niches in hydrothermal vent deposits vary not only across vent fields but also within any individual vent field.

Geologic processes such as significant breaks in ridge axes (i.e., transform faults) or hotspots in hydrothermal activity can provide barriers for dispersal and hence influence diversification and speciation. Such distribution patterns influenced by geologic processes have been shown to play a significant role in the biogeographical patterns of deep-sea hydrothermal vent fauna (Van Dover et al., 2002), but whether similar factors influence microbial distribution patterns is less clear. Microbial biogeographical diversity patterns have been reported for terrestrial geothermal springs (Papke et al., 2003; Whitaker et al., 2003; Takacs-Vesbach et al., 2008; Wagner, 2010), yet there are relatively few studies that report the biogeography of microorganisms at deep-sea hydrothermal vents (Holden et al., 2011; Huber et al., 2006; Flores et al., 2011b). Here, we investigated the occurrence, abundance, and phylogenetic diversity of the DHVE2 in hydrothermal vent deposits from several geochemically distinct and spatially distant vent fields to explore whether the distribution patterns of this lineage are influenced by geographic separation.

MATERIALS AND METHODS

SAMPLE COLLECTION

Deep-sea hydrothermal mineral deposits were collected with the HOV Alvin or ROV Jason II during research cruises to the East Pacific Rise (EPR) in 2007, the Mid-Atlantic Ridge (MAR) in 2008, the ELSC in 2009, and the GB in 2009. Once shipboard, individual samples were processed and stored anaerobically as previously described (Götz et al., 2002; Moussard et al., 2004; Reysenbach et al., 2006).

QUANTITATIVE POLYMERASE CHAIN REACTION

DNA from environmental samples was extracted from homogenized mineral deposits [≈1.6–3.2 g (w/w)] using the Ultra Clean Soil DNA Isolation Kit (MO BIO Laboratories) according to the modified protocol of Reysenbach et al. (2006). Quantitative PCR (qPCR) was performed according to manufacturer’s instructions using the Quantitect SYBR green PCR kit (Qiagen, Inc., Valencia, CA, USA) and 0.8 μM final primer concentrations, with melting curves run at the end of each reaction to ensure product specificity. Primers and thermocycling conditions were followed according to Reysenbach et al. (2006). Standard curves (10⁷–10¹⁰ gene copies/μl) for both Archaea and the DHVE2 were generated from a plasmid containing the nearly full-length 16S rRNA gene sequence of A. boonei T469T. All standards and samples were run in at least duplicate reactions. Gene copy numbers presented were averaged across replicates and normalized by the amount of material in grams (w/w) extracted.

LOCAL SIMILARITY ANALYSIS

The variable region 4 (V4) of archaeal 16S rRNA genes from 57 deposits from the MAR (Flores et al., 2011a), ELSC (Flores et al., in revision), and GB (Reysenbach, unpublished data)
were amplified, pyrosequenced, trimmed, aligned, and clustered at 97% sequence similarity as previously described (Flores et al., 2011a). DHVE2 operational taxonomic units (OTUs) were identified by performing BLAST searches (Altschul et al., 1990) using representative sequences of each OTU against the 16S rRNA gene of A. boonei T469, selecting OTUs with greater than 95% sequence similarity to A. boonei T469 and manually aligning sequences and generating phylogenetic trees in a custom ARB database (Ludwig et al., 2004). For local similarity analysis (LSA), a technique that explores co-variables of microbial species (or OTUs) to one another (Ruan et al., 2006), all OTUs with greater than 100 sequences, including three DHVE2 OTUs, were normalized and imported into the LSA compute tool (http://meta.cmb.ucsd.edu/). Results of LSA were trimmed to include only the two most prevalent DHVE2 OTUs (ID no.’s DHVE2-727 and DHVE2-1148), and other OTUs that were positively and negatively correlated ($P < 0.05$). Visualization of the resulting interaction network was performed using Cytoscape (Shannon et al., 2003). Correlated OTUs were classified to the lowest taxonomic level that had a bootstrap value of $≥50\%$ (Claesson et al., 2009) using the RDP-classifier (Wang et al., 2007). Pyrosequencing data is available for download from the MG-RAST server (Meyer et al., 2008) or by contacting the corresponding author.

**MULTI-LOCUS SEQUENCE ANALYSIS**

We used multi-locus sequence analysis (MLSA; Gevers et al., 2005) to further examine the phylogenetic divergence among isolates. The protein-coding genes chosen for MLSA were: DNA repair and recombination protein RadA, rada; ATP synthase, A subunit, atpa; DNA-directed RNA polymerase subunit B, rpoB; translation elongation factor aEF-2, Ef-2; and preprotein translocase, SecY subunit, secY. These genes were chosen because they were previously used to investigate the relationships between taxa within the Halobacteriales (Papke et al., 2011). The protein-coding genes were distributed around the genome of A. boonei T469 (Table A2 in Appendix).

Primers for the PCR amplification and sequencing of the protein-coding loci were designed based on the protein-coding genes in A. boonei T469 (NCBI # PRJNA34333), and related Thermoplasmatales with sequenced genomes; Thermoplasma acidophilum (Accession: PRJNA61573), Thermoplasma volcanium (NCBI # PRJNA35129), Picrophilus torridus (NCBI # PRJNA36697), and Ferroplasma acidarmanus (NCBI # PRJNA35151). Initial PCR primers were designed by using the oligonucleotide design tool from IDT SciTools (Integrated DNA Technologies), and then modified based on the nucleotide and amino acid conservation at potential primer regions (**Table 1**). Primers were supplied by Invitrogen (Life Technologies). Thermocycler conditions for the amplification of the protein-coding loci were 94°C for 2 min; 30 cycles of: 94°C for 45 s, annealing temperature (**Table 1**) for 45 s, 72°C for extension; then 72°C for 5 min. Annealing temperatures were optimized for each primer set (data not shown). PCR products were purified using the UltraClean PCR Clean-Up Kit (MO BIO Laboratories) according to manufacturer’s instructions. Purified PCR products were used as templates for Sanger sequencing reactions. Electropherograms of the protein-coding loci sequencing reads were analyzed and assembled using the software SeqMan (DNASTAR, Inc.).

Nucleotide polymorphism and DNA sequence variation of the protein-coding loci were calculated using the software DnaSP v 5.10.01 (Librado and Rozas, 2009) and MEGA v 5.04 (Tamura et al., 2011), respectively, using only unambiguous nucleotide positions for a diversity of Archaea (789 nt).

**Table 1 | Primers and thermocycler conditions for the amplification of protein-coding loci from DHVE2 isolates.**

| Protein-coding loci | Primer name | Primer sequence | Annealing temperature (°C) | Thermocycler extension time |
|---------------------|-------------|-----------------|---------------------------|-----------------------------|
| rada                | rada_F313a  | GGTGGTTAGAACACAGGCCATA TTDAGYAACTGTGCCTCNGC | 57 or 64 | 24 s |
| rpaB                | rpaB_973Fa  | AAGAGATTGCAGCAAGGCAAGG | 55 | 1 min, 1 s |
| atpa                | atpa_1496Fa | GCRCTCTGCTGAARAAATCTYTC | 50 | 40 s |
| secY                | secY_714Fa  | CTGNNTTYTCTKAGGAYGARG | 49 | 1 min, 2 s |
| EF2                 | EF2_364Fb   | GTWAVGARTGNTGNACCCACAC  | 53 | 48 s |
| EF2                 | EF2_1122Fb  | TGAACNOCNGARCCCGCWATWGC | 53 | 48 s |
et al., 2011). Metrics calculated were: $G + C$ mol%; number of nucleotide sequence alleles, $n_s$; the number of polymorphic nucleotide sites, $S_n$; the total number of mutations, $e$; the nucleotide diversity, $P_i$; and the number of inferred primary protein sequence alleles, $n_{pp}$; and the number of polymorphic amino acid residues, $S_{pp}$. Synonymous and non-synonymous positions and substitutions were examined using DnaSP v5.10.01 (Librado and Rozas, 2009). MEGA v5.04 (Tamura et al., 2011) was used to evaluate models for nucleotide substitution for each protein-coding locus and to construct phylogenetic trees. The model having the lowest goodness-of-fit Bayesian Information Criterion (BIC) value was used to generate a maximum-likelihood bootstrap consensus tree based on 100 replicates. The initial tree for the maximum-likelihood analysis was constructed automatically and the Nearest-Neighbor-Interchange heuristic search method was used to search for topologies that fit the data better. In all analyses of sequence diversity or phylogeny, the sequence length homologous among all isolates was utilized.

### RESULTS AND DISCUSSION

#### OCCURRENCE AND RELATIVE ABUNDANCE OF THE DHVE2

To determine the occurrence and relative abundance of the DHVE2 in deposits from geologically distinct vent fields, archaeal and DHVE2 16S rRNA genes were quantified using qPCR. Archaeal 16S rRNA genes were successfully amplified from 130 samples. Deposits from Tui Malila along the ELSC had, on average, the lowest archaeal copy number ($8.35\times10^6$ copies/g (w/w)) while TAG along the MAR had the highest archaeal copy number ($9.78\times10^7$ copies/g (w/w); Table 2). Although these values cover a wide range, they are similar to archaeal abundances that have been reported from other hydrothermal vent deposits (Takai et al., 2001; Schrenk et al., 2003; Nakagawa et al., 2005; Zhou et al., 2009). Using group specific primers, the DHVE2 were observed at all vent fields but in only 60% (78/130) of samples analyzed. At individual vent fields, the DHVE2 were most frequently observed at Mariner (80% of samples), EPR (77.8%), and TAG (75%). In contrast, they were detected in less than 50% of samples from Tui Malila (37.5%), TowCam (42.9%), and the GB (48.1%; Table 2). While their occurrence varied within an individual vent field, these results clearly illustrate the ubiquity of the DHVE2 at deep-sea vents and suggest that differences in the geological properties that influence end-member fluid pH over the ranges we examined do not inhibit colonization by the DHVE2 at these vent sites. For example, the end-member fluid pH at Mariner is around 2.5 while at GB the fluids are around pH 4.5. Yet niches are still available for colonization of the DHVE2 at both sites. Assuming all members of the DHVE2 are thermoacidophilic, then thermoacidophily is a common ecological strategy in deep-sea hydrothermal ecosystems.

In samples where the DHVE2 were not detected, the average archaeal abundance was significantly lower, at $3.64\times10^5$ copies/g (w/w), than in deposits where the DHVE2 were observed, which averaged $3.34\times10^7$ copies/g ($P = 0.002$, one-tailed $T$-test; data not shown). Additionally, deposits where the DHVE2 were absent were typically newly formed, thin-walled structures without an obvious biofilm on the exterior of the deposit. Previous work demonstrated that, while Archaea are typically the initial colonizers of newly formed vent deposits, they are primarily autotrophic with later colonization by heterotrophic Archaea and Bacteria (Page et al., 2008). Consequently, the occurrence of the DHVE2 in an individual deposit may be dependent upon the presence of a mature microbial community from which to scavenge fermentable peptides (Reysenbach and Flores, 2008). Older deposits also

| Table 2 | Results of qPCR assays to determine the occurrence and relative abundance of the DHVE2 in hydrothermal vent deposits collected between 2006 and 2009 from several different vent fields. |
| --- | --- | --- |
| **Eastern Lau Spreading Center** | **Average archaeal 16S rRNA gene copies per gram of deposit (SEM)** | **Average DHVE2 16S rRNA gene copies per gram of deposit (SEM)** | **Average proportion of DHVE2 16S rRNA gene copies (%)** |
| Kilo Moana ($n=8$, 62.5%) | $2.86 \times 10^7$ (1.25 \times 10^7) | $1.74 \times 10^5$ (1.50 \times 10^5) | 0.38 |
| Tow Cam ($n=7$, 42.9%) | $1.91 \times 10^7$ (1.08 \times 10^7) | $5.39 \times 10^6$ (3.32 \times 10^6) | 1.21 |
| Tahí Moana ($n=11$, 63.8%) | $1.51 \times 10^7$ (2.58 \times 10^6) | $5.65 \times 10^5$ (2.02 \times 10^5) | 2.39 |
| ABE ($n=11$, 63.6%) | $7.25 \times 10^6$ (2.78 \times 10^6) | $1.58 \times 10^6$ (6.17 \times 10^5) | 1.39 |
| Tui Malila ($n=6$, 37.5%) | $8.35 \times 10^5$ (2.59 \times 10^5) | $2.30 \times 10^5$ (9.22 \times 10^4) | 12.88 |
| Mariner ($n=15$, 80.0%) | $3.14 \times 10^6$ (2.11 \times 10^6) | $1.62 \times 10^5$ (7.53 \times 10^4) | 12.69 |
| **Mid-Atlantic Ridge** | | | |
| Lucky Strike ($n=10$, 70%) | $6.41 \times 10^5$ (4.22 \times 10^5) | $1.34 \times 10^5$ (5.98 \times 10^4) | 14.34 |
| Rainbow ($n=12$, 66.7%) | $2.16 \times 10^5$ (7.15 \times 10^4) | $4.49 \times 10^4$ (1.27 \times 10^4) | 0.14 |
| TAG ($n=4$, 75.0%) | $9.78 \times 10^5$ (6.98 \times 10^5) | $5.19 \times 10^5$ (5.04 \times 10^5) | 0.57 |
| Guaymas Basin ($n=27$, 48.1%) | $5.36 \times 10^5$ (2.37 \times 10^5) | $1.52 \times 10^5$ (5.03 \times 10^4) | 1.36 |
| East Pacific Rise ($n=9$, 77.8%) | $4.08 \times 10^5$ (1.62 \times 10^5) | $2.71 \times 10^5$ (1.96 \times 10^4) | 7.26 |

SEM, standard error of the mean.

*Average proportion of DHVE2 was calculated for only samples with positive amplification for both archaea and the DHVE2.

*Numbers in parentheses indicate the total number of samples with positive archaeal amplification and the percentage of those with positive amplification of the DHVE2.
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generally have more defined walls and fluid conduits that would help isolate the hydrothermal fluids from seawater allowing for less mixing of seawater and more conductive cooling of the fluids.

Quantitative PCR was also used to determine the proportion of the DHVE2 in the archaeal communities. Within individual vent fields, the average percentage of DHVE2 16S rRNA gene copies in the archaeal community ranged from 0.14% at Rainbow to 14.74% at Lucky Strike. Deposits from Mariner (12.69%), Tui Malila (12.88%), and EPR (7.26%) also had, on average, a high percentage of DHVE2 sequences within their archaeal communities (Table 2). This level of relative abundance is in agreement with previous reports (Reysenbach et al., 2006; Nunoura and Takai, 2009) and implies that, when the DHVE2 are present, they can be a significant component of the archaeal community. However, it is difficult to compare their relative abundance to other archaeal groups from previous studies because of differences in the methods employed to determine abundances.

In general, individual samples having the highest proportion of DHVE2 gene copies were typically flanges (Figure 2), although some exceptions were noticed (e.g., chimney sample EPR07-75). As fluids in flanges are conductively cooled with little seawater mixing, these fluids remain acidic as they cool to habitable temperatures and percolate vertically through the structure generating relatively larger thermoacidic zones than predicted in vertical chimney structures. Similar situations could conceivably develop in thin-walled, chalcopyrite-lined chimneys (like Mariner-1652). In a recent study, the DHVE2 accounted for nearly 46% of the archaeal 16S rRNA gene diversity associated with a sample from a flange deposit from the Yonaguni Knoll IV hydrothermal field in the western Pacific Ocean (Nunoura and Takai, 2009). Our data further support the observation that flanges may be "hotspots" for the DHVE2 and likely, other thermoacidophiles.

Table 3 | Results of local similarity analysis illustrating the co-occurrence of the DHVE2 with other archaeal OTUs.

| OTUs classified as DHVE2 | Number of sequences | Percentage of deposits | Lowest taxonomic classification with a bootstrap value greater than 50% |
|-------------------------|--------------------|------------------------|------------------------------------------------------------------|
| 727                     | 3557               | 89.5                   | Euryarchaeota (98)                                               |
| 1148                    | 1389               | 65.0                   | Euryarchaeota (92)                                               |
| 1137                    | 120                | 10.5                   | Euryarchaeota (100)                                              |

| OTUs positively correlated with 727 and 1148 |
|---------------------------------------------|
| 421                                         | 538                  | 38.6                   | Archaea (85)                                                     |
| 544                                         | 171                  | 38.6                   | Ferroglobus (55)                                                 |
| 547                                         | 190                  | 15.8                   | Euryarchaeota (93)                                               |
| 593                                         | 2204                 | 65.0                   | Euryarchaeota (93)                                               |
| 648                                         | 134                  | 70.0                   | Euryarchaeota (87)                                               |
| 677                                         | 101                  | 29.8                   | Euryarchaeota (52)                                               |
| 739                                         | 101                  | 40.4                   | Euryarchaeota (91)                                               |
| 927                                         | 990                  | 70.2                   | Thermomonomonas (55)                                             |
| 1026                                        | 145                  | 19.3                   | Euryarchaeota (72)                                               |
| 1248                                        | 535                  | 22.8                   | Euryarchaeota (65)                                               |

| OTUs negatively correlated with 727 and 1148 |
|---------------------------------------------|
| 461                                         | 3539                 | 71.9                   | Archaeoglobus (98)                                              |
| 549                                         | 7143                 | 68.4                   | Thermoproteaceae (64)                                           |
| 594                                         | 6680                 | 91.2                   | Aeropyrum (53)                                                  |
| 638                                         | 5105                 | 77.2                   | Desulfurococcales (98)                                          |
| 738                                         | 1371                 | 68.4                   | Staphylothermus (99)                                             |

FIGURE 2 | Percentage of DHVE2 16S rRNA gene sequences in the archaeal communities of hydrothermal vent deposits from several different vent fields as determined using qPCR. →, Indicates a chimney sample with a high proportion of DHVE2 sequences while * indicates two samples collected from a single flange deposit and illustrates the spatial heterogeneity of the DHVE2. Abbreviations are: Rb, Rainbow; TaM, Tai Moana; TC, Tow Cam; TuM, Tui Malila; LS, Lucky Strike; Guay09, Guaymas Basin 2009; KM, Kilo Moana; EPR07, East Pacific Rise 2007.
Spatial variability on and in a deposit can be shaped by a number of factors including heterogeneous wall thickness, deposit mineralogy, and fluid flow rate. For most of the deposits collected and analyzed in this study, only the outer few millimeters were sampled, as this is where the majority of microorganisms are detected (Takai et al., 2001; Schrenk et al., 2003; Nakagawa et al., 2005; Kormas et al., 2006; Nunoura and Takai, 2009). However, for some of the deposits, we sampled exterior and interior sections of chimneys and for flanges, different spatial areas on the top, bottom and edge. As a result of this sampling strategy, we have paired samples from a few deposits that illustrate the spatial variability of the DHVE2 associated with individual deposits. Spatial variability on a deposit was best illustrated by a flange structure collected from the Tui Malila vent field along the ELSC. On the bottom of this deposit, the DHVE2 comprised over 70% of the archaeal 16S rRNA gene sequences detected (Tui Malila-1059) while they were undetectable on the edge (Tui Malila-1066; Figure 2).

**CO-OCCURRENCE PATTERNS OF THE DHVE2 WITH OTHER ARCHAEA**

In previous studies, barcoded pyrosequencing was used to characterize the archaeal communities of numerous vent deposits from geochemically and geographically distinct hydrothermal vent fields MAR (Flores et al., 2011a), ELSC (Flores et al., in revision) and GB (Reysenbach, unpublished data). These large data sets provided an opportunity to examine the co-occurrence of the DHVE2 with other archaeal lineages using LSA. In total, three OTUs were identified as the DHVE2 (ID no.’s DHVE2-727, DHVE2-1148, and DHVE2-1137) and contained 3557, 1389, and 120 sequences, respectively (Table 3). DHVE2-727 and DHVE2-1148 were present in 89 and 65% of samples, respectively, while DHVE2-1137 was present in only 10% of samples. Using LSA, we found that the occurrence of DHVE2-727 and DHVE2-1148 were positively correlated with one another but not with DHVE2-1137 (Figure 3). Due to the low abundance, relatively rare occurrence and lack of correlation with the...
other DHVE2, results of LSA including DHVE2-1137 are not presented.

The majority (9/10) of positively correlated OTUs were Euryarchaeota (Figure 3; Table 3), but most (7 of 9) could not be classified below the phylum making it difficult to speculate on potential ecological relationships between these OTUs and the DHVE2. Some may be involved in syntrophic relationships with the DHVE2 as has been proposed for the fermentative Thermococcales (Bonch-Osmolovskaya and Stetter, 1991; Rinker and Kelly, 2000). Others may share the acidophilic strategy with the DHVE2 but utilize different carbon and/or energy sources that would allow all to co-exist in the same biotope. For example, the two OTUs that could be identified below the phylum level were related to Thermogymnomonas and Ferroglobus (Table 3). Described species of these two genera have somewhat complementary non-competing lifestyles to the DHVE2 representative, the peptide-utilizing anaerobic acidophile, A. boonei T469T. Thermogymnomonas is a an aerobic sugar-utilizing thermoacidophile (Itoh et al., 2007) and therefore would occupy a slightly different acidic niche than A. boonei T469T. The only described Ferroglobus species, F. placidus uses a range of electron donors and acceptors, and can reduce (Tor et al., 2001) and oxidize (Hafenbradl et al., 1996) iron, which could provide relatives of this OTU metabolic flexibility in the dynamic hydrothermal environment. Because iron solubility is greater at lower pH, the acidic niche may favor these potential iron oxidizers.

In contrast to the positively correlated OTUs, all negatively correlated OTUs could be confidently classified to at least the order level (Figure 3; Table 3). Some of the negatively correlated OTUs were related to the thermophilic neutrophiles, Archaeoglobus (Stetter, 1988; Burggraf et al., 1990; Seeher et al., 1994) and Aeropyrum (Sako et al., 1996). Since no known acidophilic members belong to these genera, the negatively correlated OTUs may require different physical conditions (e.g., neutral pH, more oxidizing conditions) that do not permit anaerobic thermoacidophiles to grow. Thus, the positively correlated OTUs may share the same acidophilic strategy of the DHVE2 and are able to co-exist because of different carbon and/or energy requirements, while the negatively correlated OTUs may require different physical conditions.

**PHYLOGENETIC DIVERSITY OF CULTURED DHVE2**

To expand the cultured diversity of the DHVE2, numerous enrichment cultures targeting thermoacidophiles were initiated from samples collected in 2006 to 2009. In total, 12 axenic DHVE2 isolates were obtained with 4 from the MAR (2 from Lucky Strike, 1 from Rainbow, 1 from TAG), 6 from the ELSC (1 from Tui Malila, 5 from Mariner), and 2 from the EPR (Table A3 in Appendix). All isolates grew well (overnight growth) under anaerobic, thermoacidophilic conditions but optimal growth conditions were not determined. No isolates were obtained from the GB despite the presence of similar sequences in the pyrosequencing dataset (Table 2) and detection of the DHVE2 (by amplification with DHVE2 specific

![FIGURE 4 | Neighbor-joining tree based on 16S rRNA gene sequence comparisons of novel DHVE2 isolates and other archaeal families. Note that strains MAR08-276, MAR08-307, and MAR08-361 were not included in MLSA analysis as ambiguities were observed in protein-coding genes but not in 16S rRNA gene sequences. Bootstrap percentages above 50% are shown for the neighbor-joining analysis (based on 500 replicates) and for the maximum-likelihood analysis (based on 100 replicates). The phylogenetic tree was generated considering only unambiguously aligned nucleotide positions for a diversity of Archaea \(n = 789\). New isolates are shown in bold. The scale bar represents 0.01 changes per nucleotide position. All 16S rRNA gene sequences from the DHVE2 isolates were deposited in the European Nucleotide Archive database under accession numbers FR865176 to FR865190.](www.frontiersin.org)
FIGURE 5 | Maximum-likelihood bootstrapped phylogenetic trees of various protein-coding genes from DHVE2 isolates. (A) atpA consensus tree constructed using the Kimura 2-parameter model (Kimura, 1980) taking into account a gamma distribution for the substitution rate. (B) rpoB consensus tree constructed using the Tamura-Nei model (Tamura and Nei, 1993). (C) secY consensus tree generated using Tamura 3-parameter model (Tamura, 1992). (D) EF-2 consensus tree generated as in (C). (E) radA consensus tree generated as in (A). Bootstrap values greater than 60 are shown at nodes in each tree. Branch lengths are the number of substitutions per site. Gene sequences from Thermoplasma acidophilum were used as outgroups in all trees [Gene ID’s (A) = 1455676, (B) = 1456006, (C) = 1456739, (D) = 1456055, (E) = 1456613].
PCR primers) in two enrichment cultures (data not shown). Manipulating the pH, temperature, and organic substrates in order to isolate the DHVE2 from GB samples were unsuccessful as Thermococcus species typically outgrew the DHVE2.

With the exception of strain Lau09-1128, all isolates shared greater than 97% 16S rRNA gene sequence similarity with one another and A. boonei T469\(^1\) (Figure 4). The high 16S rRNA sequence similarity is comparable to the diversity reported for Thermococcus species from different vent fields (e.g., Huber et al., 1995, 2006; Canganella et al., 1998; Holden et al., 2001) but quite different from deep-sea vent thermophilic Deltaproteobacteria, which were clearly differentiated based on their 16S rRNA gene sequences (Flores et al., 2011b). Despite the overall high 16S rRNA gene sequence similarity of all cultured DHVE2, the isolates nonetheless clustered together based on the vent field of isolation (Figure 4). Other thermophilic archaeal lineages, most notably the Sulfolobales (Whitaker et al., 2003; Reno et al., 2009) and Thermococcales (Huber et al., 2006), did not exhibit such

| Protein-coding locus | Set | N  | Length | \(n_a\) | \(S_{nt}\) | \(\text{Eta}\) | \(P_i (\times 100)\) | \(n_{pp}\) | \(S_{pp}\) |
|---------------------|-----|----|--------|--------|---------|--------|-----------------|-------|--------|
| radA\(^1\)         | All | 10 | 334    | 10     | 90      | 104    | 14.33           | 2     | 6      |
|                    | ELSC| 6  | 334    | 6      | 23      | 24     | 2.99            | 1     | 0      |
|                    | MAR | 4  | 334    | 4      | 11      | 11     | 1.70            | 1     | 0      |
| EF-2\(^2\)         | All | 10 | 685    | 10     | 158     | 175    | 11.38           | 4     | 12     |
|                    | ELSC| 6  | 685    | 6      | 34      | 36     | 2.10            | 2     | 1      |
|                    | MAR | 4  | 685    | 4      | 31      | 31     | 2.31            | 2     | 1      |
| secY               | All | 12 | 868    | 10     | 263     | 341    | 15.12           | 4     | 23     |
|                    | ELSC| 6  | 868    | 4      | 40      | 42     | 2.27            | 1     | 0      |
|                    | EPR | 2  | 868    | 2      | 7       | 7      | 0.81            | 1     | 0      |
|                    | MAR | 4  | 868    | 4      | 51      | 53     | 3.23            | 2     | 1      |
| rpoB               | All | 12 | 894    | 12     | 299     | 361    | 15.67           | 10    | 36     |
|                    | ELSC| 6  | 894    | 6      | 36      | 37     | 1.72            | 4     | 3      |
|                    | EPR | 2  | 894    | 2      | 9       | 9      | 1.01            | 2     | 1      |
|                    | MAR | 4  | 894    | 4      | 50      | 51     | 3.02            | 4     | 6      |
| atpA               | All | 12 | 585\(^6\) | 11    | 173     | 219    | 14.29           | 7     | 12     |
|                    | ELSC| 6  | 592    | 5      | 35      | 35     | 2.89            | 3     | 2      |
|                    | EPR | 2  | 592    | 2      | 7       | 7      | 1.18            | 2     | 1      |
|                    | MAR | 4  | 585\(^6\) | 4     | 26      | 26     | 2.25            | 2     | 1      |

\(N\), the number of sequences of the particular set included in the analysis.

Length, the length of the protein-coding loci examined.

\(n_a\), the number of nucleotide sequence variants, i.e., alleles.

\(P_i\), the average number of nucleotide differences per 100 nt sites between sequences.

\(\text{Eta}\), the total number of mutations.

\(S_{nt}\), the number of segregating (polymorphic) nucleotide sites.

\(n_{pp}\), the number of deduced primary protein sequence variants.

\(S_{pp}\), the number of segregating (polymorphic) deduced amino acid residue sites.

Strain Lau09-1128 was not included in any of these analyses, protein-coding loci were not obtained from all isolates: \(^1\) No radA locus was obtained for EPR07-159 or EPR07-39; \(^2\) No EF-2 locus was obtained for EPR07-159 or EPR07-39.

| Populations compared | Protein-coding locus |
|----------------------|----------------------|
|                      | EF-2    | secY    | radA    | rpoB    | atpA    |
| MAR vs. ELSC         | 0.1945  | 0.2161  | 0.2456  | 0.2275  | 0.2011  |
| MAR vs. EPR          | NA      | 0.1993  | NA      | 0.2113  | 0.1953  |
| ELSC vs. EPR         | NA      | 0.2214  | NA      | 0.2269  | 0.2071  |

The number of base differences per site from averaging over all sequence pairs between groups are shown. SE estimate(s) are shown in parentheses and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted in MEGA4.1. NA, no comparison performed between regional sets of isolates.

Table 4 | DNA polymorphism and nucleotide sequence characteristics of the five protein-coding loci examined from the DHVE2 isolates.

Table 5 | Nucleotide divergence between DHVE2 isolates from different regions.
clear biogeographical separation based only on 16S rRNA gene sequences and required MLSA to resolve the biogeographical relationships amongst strains.

MULTI-LOCUS SEQUENCE ANALYSIS
Likewise, we used MLSA to further assess the phylogenetic divergence between the DHVE2 isolates. Protein-coding loci were successfully amplified and sequenced from most, but not all of the 12 isolates. In the MLSA of members of the Halobacteriaceae, Papke et al. (2011) were similarly unable to obtain amplification from all strains. In our study, the radA and EF-2 loci were not obtained from strains Laut09-1128, EPR-159, and EPR-39, while secY could not be amplified from strain Laut09-1128. Subsequent phylogenetic analyses revealed that all sequences from strain Laut09-1128 were quite divergent (Figures 5A,B). All protein-coding loci sequences were submitted to GenBank (EF-2, JN375640 to JN375648; atpA, JN375649 to JN375660; secY, JN375661 to JN375671; rpoB, JN375672 to JN375682; radA, JN375683 to JN375692).

Remarkably high variation between geographic regions within the protein-encoding genes was observed (Table 4). The average nucleotide p-distance values, the proportion of nucleotide sites at which two sequences were compared, are different considering sequences from strains from different geographic regions (Π), varied from 0.19 to 0.25 for the different protein-coding loci (Table 5). The between-region Π values of 0.009–0.070 were observed in the MLSA study of Sulfolobus isolates with ≥99.8% 16S rRNA gene sequence similarity from terrestrial geothermal hot springs of Iceland, North America, and Kamchatka, Russia (Whitaker et al., 2003). There were a total of 983 variable nucleotide sites over 3366 bp from five protein-coding loci examined (Table 4). If the radA and EF-2 loci from strains EPR-159 and EPR-39 had been obtained the total number of variable nt sites would likely be even greater. By comparison, within 78 Sulfolobus strains, there were 124 variable nucleotide sites over 4111 bp from eight protein-coding loci (Whitaker et al., 2003), and among 106 Thermooaerrobacter uzonensis strains with ≥98% 16S rRNA sequence similarity, there were 145 variable protein-coding positions over a total of 8005 nt sites across eight protein-coding loci (Wagner, 2010). Thus, while the 16S rRNA similarity between all DHVE2 isolates (except strain Laut09-1128) is high enough that they would likely be considered the same species, they are extremely divergent considering several protein-coding loci and may actually represent different species.

There were consistent differences in the G + C% content of the protein-coding loci from the DHVE2 isolates from different regions with the G + C% values from the ELSC < EPR < MAR (Figure 6). For example, for the rpoB locus the average G + C% for isolates were 42.3% for the ELSC, 45% for EPR, and 49.1% for MAR. Differences in the GC content among related strains have been reported in previous studies and have been attributed to reductive evolution and gene loss (Rocap et al., 2003). While it is possible that gene loss or gain may have influenced the overall nucleotide composition among the DHVE2 isolates, it is more likely that the GC content differences observed between the DHVE2 isolates are due to regional codon usage bias differences (Ermolaeva, 2001). This view is supported by the high proportion of synonymous nucleotide substitutions relative to the number of possible synonymous nucleotide positions that were observed in the pairwise analyses of the protein-coding loci from DHVE2 strains from different regions (Figure 7). For example, considering the radA locus (334 bp length) from the ELSC and MAR isolates, there are an average of 77.7 possible silent nucleotide substitution sites and an average of 67.1 actual silent nucleotide substitutions among the 20 pairwise comparisons (Figure 7A). Considering the same radA locus, there are only six amino acid residue differences (Table 4).

The slowly evolving 16S rRNA gene, as well as all of the protein-coding loci, revealed regional clustering patterns for the DHVE2 isolates (Figures 4 and 5) suggesting an early origin of the differentiation between ELSC, EPR, and MAR populations. Although we only had a limited number of isolates, these observations suggest divergent evolution of geographically isolated DHVE2 populations, i.e., allopatry (Whitaker, 2006). Additionally, some evidence for biogeographical patterns within oceanic regions was also noted. For example, most of the protein-coding loci from strain MAR-641, obtained from the TAG vent field of the MAR, are phylogenetically different from the other strains obtained from the Lucky Strike and Rainbow vent sites also along the MAR (Figure 5). Intra-regional differences have been observed in other studies of the spatial diversity patterns of microorganisms from thermal environments (Petursdottir et al., 2000; Hreggvidsson et al., 2006; Takacs-Vesbach et al., 2008; Wagner, 2010). With additional DHVE2 strains and genomes, the global patterns of diversity of the DHVE2 could be explored in greater detail. Based on the variation observed in the protein-coding genes, which are part of the core genome for this lineage, extensive differences in the variable genome (Tettelin et al., 2008) are also expected.

CONCLUSION
Results from this study show that the DHVE2 are ubiquitous in deep-sea hydrothermal environments and tend to co-occur with other Euryarchaeota. Phylogenetic analyses of the 16S rRNA genes and protein-coding loci from 12 different DHVE2 isolates revealed clear biogeographical clustering patterns indicative of
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FIGURE 7 | Synonymous and non-synonymous nucleotide positions and substitutions in protein-coding loci among DHVE2 isolates from different regions. (A) Isolates from the MAR compared to isolates from the ELSC (B) isolates from the ELSC compared to isolates from the East Pacific Rise; (C) isolates from the MAR compared to the EPR. Mean values shown on graph, range given with bars. Abbreviations are: Pos, possible synonymous or non-synonymous nucleotide positions; Dif, actual synonymous or non-synonymous differences.
allopatric speciation. Assuming that all DHVE2 are thermoacidophilic, then thermoacidophilic is an important physiological strategy for some microorganisms in these ecosystems. Factors that seemed to influence the occurrence and abundance of the DHVE2 within an individual vent field include the age of the vent deposit (as this is indicative of the maturity of the microbial community, Page et al., 2008), fluid mixing style, and type of vent structure (chimney vs. flange). Other factors like deposit mineralogy, grazing by eukaryotes, and viruses may also be influencing the biogeography of the DHVE2 but were not examined as part of this study.

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APPENDIX

Table A1 | Summary of 16S rRNA gene sequences previously detected in marine hydrothermal environments.

| Accession number | Clone name  | Vent field                                      | Reference |
|------------------|-------------|------------------------------------------------|-----------|
| AB247823         | pLM14A-5    | Mariner – Eastern Lau Spreading Center          | Unpublished |
| AB052986         | pPACMA-Q    | Manus Basin                                    | Takai et al. (2001) |
| AB052983         | pPACMA-M    |                                                |           |
| AB052990         | pPACMA-W    |                                                |           |
| AB052985         | pPACMA-P    |                                                |           |
| AB366574         | SSM0040-14  | Suiyo Seamount, Izu-Ogasawara Arc               | Kimura et al. (2010) |
| AB019740         | pSSMCA108   |                                                | Takai and Horikoshi (1999) |
| AB019739         | pMC2A10     | Myojin Knoll, Izu-Ogasawara Arc                 | Takai and Horikoshi (1999) |
| AB019741         | pISA12      | Iheya Basin, Middle Okinawa Trough             | Takai and Horikoshi (1999) |
| AB019742         | pISA42      |                                                |           |
| AB175693         | IACC-11     |                                                | Nakagawa et al. (2005) |
| AB197209         | IAP6.5m-12  |                                                | Unpublished |
| AB302005         | P816_a_1.07 | Yonaguni Knoll, Southern Okinawa Trough        | Nunoura et al. (2010) |
| AB235350         | pYK04-8A-26 |                                                |           |
| AB611429         | HTM1039Pn-A31 |                                            |           |
| AB611418         | HTM1039Pn-A12 |                                        |           |
| AB611448         | HTM1039Pn-A103 |                                        |           |
| AB611404         | HTM871W-A1  |                                                |           |
| AB611358         | HTM873S-A1  |                                                |           |
| AB611414         | HTM1039Pn-A8 |                                                |           |
| AB611339         | HTM866S-A3  |                                                |           |
| AB611424         | HTM1039Pn-A24 |                                        |           |
| AB611425         | HTM1039Pn-A26 |                                        |           |
| AB611367         | HTM873I-A23 |                                                |           |
| AB611430         | HTM1039Pn-A32 |                                        |           |
| AB611375         | HTM1039S-A21 |                                            |           |
| AB611386         | HTM1036S-A21 |                                            |           |
| EU107487         | A10         | Snail surface – Okinawa Trough                 | Unpublished |
| AB167485         | TOTO-A6-1   | Toto Cladera – Marian Arc                      | Nakagawa et al. (2006) |
| AB167482         | TOTO-A1-28  |                                                |           |
| AB167498         | TOTO-ISCS-A45 |                                        |           |
| AB167486         | TOTO-A6-12  |                                                |           |
| AB239232         | Pccs1A26    | Southern Mariana Trough                        | Kato et al. (2010) |
| AB239231         | Pccs1A25    |                                                |           |
| AB239234         | Pccs1A38    |                                                |           |
| EU574651         | CPA_17      | Northern Mariana Backarc                       | Unpublished |
| AM749972         | 8S4e_arc05A | Kermadec Arc – New Zealand                     | Stott et al. (2008) |
| AB177273         | ODP1251A15.24 |                                        | Inagaki et al. (2006) |
| AF355836         | 33-P120A99  | Juan de Fuca Ridge                             | Huber et al. (2002) |
| DQ118404         | Fosmid Alv-FOS4 |                                        | Moussard et al. (2006b) |
| DQ082975         | FOS2        |                                                |           |
| DQ082978         | FOS3        |                                                |           |
| DQ082980         | metF2       |                                                |           |
| DQ082964         | met50       |                                                |           |
| DQ082953         | met21       |                                                |           |
| DQ082954         | met24       |                                                |           |
| DQ082969         | metF8       |                                                |           |
| DQ082955         | met43       |                                                |           |
| DQ118403         | Fosmid Alv-FOS1 |                                        | Moussard et al. (2006b) |

(Continued)
Table A1 | Continued

| Accession number | Clone name | Vent field | Reference       |
|------------------|------------|------------|-----------------|
| AF526965         | pEPR193    |            | Nercissian et al. (2003) |
| AF526964         | pEPR195    |            |                 |
| AF526963         | pEPR122    |            |                 |
| AF526962         | pEPR719    |            |                 |
| AF526961         | pEPR707    |            |                 |
| AY672495         | CH8_7a_Arc | 9’N East Pacific Rise | Kormas et al. (2006) |
| AF356635         | G26-C56    | Guaymas Basin | Unpublished |
| AF356637         | G26_C73    |            |                 |
| AF068820         | VC2.1 Arc13 | Snake Pit – Mid-Atlantic Ridge | Reysenbach et al. (2000) |
| AB496479         | pMARA06_14 | Lucky Strike – Mid-Atlantic Ridge | Unpublished |
| FM863771         | T48R       | TAG – Mid-Atlantic Ridge (Rimicaris gut) | Unpublished |
| FM863772         | T14R       |            |                 |
| FM863773         | T22R       |            |                 |
| AY251065         | FT17A09    | Central Indian Ridge | Hoek et al. (2003) |
| AY251064         | FT17A03    |            |                 |

All listed sequences are greater than 600bp in length and share greater than 94% sequence similarity with Aciduliprofundum boonei T469T.

Table A2 | Location of the protein-coding loci in the genome of A. boonei T469T.

| Locus | Gene description | NCBI Gene ID | Genomic position |
|-------|------------------|--------------|------------------|
|       |                  |              | Start           | End          |
| radA  | DNA repair and recombination protein RadA | 8827373 | 426732 | 427706 |
| atpA  | ATP synthase, A subunit | 8828224 | 1209531 | 1211279 |
| rpoB  | DNA-directed RNA polymerase subunit B | 8828049 | 1047786 | 1051391 |
| EF2   | Translation elongation factor aEF-2 | 8827008 | 71186 | 73390 |
| secY  | Preprotein translocase, SecY subunit | 8828467 | 1443463 | 1445262 |
### Table A3 | Hydrothermal vent deposits from which new DHVE2 isolates were obtained.

| Isolate name | Vent field of isolation (deposit type) | Location | Depth (m) |
|--------------|----------------------------------------|----------|-----------|
| Mar08-237a   | Rainbow (chimney)                      | 36°13.75625′N 33°54.11541′W | 2275 |
| Mar08-276*   | Lucky Strike (chimney)                  | 37°17.32494′N 32°16.50738′W | 1617 |
| Mar08-307*   | Lucky Strike (flange)                   | 37°17.3250′N 32°16.5058′W | 1624 |
| Mar08-339    | Lucky Strike (chimney)                  | 37°17.45770′N 32°16.91245′W | 1730 |
| Mar08-361*   | Lucky Strike (flange)                   | 37°17.4528′N 32°16.9161′W | 1730 |
| Mar08-368    | Lucky Strike (chimney)                  | 37°17.4998′N 32°16.6715′W | 1721 |
| Mar08-641    | TAG (chimney)                           | 26°8.2043′N 44°49.5283′W | 3621 |
| EPR07-39     | 9°N (chimney)                           | 9°50.31366′N 104°17.48471′W | 2510 |
| EPR07-159    | 9°N (chimney)                           | 9°50.2876′N 104°17.4721′W | 2507 |
| Lau09-652    | Mariner (flange)                        | 22°10.82942′S 176°36.10868′W | 1919 |
| Lau09-654    | Mariner (flange)                        | 22°10.82942′S 176°36.10868′W | 1919 |
| Lau09-664    | Mariner (chimney)                       | 22°10.80806′S 176°36.05652′W | 1915 |
| Lau09-781    | Mariner (chimney)                       | 22°11.2751′S 176°36.0755′W | 1919 |
| Lau09-1128   | Tui Malila (flange)                     | 22°0.1708′S 176°34.1086′W | 1883 |
| Lau09-cd1713 | Mariner (flange)                        | 22°10.79920′S 176°36.06771′W | 1911 |

*Denotes isolates that may not be pure as ambiguities were observed in protein-coding genes but not in 16S rRNA gene sequences.