

Typing of *Legionella* strains isolated from environmental samples in Crete, Greece, during the period 2004–2011

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ABSTRACT

In Greece, standard tests are performed in watering and cooling systems of hotels. A total of 1,494 water samples were collected during 2004–2011 from 124 hotels from four regions in Crete (Greece). Samples were tested for the presence of *Legionella* spp.; 103 isolates were identified and typed by polymerase chain reaction (PCR)-sequencing and sequence-based typing (SBT) (in case of *L. pneumophila* sg 1). Of those, 48 belonged to various serogroups of *L. pneumophila* (sg 1, 2, 3, 5, 6, 8, 12, 13, and 15), 32 were characterized as *L. anisa*, 17 as *L. taurinensis* and there was a single occurrence of *L. quinlivanii*, *L. maceachernii*, and *L. oakridgensis*. In the case of *L. pneumophila* SG1, one prevalent sequence type was revealed (ST37). The variability of *Legionella* spp. observed questions the existence of a single ST of the *L. pneumophila* sg1 species and leads towards the need for a genetic level investigation of all Legionnaires' disease cases.

Key words | Greece, *Legionella pneumophila*, *Legionella* spp., SBT, typing

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INTRODUCTION

Legionnaires' disease is caused by bacteria of the genus *Legionella*; these are gram-negative facultative intracellular bacteria that favor moist environments where the occurrence of free living amoeba and protozoa is higher (Diederens 2008). Aquatic, or moist, environments are the main gateway for human infection since this is usually a result of contaminated aerosols inhalation. Such conditions can be met in spa pools, water systems and wet cooling systems (Bartram *et al.* 2007), while large water pipe-line networks serve as the prevalent environment for developing biofilms of *Legionella* (Borella *et al.* 2005).

The majority of incidents of Legionnaires' disease are attributed to *L. pneumophila* serogroup 1 strains; however, other serogroups of species have also been implicated in human disease. At present, the method of choice for

epidemiological subtyping is the web-based sequence-based typing scheme (SBT) (Gaia *et al.* 2005; Ratzow *et al.* 2007).

In Greece, based on the current regulations for Legionellosis control, standard tests are performed in the watering and cooling systems of hotel units, as well as investigations of Legionellosis incidents, as part of the surveillance scheme. Herein, we present the results of Legionellosis control in the vicinity of the island of Crete, Greece, in the period between 2004 and 2011. Nine *L. pneumophila* serogroups together with the presence of five non-*pneumophila* species (*L. taurinensis*, *L. anisa*, *L. quinlivanii*, *L. oakridgensis*, *L. maceachernii*) were recorded. The parallel use of MALDI-TOF mass spectrometry could provide great assistance on the rapid identification of *Legionella* spp.

MATERIALS AND METHODS

Sampling

A total of 1,494 water samples were collected during the period 2004–2011 from 124 hotels from four regions (Lasithi, Heraklion, Rethymno, Chania) in Crete (Greece), both on the basis of routine surveillance and following a human case of *Legionella* infection.

In each hotel, eight to 15 water samples were collected. Collection sites were dependent on the hotel establishments and most commonly included: one water sample (tap water) at the inlet of each hotel water system, one right after the main water supply tank, one before and one after the boiler and one water sample from each of the closest and the furthest rooms from the boiler. From these rooms a direct cold and a hot water sample (from room showers) were collected, together with cold and hot samples after the water was flushed for 1 min. At each sampling point, water samples were collected in sterile borosilicate 1 L glass bottles. To neutralize the residual-free chlorine, sodium thiosulfate was added to the bottles. All water samples were stored at 4 °C and processed within 24 h of collection.

Plate culture method

Isolation of *Legionella* from water samples was performed by culture according to International Standard method ISO 11731 (International Standards Organization, 1998) and ISO 11731-2 (International Standards Organization, 2004). Suspected colonies were randomly chosen for subculture on BCYE and GVPC agar.

Direct fluorescent antibody (DFA)/agglutination test

Isolated colonies were identified using a DFA test (MONO-FLUO™ *L. pneumophila* IFA test kit, Biorad) that can detect *L. pneumophila* serogroup 1, an agglutination test (SLIDEX® Legionella-Kit, Biomérieux, Craonne, France), which allows the discrimination of *L. pneumophila* serogroup 1 from serogroups 2–14 and *L. anisa*, while for the exact detection of each serogroup individual latex polyclonal reagents were used (Pro-lab, Richmond Hill, Canada).

DNA extraction/polymerase chain reaction (PCR) amplification

DNA from suspected colonies was extracted using the Tissue kit (Qiagen, Hilden, Germany), as proposed by the manufacturer. DNA extracts were stored at –20 °C until analysis. DNA extracts were screened using primers 8UA and 1485B targeting a 1477-bp portion of the *Legionella* 16S *rRNA* gene, as previously described (Ratcliff et al. 1998). Additionally, the *mip* gene was amplified using the LegmipF/R primers, which produce a variable product of approximately 700 bp, as described by Ratcliff et al. (1998). Both amplification protocols were performed as described by the aforementioned authors.

Sequence-based typing

Samples that were identified as *L. pneumophila* sg 1 were processed by SBT using loci of the *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* genes according to the EWGLI scheme (Gaia et al. 2005; Ratzow et al. 2007). Automated contig-assembly and base-calling of DNA sequence traces were performed by using the EWGLI sequence quality tool (Ewing & Green 1998). All data on the database of SBT were accessed from the EWGLI website (www.ewgli.org).

PCR sequencing

Positive PCR products were purified (PCR product purification kit, Qiagen, Germany) and were directly sequenced using the sequencer CEQ 8000 Beckman Coulter (Bioanalytica – Genotype, Athens); in case of SBT, the primers described in the EWGLI database were used; in the case of 16S *rRNA* the sequencing primers used (907B, 519A) have been described (Ratcliff et al. 1998). All PCR products were sequenced twice to verify the revealed sequences. The sequences revealed were processed using Chromas v1.49 and *Lasergene* Ver.7.1 software for viewing the chromatograms and editing of the retrieved nucleotide sequences, and nucleotide BLASTn for investigating the homology of the obtained sequences compared to the NCBI databases.

PHYLOGENETIC AND ALLELIC DIVERSITY ANALYSES

Multiple sequence alignments of concatenated DNA sequences and phylogram construction were carried out with MEGA4 (Tamura et al. 2007) and ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html). The evolutionary history of the phylogenetic trees was inferred using the Neighbor-Joining method (Larkin et al. 2007). The bootstrap consensus trees were inferred from 1,000 replicates which were taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

MALDI-TOF MASS SPECTROMETRY

MALDI Biotyper (Microflex LT MALDI-TOF mass spectrometer) (Bruker Daltonics, Leipzig, Germany) equipped with a microSCOUT ion source, was used for identification of individual *Legionella* colonies against the microbial database (v3.1.2.0). Spectra were recorded using the flexControl software with the default parameters set by the manufacturer for optimization (Bruker Daltonics, Leipzig, Germany). For each spectrum, 240 laser shots were collected and analyzed (6 × 40 laser shots from different positions of the target spot). All identifications were evaluated according to the manufacturer scoring scheme.

RESULTS

Of the 103 isolates, 48 belonged to *L. pneumophila* sg1 (8/103), sg2 (2/103), sg3 (13/103), sg5 (1/103), sg6 (15/103), sg8 (3/103), sg12 (3/103), sg13 (1/103), sg15 (1/103), *L. anisa* (32/103), *L. taurinensis* (17/103), *L. maceachernii* (1/103), *L. quinlivanii* (1/103), and *L. oakridgensis* (1/103) (Table 1).

The sequence based typing scheme was performed for all *L. pneumophila* serogroup 1 isolates using seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA*), which revealed a prevalent sequence type with allelic profile ST

3,4,1,1,14,9,11. The *mompS* (of all isolates) and *proA* of the second isolate were designated as putative new alleles.

All genes were homologous to *L. pneumophila* subsp. *pneumophila* str. Philadelphia 1 (AE017354). In all *L. pneumophila* SG1 isolates the genes *flaA*, *pilE*, *asd*, *mip* and *proA* were 100% identical to those of AE017354. An exception was *proA* of isolate 2 which showed 99% maximum identity to AE017354. The *mompS* gene of all isolates had 99% identity to AE017354 and, again, in all isolates the *neuA* gene was 100% identical to *L. pneumophila* serogroup 1 ORF1-4 DNA and *lag-1* gene (LPS synthesis cluster), strain uppsala 3 (AM932053) and just 97% to AE017354. A Neighbor-Joining tree with bootstrap analysis was created in order to see the phylogenetic relations of the STs detected herein and already published ones. All of our isolates were closely related to ST37 with isolate 2 showing greater divergence (Figure 1).

Concerning the whole group of the pneumophila isolates, we could not find any association between the nucleotide sequences of the *mip* or 16SrRNA genes and the serogroup of the pneumophila species. A slightly different grouping scheme was observed when aligning the sequences of the *mip* gene compared to the groupings formed when aligning with the 16SrRNA gene (Supplementary Figure 1(a) and 1(b), available online at <http://www.iwaponline.com/wh/011/015.pdf>). Most of the sequences in both gene occasions were either 100% homologous to already published sequences or were found to have very few polymorphisms. The only exception, where a low degree of similarity was observed, was the *mip* gene sequences of three of the pneumophila isolates. Two of these isolates were detected in the same hotel while the third was detected in a different hotel. The two hotels are separated by approximately 220 km and are located on opposite sides of the island, one is on the far north-west part of the island and the other is on the south-east part. All three isolates were *L. pneumophila* serogroup 8 and their *mip* gene shared homology of 88.92% with *L. pneumophila* strain CDC F1053 (AF095218.1) while their 16SrRNA gene sequences shared homology of 98.23% with *L. pneumophila* subsp. *pascullei* strain U8W; ATCC 33737 (NR_041742.1). When creating Neighbor-Joining trees, for both of the genes, the placement of this pneumophila isolate was in a different node but close to the cluster of the pneumophila species reported herein and of published genomes.

Table 1 | Month, year, prefecture, collection site, species and serogroups of the *Legionella* isolates

Month	Year	Prefecture	ID	Collection site Source	Isolated <i>Legionella</i> Description	Species	Serogroups
April	2004	Heraklion	2,362	Room	Hot water	Anisa	
May	2004	Heraklion	2,720	Room			1
May	2005	Heraklion	2,722	Swimming pool	Douche	Anisa	
June	2006	Heraklion	2,993	Swimming pool	Douche	Anisa	
* June	2006	Chania	3,145	Swimming pool	Douche	Anisa	
June	2006	Chania	3,147	Room	Cold water	Anisa	
June	2007	Chania	3,149	Room	Hot water	Anisa	
June	2007	Chania	3,150	Room	Hot water	Anisa	
June	2007	Chania	3,157	Room	Cold water	Anisa	
August	2008	Heraklion	3,986	Swimming pool/waterfall		Anisa	
August	2008	Heraklion	3,990	Room	Hot water	Anisa	
September	2008	Lasithi	4,017	Room	Hot water	Anisa	
* September	2008	Lasithi	4,029	Room	Hot water	Taurinensis	
September	2008	Lasithi	4,030	Room	Hot water	Taurinensis	
September	2009	Lasithi	4,034	Room	Hot water	Taurinensis	1
September	2009	Lasithi	4,035	Room	Cold water	Anisa	
September	2009	Lasithi	4,036	Room	Cold water	Anisa	
September	2010	Lasithi	4,037	Swimming pool	Douche	Taurinensis	
September	2010	Lasithi	4,038	Drilling		Anisa	
September	2010	Lasithi	4,042	Room	Cold water	Taurinensis	
September	2010	Rethymno	4,067	Boiler		Anisa	
September	2011	Rethymno	4,076	Room	Hot water		6
September	2011	Rethymno	4,082	Air cooling system		Anisa	
September	2011	Heraklion	4,109	Water entering hotel		Taurinensis	
* September	2011	Heraklion	4,111	Boiler		Taurinensis	
September	2011	Heraklion	4,133	Tank		Taurinensis	
September	2004	Heraklion	4,135	Swimming pool	Douche	Anisa	
September	2004	Heraklion	4,136	Room	Hot water	Taurinensis	
September	2004	Heraklion	4,140	Swimming pool	Douche	Taurinensis	
September	2005	Heraklion	4,148	Room	Cold water	Anisa	
September	2005	Heraklion	4,149	Room	Hot water	Anisa	
September	2005	Heraklion	4,150	Room	Hot water	Anisa	
September	2005	Heraklion	4,152	Room	Cold water	Anisa	
September	2007	Heraklion	4,205	Water entering hotel		Anisa	
September	2007	Heraklion	4,206	Swimming pool	Douche	Anisa	
September	2007	Heraklion	4,207	Swimming pool	Douche	Anisa	
September	2007	Heraklion	4,211	Tank		Anisa	
September	2006	Heraklion	4,212	Tank		Anisa	
* September	2006	Heraklion	4,216	Room	Hot water		2

(continued)

Table 1 | continued

Month	Year	Prefecture	ID	Collection site Source	Isolated <i>Legionella</i> Description	Species	Serogroups	
*	September	2006	Heraklion	4,220	Room	Cold water	Anisa	1
	September	2006	Heraklion	4,222	Room	Hot water	Anisa	
	September	2009	Heraklion	4,223	Room	Cold water	Anisa	
	September	2009	Heraklion	4,227	Hose		Quinlivanii	3
	September	2009	Chania	4,232	Room	Cold water	Oakridgensis	
	September	2009	Chania	4,234	Room	Hot water	Anisa	
*	September	2011	Chania	4,235	Room	Hot water		2
*	October	2007	Heraklion	4,266	Collecteur			1
	October	2007	Heraklion	4,267	Boiler			6
	October	2007	Heraklion	4,268	Boiler			6
*	October	2009	Heraklion	4,271	Room	Hot water		12
*	October	2009	Heraklion	4,272	Room	Hot water		6, 12
	October	2009	Heraklion	4,273	Room	Cold water		1
	October	2009	Heraklion	4,275	Room	Hot water		12
	October	2011	Rethymno	4,284	Room	Hot water		6
	October	2011	Rethymno	4,286	Room	Cold water		6
	October	2011	Rethymno	4,292	Room	Hot water		6
	October	2011	Rethymno	4,296	Room	Hot water		6
	October	2005	Rethymno	4,297	Room	Hot water		6
	October	2005	Rethymno	4,300	Boiler			6
	October	2005	Rethymno	4,301	Boiler			6
*	October	2005	Rethymno	4,302	Air cooling system		Taurinensis	1
	October	2008	Rethymno	4,303	Swimming pool	Douche		6
*	October	2008	Lasithi	4,316	Room	Cold water		8
*	October	2010	Lasithi	4,317	Room	Hot water		8
*	October	2010	Lasithi	4,360	Water entering hotel			8
	October	2010	Lasithi	4,368	Room	Cold water		1
	November	2011	Rethymno	4,416	Room	Cold water		6
	November	2011	Rethymno	4,422	Room	Hot water	Anisa	
*	November	2011	Rethymno	4,424	Swimming pool	Douche		6
	November	2011	Rethymno	4,428	Boiler			5, 15
	November	2010	Heraklion	4,436	Tank		Anisa	3
	November	2010	Heraklion	4,437	Boiler			3, 6
	November	2010	Heraklion	4,438	Filter from boiler			3
	November	2010	Heraklion	4,439	Room	Hot water		3
	November	2011	Heraklion	4,440	Room	Cold water	Taurinensis	3
	November	2011	Heraklion	4,441	Room	Cold water	Taurinensis	3
	November	2011	Heraklion	4,442	Room	Cold water	Taurinensis	3
	November	2004	Heraklion	4,443	Room	Hot water	Taurinensis	3, 13

(continued)

Table 1 | continued

Month	Year	Prefecture	ID	Collection site Source	Isolated <i>Legionella</i> Description	Species	Serogroups
November	2011	Heraklion	4,444	Room	Cold water	Taurinensis	3
November	2005	Heraklion	4,445	Room	Hot water	Taurinensis	3
November	2011	Heraklion	4,446	Room	Cold water		1, 3
November	2011	Heraklion	4,447	Filter from boiler			3
November	2011	Rethymno	4,475	Water entering hotel		Maceachernii	

The asterisk (*) indicates the isolates that were used in order to construct the phylogenetic tree.

Supporting were the values of a bootstrap analysis (1000 replicates) that was performed for both trees where, especially for the case of the *mip* gene, it was 100 (data not shown). For a more solid perspective the sequences of the two genes were concatenated and a new NJ tree with bootstrap analysis was created (Figure 2). The resulting tree placed the pneumophila isolate again in the same allocation as with the previous individual gene trees with the highest bootstrap value.

All isolates were analyzed with MALDI Biotyper and results were evaluated based on the scoring scheme as suggested by the manufacturer. *L. pneumophila* sgs 1, 2, 3, 5, 6, 8, 12, 13 and 15 were identified at the genus level (*L. pneumophila*) with scores ranging from 2.013 to 2.389. All *L. anisa* isolates and the single *L. oakridgensis* isolate were securely identified (scores from 2.084 to 2.452). Of the remaining isolates, none could be securely identified as the spectra for *L. quinlivanii*, *L. taurinensis* and *L. maceachernii* were not present at the database provided by the MALDI Biotyper manufacturer.

DISCUSSION

The major drawback in the investigation of the *Legionella* epidemiology and ecology is the method that most European countries use to detect cases of Legionellosis. Based on EWGLINET data and case definition, in 2009, 85.7% of the reported Legionellosis cases were based on the result of the urinary antigen test alone (Joseph et al. 2010). Since most laboratories do not attempt the isolation of the *Legionella* spp., there are only few data on the Eco-epidemiology of existing strains and, in the case of *L. pneumophila*, of STs.

SBT of *L. pneumophila* sg1 is a powerful genotypic method applied towards the examination of the ecology and epidemiology of the different STs. It is a simple laboratory method that retrieves unambiguous results, easily interpreted (Gaia et al. 2003, 2005; Amemura-Maekawa et al. 2005; Scaturro et al. 2005). It was adopted as an international standard and is widely used by the members of the EWGLI as it is a simple, rapid and discriminatory typing method. Furthermore, it allows the exchange of data between laboratories (Chasqueira et al. 2009).

In Greece there were no previous data published concerning the presence of the *Legionella* spp. in the vicinity of the island of Crete. In the current research we tested environmental samples which gave results that could be considered as representative of the prevalent species and profiles of *Legionella* in the island. Although it is not certain if the results reflect the true state situation about the variety of the strains present in the specific area, still they describe the prevalence of *L. pneumophila* ST37 and the increased variability of non-pneumophila species even in small geographic areas.

Based on our results we can speculate that the prevalent ST of *L. pneumophila* sg1 in Crete is described by the 3,4,1,1,14,9,11 profile (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA* genes, respectively). This profile is, phylogenetically, closely related to ST37 that is currently present to the EWGLINET database (Figure 1). Closely related to ST37 is also isolate 2 which shows a higher degree of divergence. Based on the phylogenetic tree, we could assume that the prevalent *L. pneumophila* strain in Crete has derived from a common ancestor with *L. pneumophila* ST37 (already implicated in human disease in the UK) (Harrison et al. 2009).

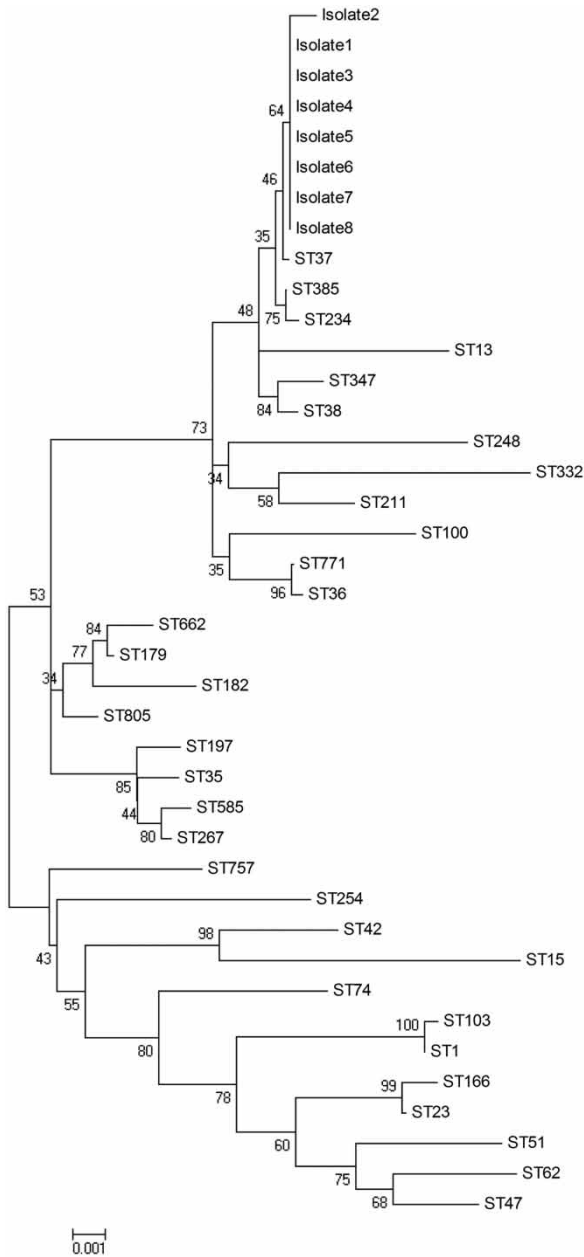


Figure 1 | Evolutionary relationships of the isolated strains against published STs. Concatenated sequences, of the genes used in the SBT scheme, were used for the tree construction. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset.

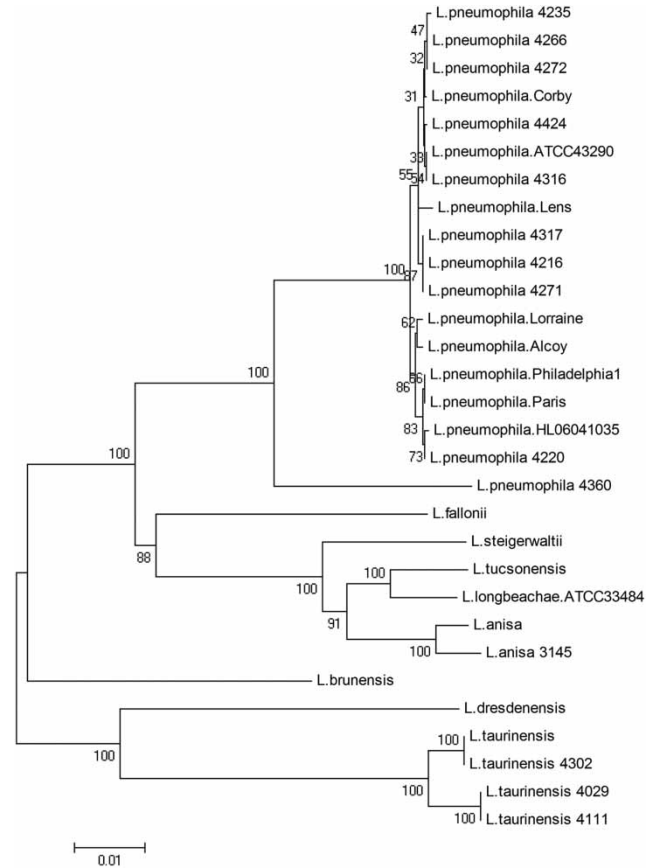


Figure 2 | Neighbor-Joining tree of *mip* and 16SrRNA concatenated sequences. The sequences of both genes (*mip* and 16SrRNA) were concatenated and the evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Isolates with identical gene sequences are represented once and can be identified by the four digit isolate number at the end. Isolate *L. pneumophila* 4360 was presented in a distinct branch supported by a high bootstrap value.

A quite interesting finding was also the case of three of the *L. pneumophila* isolates that were isolated from two different areas of the island and presented a low degree of similarity with any other published sequences. As mentioned in the Results section, these three isolates gave a positive agglutination reaction with serogroup 8 antisera and when examined phylogenetically (Figure 2, representative sequence 4360) they were allocated in the wider pneumophila cluster but in a distinct branch. *L. pneumophila* serogroup 8 has been recorded as human cases (Tang & Toma 1985; Senn et al. 1987; Kawanami et al. 2011), as a pseudo-outbreak in a bronchoscopy suite due to

a contaminated ice machine (Schuetz *et al.* 2009), while death has been reported once following co-infection with *Aspergillus fumigatus* (Guillouzouic *et al.* 2008). Thus, further investigation of these isolates is of great interest and public health importance.

Concerning the isolates belonging to the other serogroups (sg 2, 3, 5, 6, 12) most of them have been implicated in human cases in the past involving various clinical manifestations ranging from pneumonia, ulcerative colitis, pericarditis, esophageal perforation or even death (Cordes *et al.* 1981; Thacker *et al.* 1987; Franzin *et al.* 1989; Meigh *et al.* 1989; Tram *et al.* 1990; Fendukly *et al.* 2007; Chien *et al.* 2010; Grattard *et al.* 2010; Kusnetsov *et al.* 2010; Mencacci *et al.* 2011).

To our knowledge, this is the first report commenting on the presence of *L. pneumophila* sg 15 on environmental water, or even bibliographically in general. *L. anisa* has already been detected in water systems (Yu & Stout 2004; Stout *et al.* 2007; Svarrer & Uldum 2011) and has been used as an indicator of the presence of *L. pneumophila* (van der Mee-Marquet *et al.* 2006). In the case of human infection it primarily causes Pontiac fever while other clinical manifestations have also been described (Jones *et al.* 2003; Tanabe *et al.* 2009). Human disease has been attributed twice to *L. oakridgensis* (Tang *et al.* 1985; Lo Presti *et al.* 2000) since its first description (Orrison *et al.* 1983). A few reports have also linked *L. maceachernii* to human disease (Merrell *et al.* 1991; Dumoff *et al.* 2004; van Dam *et al.* 2006; Chee & Baddour 2007) or even death (Wilkinson *et al.* 1985; Thomas *et al.* 1992). *L. taurinensis* (Lo Presti *et al.* 1999; Franzin & Gioannini 2000) and *L. quinlivanii* have just been described twice (Wilkinson *et al.* 1990; Birtles *et al.* 1991).

On the current survey, we detected new putative alleles of *mompS* and *proA* and described, for the first time, some of the prevalent species of *Legionella* in a preferred tourism destination, the island of Crete, Greece. The process of identification of both *L. pneumophila* and non-*L. pneumophila* species could be implemented by MALDI-TOF technology in the near future. However, due to the variability present in this genus, many spectra need to be added in the database in order to obtain more secure results.

Knowing the strains of non-*L. pneumophila* species and the STs of the *L. pneumophila* sg 1 strains is essential,

especially whenever there are human cases related to tourism. Under such circumstances, a simple urine antigen test alone should be considered insufficient to release accusations against a hotel unit, even if there are isolations of *Legionella* strains from its water system. Culture and characterization of the *Legionella* spp. isolated from the patients should be encouraged in order to define if the infection has been caused by the same strain/ST detected in the hosting vacation unit. In any case, *L. pneumophila* other than serogroup 1 and other species should be included in the differential diagnosis of patients with suspected atypical community-acquired pneumonia or suffering from Legionnaires' disease.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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