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Impacts
• From 2008 to 2011, 11 human cases of Lyme Borreliosis were reported in the Verbano-Cusio-Ossola (VCO) province, north-eastern Piedmont, Italy.
• The abundance and altitudinal distribution of ticks in VCO and B. burgdorferi s.l. in these vectors were examined. Phylogenetic analyses using ospC gene sequences were performed and 14 of 34 positive samples formed a clade with ospC reference sequences of human invasive strains.
• The study supports the need for providing the right information to the institutions, physicians, forestry workers and visitors to adopt proper precautions during season at risk and to protect public health.

Keywords:
Acarologic risk; Borrelia burgdorferi sensu lato; Ixodes ricinus; tick; zoonosis

Summary

Following reports of human cases of Lyme borreliosis from the Ossola Valley, a mountainous area of Piemonte, north-western Italy, the abundance and altitudinal distribution of ticks, and infection of these vectors with *Borrelia burgdorferi* sensu lato were evaluated. A total of 1662 host-seeking *Ixodes ricinus* were collected by dragging from April to September 2011 at locations between 400 and 1450 m above sea level. Additional 104 *I. ricinus* were collected from 35 hunted wild animals (4 chamois, 8 roe deer, 23 red deer). Tick density, expressed as the number of ticks per 100 m^2, resulted highly variable among different areas, ranging from 0 to 105 larvae and from 0 to 22 nymphs. A sample of 352 ticks (327 from dragging and 25 from wild animals) was screened by a PCR assay targeting a fragment of the 16S rRNA gene of B. burgdorferi s.l. Positive samples were confirmed with a PCR assay specific for the 5S-23S rRNA intergenic spacer region and sequenced. Four genospecies were found: *B. afzelii* (prevalence 4.0%), *B. lusitaniae* (4.0%), *B. garinii* (1.5%) and *B. valaisiana* (0.3%). Phylogenetic analysis based on the ospC gene showed that most of the *Borrelia* strains from pathogenic genospecies had the potential for human infection and for invasion of secondary body sites.

Introduction

Lyme Borreliosis (LB) is a multisystem disorder affecting humans and domestic animals. It is caused by spirochetes belonging to the *Borrelia burgdorferi* species complex (*Borrelia burgdorferi* sensu lato, s.l.), including 19 genospecies with a worldwide distribution (Mannelli et al., 2012). In Europe, five genospecies, *B. burgdorferi* sensu stricto (s.s.), *B. garinii, B. afzelii, B. spielmanii, B. bavariensis*, are considered pathogenic for humans. Others, like *B. valaisiana, B. lusitaniae, B. bissettii* and *B. finlandensis* are of uncertain pathogenicity (EUCALB, 2013). LB is the most common tick-borne infection in Europe, and it is transmitted by the bite of ticks of the genus *Ixodes*, mainly *Ixodes ricinus*. Small and medium-sized mammals, birds and reptiles are important for the maintenance of B. burgdorferi genospecies.
Humans are considered incompetent hosts (Jongejan and Uilenberg, 2004; Mannelli et al., 2012). Clinically, silent infection is common. Human infections often begin with a characteristic skin lesion (erythema migrans) and flu-like symptoms; in more severe forms diffuse pain, facial paresis, meningitis, severe arthritis and/or myocarditis can appear. Moreover, acrodermatitis chronica atrophicans (ACA) and severe neuropathies can be observed in late LB (Nau et al., 2009). Among domestic animals, clinical signs

associated with disease have been reported in dogs, cattle and horses (European Food Safety Authority (EFSA), 2010).
The B. burgdorferi s.l. genospecies present outer surface protein antigens called Osp (OspA and OspC) that play an essential role in the pathogenesis (Fingerle et al., 2007).
The ospC gene is highly variable, and this diversity is believed to represent different Borrelia serotypes (Wilske et al., 1995). Moreover, phylogenetic analysis has shown that ospC alleles cluster in separate groups, characterized by high inter-group (>8%) and low intragroup (<2%) genetic divergence (Wang et al., 1999). Interestingly, isolates which have shown invasiveness in humans (intended as the ability to disseminate in body districts other thanthe primary inoculation site) tend to cluster together into specific groups (Baranton et al., 2001; Lagal et al., 2003). This is not surprising, keeping in mind that OspC becomes the main surface antigen after the tick bite, and therefore, different ospC allelic variants can determine the Borrelia ability to escape the host immuno-response when the parasite actively replicates in the inoculation site. On this basis, phylogenetic analysis of ospC sequences can represent a useful tool to predict invasiveness of Borrelia isolates and their potential to induce generalized clinical manifestations.
Notification rates of LB are highly variable among European countries. The first Italian human case was reported in 1983 in the Liguria region (Crovato et al., 1985). Afterwards, infections were reported in several regions, particularly in the north-east mountain area. Friuli Venezia Giulia, Trentino Alto Adige, Liguria, Veneto and Emilia-Romagna are considered endemic regions (Matassa, 2007).
Until recently, LB was not considered a great concern in Piedmont region, north-western Italy. The disease was first reported in this region in 1990 (Ciceroni and Ciarrocchi, 1998), and 19 human cases were notified to date, according to the Regional Epidemiology Reference Service for the surveillance, prevention and control of infectious diseases (Se-REMI, personal comunication). The latest 11 cases, from 2008 to 2011, were all reported by the local health unit of Verbano-Cusio-Ossola (VCO) province, in north-eastern Piedmont. Affected patients were usually exposed to ticks during outdoor activities in the woods. The aim of this study was to determine the abundance and altitudinal distribution of ticks in the VCO province and to detect *B. burgdorferi* s.l. in these vectors. In addition, phylogenetic analyses were performed using ospC gene sequences to assess the potential for human invasiveness of pathogenic genospecies.

Materials and Methods

Ticks collection. The study was conducted from April to September 2011 in the Ossola Valley of VCO province. Investigations were carried out in the municipalities of Varzo (46°120N, 8°250E), Calasca-Castiglione (46°40, 8°70E) and Cannobio (46°40N, 08°420E), where a Lyme disease human case had been reported during 2010. A convenience sampling of study sites was performed by choosing easily accessible areas along selected roads. Sites were located between 400 and 1450 m above sea level (a.s.l.) and were characterized by deciduous forests. In each study area, the ticks were monthly collected by dragging, using a 1 m2 white flag on low vegetation and leafs. Dragging was performed on 200–400 m transects in the first dragging sessions (April–July), then 100 m transects were defined. At every session, vegetation characteristics (dominant species and relative vegetation cover), temperature and humidity were registered.
Ticks were kept alive in plastic vials, then stored at -20°C.
Ticks from wild animals killed during the 2010 hunting season, from September to December, in the same area were collected. Animals were rapidly examined, and a sample of ticks was collected from each carcass at different body sites and stored in 70% ethanol. All tick specimens collected were identified by species and stage using a stereoscopic microscope, according to standard taxonomic keys (Manilla, 1998; Cringoli et al., 2005).
Molecular analysis. A randomly selected tick sample, sufficient to detect 30% prevalence with 95% confidence level and 5% error, was screened by PCR assays. After total nucleic acid extraction performed on each individual tick with Nucleospin RNA II kit (Machery-Nagel, Du¨ren, Germany) in combination with the Nucleospin RNA/DNA Buffer set, a first screening was performed by a PCR assay targeting a 357 bp fragment of the 16S rRNA gene of *B. burgdorferi* s.l. (Marconi and Garon, 1992). The positive/doubtful DNA samples were amplified for confirmation with a PCR protocol specific for an intergenic spacer region between 5S and 23S rRNA genes (Rijpkema et al., 1995). Positive samples were sequenced and compared with sequence data available from GenBank. OspC gene amplification was carried out from positive samples by a two-step heminested strategy according to the protocol described by Wang et al. (1999). OspC sequences recorded from the GenBank and the new sequences (GenBank acc. nos. KF958708–KF958725)

were aligned using BioEdit. Phylogenetic analysis was performed by maximum likelihood method with MEGA 5.0. The nucleotide substitution model was set according to JMODELTEST2 (Darriba et al., 2012) output and was General Time Reversible with gamma variation (GTR+G). The robustness of the hypothesis was tested in 1000 nonparametric bootstrap analyses. OspC gene allelic groups were defined and numbered according to previous studies (Baranton et al., 1992; Seinost et al., 1999; Lagal et al., 2003).
Statistical analysis. Infection prevalence and 95% confidence intervals (95%CI) were calculated with R software (R Core Team, 2013). The Fisher's exact test was used to study the association among categorical variables. A negative binomial regression with robust standard errors was used to obtain estimates of mean numbers of questing I. ricinus per month, with 95%CI. Negative binomial error was used to take into account the potential overdispersion of the distribution of questing ticks among dragging sites. We applied intercept-only, generalized log-linear models using the GENMOD procedure in the SAS system (SAS version 9.2; Little et al., 2002). The logarithm of the meters of dragging transects at each site/ dragging session was included as an offset variable, to account for site to site and monthly variation in the dragging distance. Monthly estimates of the acarological risk (R, the probability of collecting at least one infected tick in a 100 m transect; Mannelli et al., 2003) were calculated for nymphs (the most likely stage to transmit B. burgdorferi s.l.). The overall infection prevalence in nymphs was used to calculate R, due to the small sample size of ticks tested per each month.
Moreover, VCO province climatic data for the last 10 years, available on the website of the Regional Agency for the Protection of the Environment (ARPA, Piemonte, 2012), were also examined.

Results

Ticks collection. A total of 1766 ticks were collected and identified as I. ricinus. Questing ticks (n = 1662) were collected by dragging in 13 study sites (four in Varzo, six in Calasca-Castiglione and three in Cannobio): 1249 larvae (75.2%), 407 nymphs (24.5%) and six adults (0.3%; three males and three females). Questing larvae peaked in May and September, while nymphs were more abundant in summer months. Mean immature tick numbers and 95% CI calculated according to the model are shown in Fig. 1; the fit of the model was good. The adults were collected in May (n = 2) and July (n = 4). Tick density was highly variable among the different dragging sites and ranged from 0 to 104 larvae and from 0 to 21 nymphs per 100 m2. I. ricinus was collected up to 1450 m a.s.l. (Table 1). The mean number of nymphs collected by 100 m2 did not show great variations up to 1000 m a.s.l. (range: 2.6–3.5 nymphs/100 m2); nymphs slightly increased between 1000–1200 m (6.1, 95%CI: 2.1–17.9) and sharply decreased at higher altitudes (0.4, 95%CI: 0.3–0.5). Moreover, 104 adult *I. ricinus* (30 males and 74 females) were collected from hunted wild animals: 4 chamois (*Rupicapra rupicapra*), 8 roe deer (*Capreolus capreolus*), 23 red deer (*Cervus elaphus*).

Molecular analysis. The PCR assay targeting the 16S fragment was performed on 352 randomly selected *I. ricinus*: 25 adults collected on wild animals and 327 questing ticks (321 nymphs and 6 adults). All tested ticks from ungulates were negative for *B. burgdorferi* s.l. Conversely, a total of 34 questing nymphs were found infected. The DNA sequencing of 32 samples showed the presence of four genospecies: *B. afzelii* (38%), *B. lusitaniae* (38%), *B. garinii* (15%) and *B. valaisiana* (3%). Two samples could not be identified at genospecies level by sequencing due to the weak signal, but their positivity was confirmed by both PCR assays. Positive samples to *B. afzelii* and *B. garinii* (n = 18), which are recognized as pathogenic genospecies, were subjected to the ospC PCR. Phylogenetic analyses included the newly generated and reference sequences, for a total of 131 ospC sequences. Maximum likelihood phylogenetic trees, showing *B. afzelii* and *B. garinii* strains isolated from humans (H) and invasive strains (Inv), are showed in Figs 2 and 3, respectively. Most of *B. afzelii* strains detected in our study (12/13) clustered with sequences identified in humans. Among them, 10 were localized in invasive groups, specifically groups A2 (n = 6), A5 (n = 1), A6 (n = 2) and A7 (n = 1). Similarly, four out of five *B. garinii* samples formed a clade with ospC reference sequences of human invasive strains, clustering with groups G5 (n = 2), G9 (n = 1) and G10 (n = 1).

Statistical analysis. Questing tick infection prevalence was 10.4% (95% CI: 7.1–13.7), and the highest value was observed in Calasca-Castiglione dragging sites (Table 2). Questing nymph infection prevalence was 10.6% (95% CI: 7.4–14.5). Positive samples were collected between 400–1200 m a.s.l., but the majority of positives were found above 600 m (96.6%, Table 3). Indeed, a significant difference in the infection

prevalence was detected between nymphs collected below and above 600 m (Fisher's exact test, P = 0.003). The overall risk of finding at least one nymph infected by B. burgdorferi s.l. in the study area, R, peaked in July (0.39, 95% CI: 0.2–0.7) (Fig. 4). According to the data reported by ARPA, over the last 10 years, the three studied municipalities showed very similar climatic conditions. The temperature seasonal average, ranged from 10 to 18°C in winter, from 16 to 20°C in spring, from 18 to 26°C in summer and from 12 to 20°C in autumn. The average seasonal accumulated rainfall was higher in the municipalities of Calasca-Castiglione and Cannobio in winter (75 mm) and in spring (200 mm), while both in summer and in autumn the municipality of Cannobio was the rainiest (200 and 300 mm, respectively). Varzo was always much less rainy than others (50 mm in winter and 175 mm in the rest of the year). The permanence of the snowpack from 2000 to 2010, showed a duration that ranged from 1 to 120 days in all examined municipalities.

Discussion. This study confirmed the circulation of *B. burgdorferi* s.l. in questing ticks in the Ossola Valley. *I. ricinus*, the main vector of several agents of medical and veterinary importance, was the only tick species collected in our study. In the past, *I. ricinus* was generally found in Italy in deciduous forests below 1000 m a.s.l. but, as reported in many European countries, it is increasingly spreading in high-altitude areas (Lindgren et al., 2000; Moran-Cadenas et al., 2007a; Danielova et al., 2008; Jore et al., 2011; Martello et al., 2013). Our data confirm its presence up to 1450 m a.s.l. in the Italian side of the Western Alps, with 26% of ticks collected over 1000 m (Table 1).

The province of the VCO has a highly variable climate, thanks to a complex geography. In this area, the largest river basins of the region are present (basin of the river Toce), one of the largest lakes of the national territory (lake Maggiore) and mountains which are among the highest in the country (Monte Rosa). Alps and alpine lakes favour the formation of the humid air masses, making Ossola Valley one of the rainiest and coldest areas in Italy. Unfortunately, it is not possible to ascertain if the recent climatic trends had effect on ticks population in VCO on the basis of our tick collection, which was performed in six months of a single year. Further studies extended to a greater number of years could give more reliable information. Opinions about climate changes consequences on tick dynamics are contrasting. Some Authors state that the increase in global temperature can reduce the over-wintering mortality of vectors and new areas become suitable for infected ticks activity; moreover, warmer temperatures allow a shorter interstadial development time, increasing the tick population abundance (Githeko et al., 2000; World Health Organization Report, 2004; Daniel et al., 2009; Knap et al., 2009; Hancock et al., 2011). Likewise, climatic factors can also affect ticks along altitudinal gradient (Gern et al., 2008). On the other hand, some authors consider economic conditions and human behaviour the main causes of tickborne zoonoses (TBZ) incidence, especially for the TBE (Rogers and Randolph, 2006; Godfrey and Randolph, 2011; Medlock et al., 2013). In TBZ and vector diffusion, many factors, strongly interlinked, are involved. It is thus not easy to understand if and how climate factors may have an effect in a particular area (Beugnet and Marie, 2009). Human exposure to tickborne diseases is associated with local abundance of infected ticks, density of vertebrate reservoir hosts and climatic factors. Ticks exposure increases in the case of particular occupational (forestry work and farming) or leisure activities (hunting, mushroom collecting and berry picking) (Alciati et al., 2001). Human pressure and animal migration may also support introduction of new species of ticks from neighbouring countries.

In our study, most of the ticks collected were larvae and nymphs, only six were adults, and this result is probably due to the dragging technique used that is not suitable for the collection of adults.

No infected ticks collected from wild animals were found. As regards questing ticks, only nymphs were positive to B. burgdorferi s.l., and their infection prevalence was similar to the average of other European countries (Jouda et al., 2004). The risk of collecting at least one B. burgdorferi s.l. infected nymph in our study area had a peak in the summer months; however, no sampling was performed in June due to bad weather conditions.

We observed a significantly higher infection prevalence in ticks collected above 600 m compared with ticks collected at lower altitudes. Such result is in contrast with previous observations showing that ticks were less infected with increasing altitude (Burri et al., 2007). The presence of various B. burgdorferi genospecies was detected in the Ossola Valley. *B. lusitaniae* was the only species found in Cannobio. The majority of positive ticks (n = 26) were collected in Calasca-Castiglione, where we identified three genospecies (*B. afzelii, B. garinii and B. lusitaniae*). Local variations in the distribution of genospecies may be due to the available hosts for ticks, which act as Borrelia reservoirs (Richter et al., 2013). *B. afzelii* is mainly associated with rodents, *B. garinii* and *B. valaisiana* with birds, and *B. lusitaniae* with lizards (Mannelli et al., 2012). In Italy, the reported *B. burgdorferi* s.l. prevalence in questing ticks varies considerably among study areas:

from 17.5% to 20.6% in Veneto and Friuli Venezia Giulia (Favia et al., 2001; Rizzoli et al., 2002; Capelli et al., 2012); from 1.3% to 40.1% in Trentino Alto Adige (Cinco et al., 1998; Mantelli et al., 2006; Pecchioli et al., 2007; Nazzi et al., 2010); 18.0% in Lombardy (Pistone et al., 2010); 18.2% in Liguria (Mannelli et al., 2003); from 8.7% to 23.9% in Tuscany (Bertolotti et al., 2006; Selmi et al., 2010; Tomassone et al., 2013); 10.4% in Emilia-Romagna (Corrain et al., 2012); 27.9% in Lazio (Cinco et al., 1997); 29,9% in Marche (Pascucci and Cammà, 2010). It is difficult to compare our results with those reported in the literature given differences in tick species collected, geographic locations and climatic conditions.

Results of the phylogenetic analysis based on ospC gene indicate that the Borrelia strains circulating in ticks and belonging to known pathogenic genospecies may have the potential for human infection and possibly for the invasion of secondary body sites. Almost half of *B. afzelii* strains exhibited the typical pattern of invasive group A2, which included reference sequences associated to ACA. Interestingly, Lagal et al. (2003), who analysed a larger number of invasive *B. afzelii* strains, did not detect any ospC sequences corresponding to group A2. This may reflect geographical differences in the distribution of ospC alleles. Four *B. afzelii* strains corresponded to the invasive groups A5, A6 and A7, which were initially not considered to be invasive (Baranton et al., 2001) till the discovery of cases of ACA, multiple erythema migrans and meningitis associated with these ospC patterns (Lagal et al., 2003). *B. garinii* strains referred to invasive groups G5, G9 and G10, which includes ospC sequences associated with clinical cases of ACA and neuroborreliosis.

Although our study was carried out in a limited time period and relatively small area, it provides the first data on ticks and LB on the Italian side of the western Alps, contributing to confirm the emergence of TBZ in mountain areas in Europe.

It is important to point out the possible increased exposure of people to tick bite in the risk areas and to provide the right information to the institutions, physicians, forestry workers and visitors to adopt proper precautions during season at risk. This would be particularly important in this endemic area as the Ossola Valley, where invasive borrelia strains are present.

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Conflict of interest. There are no conflicts of interest.

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Fig. 1. Mean numbers and 95%CI of questing *Ixodes ricinus* collected by dragging in Verbano-Cusio-Ossola province, Piemonte, from April to September 2011; (a) larvae; (b) nymphs.

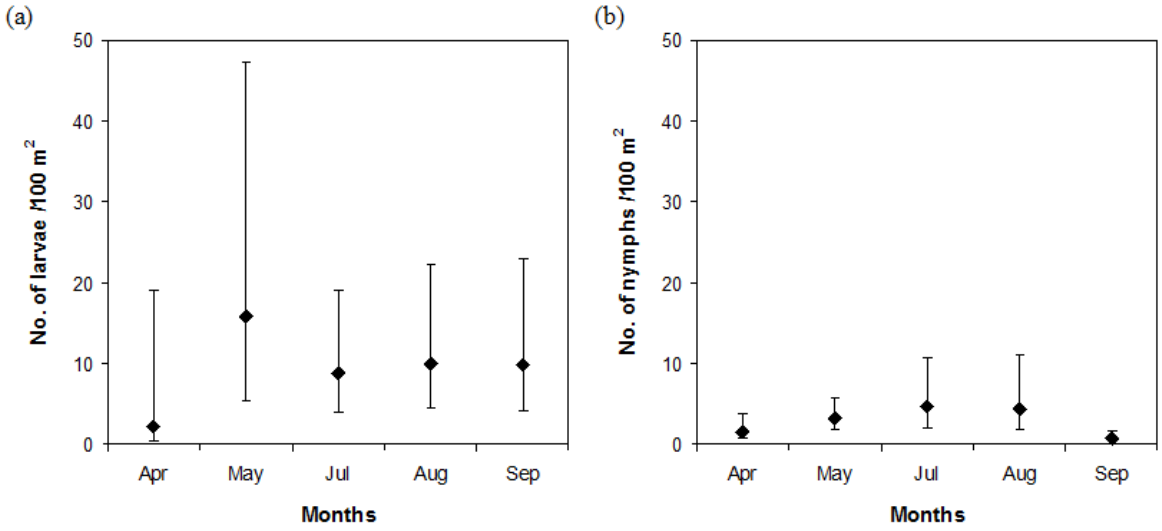

Fig. 2. Maximum likelihood tree of ospC gene sequences from 59 *Borrelia afzelii* isolates. The phylogenetic tree was obtained by an alignment of 547 bp including 13 new sequences from the study area and 46 sequences from GenBank. The isolate identifier is followed by the sequence accession number. Bootstraps (1000 replicates) values >50 are indicated at the internal nodes. The length of each pair of branches represents the distance between sequence pairs. H: human origin (erythema migrans), H Inv.: human origin, invasive phenotype (from chronic disease). *B. afzelii* isolates identified in this study are labelled by a dot. OspC groups are marked.

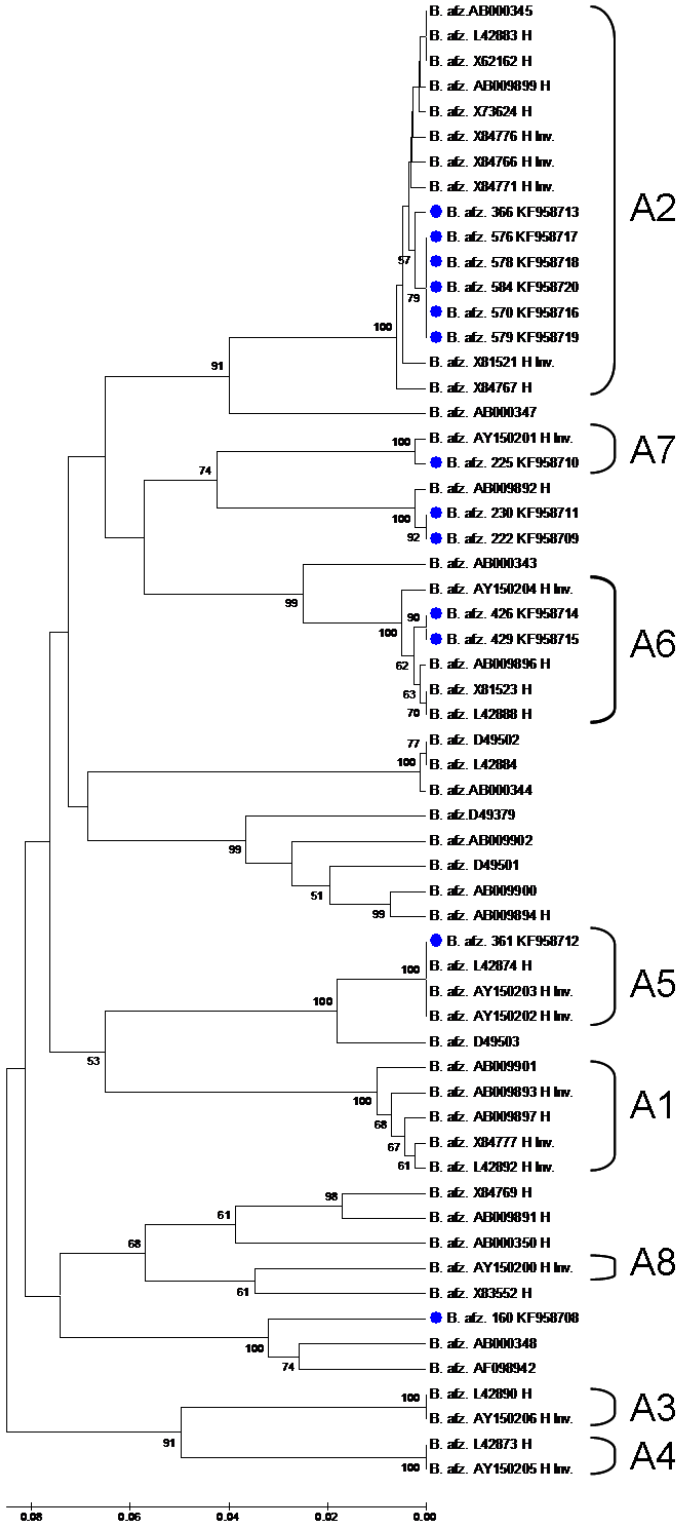

Fig. 3. Maximum likelihood tree of ospC gene sequences from 74 *B. garinii* isolates. The 48 phylogenetic tree was obtained by an alignment of 529 bp including five new sequences from the study area and 69 sequences from GenBank. The isolate identifier is followed by the sequence 50 accession number. Bootstraps (1,000 replicates) values >50 are indicated at the internal nodes. The length of each pair of branches represents the distance between sequence pairs. H: human origin (erythema migrans), H Inv.: human origin, invasive phenotype (from chronic disease). *B. garinii* isolates identified in this study are labeled by a green circle. OspC 2 groups are marked

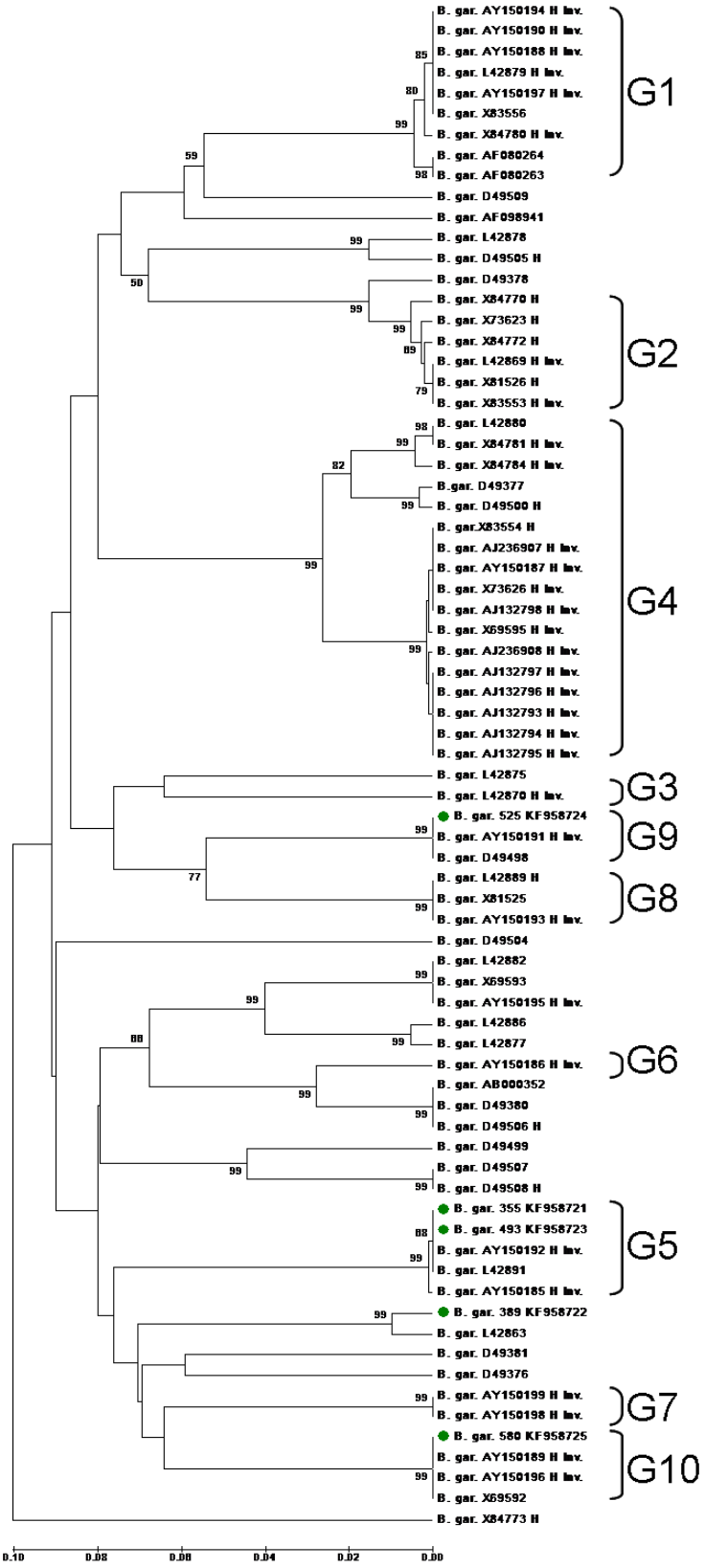

Fig. 4. Acarological risk, R (the probability of finding at least one *Ixodes ricinus* nymph infected with *Borrelia burgdorferi* s.l.), in Verbano-Cusio- Ossola study sites, Piemonte, from April to September 2011. Vertical bars represent 95% CIs.

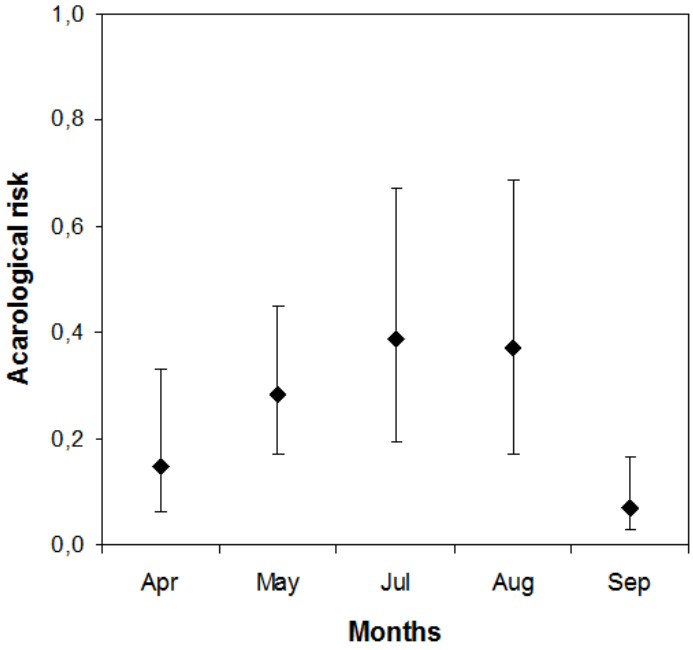

Table 1. *Borrelia burgdorferi* s.l prevalence in questing ticks collected in different municipalities at different altitudinal range; La: larvae, Ny: nymphs, Ad: adults

Elevational range (m a.s.l.)	No transects	Municipality									Total ticks			No positive / No examined ticks	*B. burgdorferi s.l.* infection prevalence (95%CI)
		Varzo			Cannobio			Calasca-Castiglione							
		La	Ny	Ad	La	Ny	Ad	La	Ny	Ad	La	Ny	Ad		
400–600	30	0	0	0	574	82	0	141	21	1	715	103	1	3/101	3.0 (0.6–8.4)
600–800	20	97	35	0	0	0	0	51	25	0	148	60	0	7/51	13.7 (5.7–26.2)
800–1000	26	0	0	0	18	14	0	95	72	3	113	86	3	12/73	16.4 (8.8–26.9)
1000–1200	32	108	58	0	20	7	0	133	83	2	261	148	2	12/94	12.7 (6.8–21.2)
1200–1450	32	12	10	0	0	0	0	0	0	0	12	10	0	0/8	0.0 (0–36.9)
Total	140	217	103	0	612	103	0	420	201	6	1249	407	6	34/327	10.4 (7.3–14.2)

Table 2. Prevalence of *Borrelia burgdorferi* s.l. genospecies in Ixodes ricinus ticks collected by dragging in 3 municipalities of VCO. NI: unidentified

Municipality	No positive / No examined ticks	*B. valaisiana* No (%)	*B. afzelii* No (%)	*B. garinii* No (%)	*B. lusitaniae* No (%)	N.I. No (%)	*B. burgdorferi* s.l. infection prevalence (95%CI)
Varzo	7/90	1 (1.1)	6 (6.6)	0	0	0	7.78 (2.24–13.31)
Cannobio	1/100	0	0	0	1 (1)	0	1.00 (0.00–2.95)
Calasca-Castiglione	26/137	0	07 (5.1)	5 (3.6)	12 (8.7)	2 (1.4)	19.00 (12.41–25.54)
Total	34/327	1 (0.3)	13 (4)	5 (1.5)	13 (4)	2 (0.6)	10.40 (7.09–13.71)

Table 3. Elevational distribution of collected *Ixodes ricinus* nymphs and prevalence to *Borrelia burgdorferi* s.l

Elevational range (m a.s.l.)	Nymphs/ 100 m^2 (95%CI)	No positive/ No examined	Prevalence (%)(95%CI)
<600	2.9 (1.3, 6.4)	3/100	3.0 (0.6, 8.5)
>600	3.3 (1.6, 6.8)	31/221	14.0 (9.7, 19.3)
Total	3.2 (1.8, 5.8)	34/321	10.6 (7.4, 14.5)