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# *Borrelia crocidurae* Infection of *Ornithodoros erraticus* (Lucas, 1849) Ticks in Tunisia

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## Abstract

Tick-borne relapsing fever (TBRF) is caused by *Borrelia* species transmitted to humans by infected *Ornithodoros* sp. ticks. The disease has been rarely described in North Africa, and in Tunisia the local transmission of TBRF seems to have disappeared or is undiagnosed. A longitudinal study was conducted in 14 sites located in four different bioclimatic zones of Tunisia to assess both the distribution of *Ornithodoros* sp. and their infection rate with the relapsing fever *Borrelia* sp. Three polymerase chain reaction methods targeting the 16S rRNA, the intergenic spacer, and the *fla* (flagellin) genes were used and phylogenetic analyses were carried out. Three hundred and fifty-eight specimens of *Ornithodoros* were collected: *O. erraticus* (previously termed "small variety") (n = 190) and *O. normandi* (n = 168). *Borrelia crocidurae* DNA was detected in 15.1% of *O. erraticus* (small variety) (24 out of the 159 randomly selected for testing) collected in rodent burrows situated in the arid and Saharan areas in southern Tunisia. Molecular analysis targeting the 16S rRNA gene and the noncoding intergenic spacer domain showed good resolution for this *Borrelia* sp., although no molecular polymorphism was evidenced according to location. In contrast, none of the 133 *O. normandi*, also randomly selected for testing, was infected by *Borrelia* sp. and these ticks were restricted to the subhumid and semiarid zones in northern Tunisia. Both *O. erraticus* (small variety) and *O. normandi* were found in Tunisia and the high *B. crocidurae* infection rate found in *O. erraticus* highlights the risk of TBRF transmission in the southern part of the country.

Key Words: *Borrelia crocidurae*—*Ornithodoros*—Tick-borne relapsing fever—Tunisia.

## Introduction

**T**ICK-BORNE RELAPSING FEVER (TBRF) is caused by several *Borrelia* species and transmitted by Argasid soft ticks of the genus *Ornithodoros*. This genus comprises about 100 species (Hoogstraal 1985) that are mostly geographically restricted and are considered to be specific vectors of a given *Borrelia* sp. As *Borrelia* sp. may persist for many years in their long-lived vectors, *Ornithodoros* sp. are considered as both vectors and reservoirs (Parola and Raoult 2001). Vertebrate reservoirs include a variety of mammals, mainly rodents, inhabiting burrows, dens, or caves. Humans are infected while in contact with *Ornithodoros* sp. in infested areas. TBRF occurs in all continents, except Antarctica and Australia, and may cause a serious disease with neurological, ocular, or reproductive complications if untreated (Cutler 2006, Cutler

et al. 2009). The reported mortality rate is up to 5% and the severity of the disease varies according to the species: *Borrelia duttonii* being associated with greater severity than *Borrelia crocidurae* (Cutler 2006, Cutler et al. 2009). TBRF is endemic in Africa and is highly prevalent in central, eastern, and southern Africa, where it is due to *B. duttonii* and transmitted by *Ornithodoros moubata* and *Ornithodoros porcinus* (Hoogstraal 1985, Cutler 2006, Cutler et al. 2009). The disease is also endemic in western and Saharan African countries such as Mauritania, Senegal, Mali, and Chad, where it is due to *B. crocidurae* and transmitted by *Ornithodoros sonrai* (formerly genus *Alectorobius*) (Cutler et al. 2009). The rising incidence reported in these regions may be related to the climatic changes (Trape et al. 1991, 1996, Vial et al. 2006). In Northwest Africa, *B. crocidurae* (including the possibly synonymous *Borrelia merionesi* and *Borrelia microti*)

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is transmitted by *Ornithodoros erraticus* previously termed as "small variety" of *O. erraticus* (Morel 1965, Hoogstraal 1985, Stanek 1995) and *Borrelia hispanica* is maintained by *Ornithodorus marocanus*, which is often reported as *O. erraticus* large variety (Hoogstraal 1985, Stanek 1995). In Tunisia, since the first human case of TBRF reported by Nicolle in 1931 (Nicolle et al. 1932), the disease has been neglected and recent epidemiological data are lacking. Local transmission of TBRF seems to have disappeared or is undiagnosed.

We conducted a longitudinal study to assess both the distribution of *Ornithodoros* sp. in Tunisia and their infection rate by relapsing fever *Borrelia* sp. Because of fastidious culture requirements, we used recently developed molecular methods that lead to rapid detection and identification of relapsing fever *Borrelia* sp. in vertebrates and vectors (Cutler et al. 1994, Marti Ras et al. 1996, Fukunaga et al. 2001, Wyplosz et al. 2005, Assous et al. 2006).

## Materials and Methods

### Collection and identification of Ornithodoros ticks

Our study was conducted between March 2005 and July 2006, in 14 sites located in four different bioclimatic zones of Tunisia (Table 1). As *Ornithodoros* sp. are endophilic ticks inhabiting small mammal burrows, the sites were selected on the basis of the presence of such burrows, mainly occupied by jird (*Meriones shawi*), gerbil (*Gerbillus campestre*), and the fat sand rat (*Psammomys obesus*) (Bernard 1970). In each site, burrows were randomly selected, regardless of their activity, because *Ornithodoros* sp. are able to survive for long periods, even up to 5 years, in the absence of their hosts. Burrows were opened to the nest, using a pickaxe. Nest soil samples were sieved through wire mesh to eliminate debris and then exposed to sunlight on a sorting tray. All ticks collected were stored into flasks containing 70% ethanol until identification and DNA extraction. For identification, we used the description of Colas-Belcour (1930) based on the camerostomal folds and the drawing of the dorsal surface (or integument) of the body. The morphology of the coxae 1 was used as an additional criterion (Fig. 1). The sex and developmental stage were determined for each tick.

### DNA extraction and polymerase chain reaction methods

Tick DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 $\mu$L of ADMH Tris-HCL; 0.5 mM EDTA PH 9.0 (AE) buffer and stored at $-20°$C until amplification. Three polymerase chain reaction (PCR) protocols were used to detect relapsing fever *Borrelia* DNA in ticks. First, a seminested protocol targeting the entire 16S rRNA gene and using the outer primers fD3 and T50 was carried out, as previously described (Marti Ras et al. 1996). The inner primers were REC4, as previously described (Marti Ras et al. 1996), and RF16SR (5′ pos 867-AGGCGCCACACTTAA CACGT-3′pos 847). REC4 and RF16SR were paired with T50 and fD3, respectively, to obtain two amplicons with a 208 bp overlap. These two contigs were aligned to obtain the sequence of the entire 16S rRNA gene (1.5 kb long) (Sarih et al. 2009). Second, a nested protocol targeting the large noncoding intergenic spacer (IGS) between the 16S and 23S rRNA genes was used (Bunikis et al. 2004). The third PCR method was a classic single PCR targeting the *fla*B (flagellin) gene (Assous et al. 2006). The PCR products were revealed by electrophoresis in a 0.8% agarose gel in Tris-borate-EDTA (TBE), stained with ethidium bromide.

### Sequence alignment and phylogenetic relationships

Amplification PCR products were sequenced by Genome Express (Meylan, France) using the same oligonucleotides as for PCR (Marti Ras et al. 1996, Bunikis et al. 2004, Assous et al. 2006, Sarih et al. 2009). All the sequences determined in this study were submitted to GenBank (accession numbers for the IGS are from GQ358160 to GQ358180 and for the 16S rRNA are from GQ358181 to GQ358200). The CLUSTAL X

TABLE 1. *ORNITHODOROS* SPECIES AND *BORRELIA CROCIDURAE* INFECTION IN TUNISIA

| Site (no.) | Bioclimatic zone | Coordinates North | Coordinates West | Number of burrows examined | Ornithodoros normandi Total (N/M/F)[a] | Ornithodoros erraticus Total (N/M/F)[a] | O. erraticus positive for Borrelia/ticks tested (%) |
|---|---|---|---|---|---|---|---|
| Jbel El Hnoucha (1) | Subhumid | 37° 00′ 90,8″ | 09° 43′ 08,1″ | 10 | 64 (46/1/17) | 6 (3/1/2) | 0/6 |
| Sabâ El Aouinet (2) | Subhumid | 36° 59′ 72,2″ | 09° 46′ 44,7″ | 5 | 29 (21/4/4) | 0 | 0 |
| Oued Remel (3) | Semiarid | 36° 06′ 92,2″ | 08° 38′ 31,24″ | 7 | 34 (19/9/6) | 0 | 0 |
| Oued Errebh (4) | Semiarid | 36° 24′ 51,74″ | 10° 08′ 08,45″ | 7 | 41 (26/9/6) | 0 | 0 |
| Hanchir el Ghort (5) | Semiarid | 36° 33′ 02,4″ | 10° 36′ 39,72″ | 7 | 0 | 0 | 0 |
| Forêt Dar Chichou (6) | Subhumid | 36° 58′ 080″ | 10° 59′ 75,2″ | 7 | 0 | 0 | 0 |
| Echahda El Gharbia (7) | Arid | 35° 16′ 13,5″ | 10° 14′ 12,3″ | 5 | 0 | 25 (19/4/2) | 5/23 (21.7) |
| Sabkhet El Khsima (8) | Arid | 35° 33′ 08,8″ | 10° 12′ 72,8″ | 5 | 0 | 4 (2/1/1) | 1/4 (25) |
| Sabkhet Bhir (9) | Arid | 35° 14′ 73,6″ | 09° 45′ 38,4″ | 15 | 0 | 65 (36/12/17) | 9/56 (16.1) |
| Sebket Echrita (10) | Arid | 35° 16′ 13,0″ | 10° 14′ 20,8″ | 7 | 0 | 15 (10/4/1) | 2/14 (14.3) |
| Jelma (11) | Arid | 35° 14′ 02,92″ | 09° 27′ 42,54″ | 6 | 0 | 0 | 0 |
| El Guettar (12) | Arid | 34° 18′ 10,27″ | 08° 59′ 32,10″ | 11 | 0 | 46 (38/4/4) | 4/35 (11.4) |
| Limaguess (13) | Saharian | 33° 45′ 57,51″ | 09° 05′ 02,22″ | 8 | 0 | 29 (25/4/0) | 3/21 (14.3) |
| Djemna (14) | Saharian | 33° 34′ 54,34″ | 09° 00′ 58,71″ | 8 | 0 | 0 | 0 |
| Total | | | | 108 | 168 | 190 | 24/159 (15.1) |

[a]Nymphs/males/females.
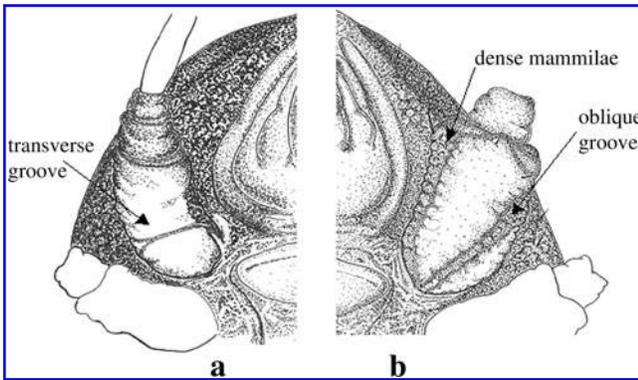
**FIG. 1.** Diagram of coxae I: (**a**) *Ornithodoros normandi*, (**b**) *Ornithodoros erraticus* (drawn by the author).

(Thompson et al. 1997) algorithm was used for sequence alignments and MEGA 4.1 (Kumar et al. 1994) software for phylogenetic analyses. Distances were calculated using the Jukes and Cantor correction (Saitou and Nei 1987) in a pairwise deletion procedure. Sequences were then analyzed by the unweighted pair group with mathematical average (Sneath and Sokal 1962) for the construction of the phylogenetic trees.

## Results

### Collected ticks

Three hundred and fifty-eight *Ornithodoros* sp. were collected in the 108 burrows examined and ticks were found in 10 out of the 14 sites investigated, from a depth of 50 cm to 2 m (Table 1). Ticks belonged to the species *O. erraticus* (previously named small variety; Lucas 1845) ($n = 190$) and *Ornithodoros normandi* ($n = 168$) and details are presented in Table 1. The distinction between these *Ornithodoros* sp. was based mainly on the features of the coxae 1 (the first segment of the leg), which presents a transverse groove in *O. normandi*, in contrast to an oblique one with a dense mammilae in *O. erraticus* (Fig. 1).

### Borrelia *sp. detection in* Ornithodoros *sp. ticks*

Among the ticks collected, 159 *O. erraticus* (small variety) (111 nymphs, 22 males, and 26 females) and 133 *O. normandi* (90 nymphs, 15 males, and 28 females) were randomly selected and tested for *Borrelia* infection. *Borrelia* DNA was detected by both the 16S and the IGS PCR in 24 *O. erraticus*, leading to an infection rate of 15.1%. The *fla* PCR method was positive in only 50% of these samples. In contrast, no *Borrelia* DNA was detected from the 133 *O. normandi* tested. The infection rates of *O. erraticus* ticks were 11.7% for nymphs (13 positive out of 111), 27.3% for males (6 positive out of 22), and 19.2% for females (5 positive out of 26). No significant difference was observed between the infection rates of adults and nymphs ($\chi^2 = 3.28$; $p = 0.07$) and between males and females ($\chi^2 = 0.44$; $p = 0.5$). The *O. erraticus* infected by *Borrelia* sp. were collected only in the arid and Saharan areas (site no. 7–10, 12, and 13 in Table 1), whereas no *Borrelia* DNA was detected in the ticks collected in the subhumid and semiarid areas in northern Tunisia, even in Jbel El Hnoucha (site no. 1 in Table 1) where *O. erraticus* ticks were found.

The entire 16S rRNA sequences were available from 20 out of the 24 PCR products obtained from *O. erraticus* (small variety) and *B. crocidurae* was identified from all these sequences by BLAST (Basic Local Alignment Search Tool) analyses. All 16S rRNA sequences were submitted to GenBank (accession no. GQ358181 to GQ358200). They demonstrated 99.93% (1 nucleotide [nt] difference) to 100% identity with *B. crocidurae* DQ057990 from GenBank. This polymorphism (1 nt difference) was illustrated on the phylogenetic tree by the shallow division in two close branches, which comprised all our *B. crocidurae* Tunisian sequences (Fig. 2). We noted only very small differences between African species, confirming previous results (for examples, 1 or 2 nt difference between our *B. crocidurae* Tunisian sequences and *B. duttonii* Ly AF107364, and 2 or 3 nt difference between *B. crocidurae* and *B. hispanica* DQ057988) (Scott et al. 2005, Wyplosz et al. 2005, Sarih et al. 2009) (Fig. 2). In the IGS analysis, all the 21 *Borrelia* sequences obtained from the Tunisian *O. erraticus* were grouped in a single cluster with the *B. crocidurae* DQ000287 (Fig. 3). These sequences were submitted to GenBank (accession no. GQ358160 to GQ358180). As expected, the IGS sequences showed larger intraspecies and interspecies polymorphisms than the 16S rRNA sequences, with the exception of *Borrelia recurrentis* and *B. duttonii* East African species, which cannot be separated by their IGS sequence as previously shown (Scott et al. 2005). However, no molecular polymorphism according to the location has been observed among our *B. crocidurae* sequences (Figs. 2 and 3). Because of the inferior sensitivity of the *fla* PCR, only three *fla* sequences could be determined, leading to *B. crocidurae* identification.

## Discussion

During our investigation, 108 burrows, in four bioclimatic zones of Tunisia, were opened and investigated for the presence of soft ticks of the genus *Ornithodoros*. We collected 358 *Ornithodoros* sp. belonging to *O. normandi* Larrousse and *O. erraticus* Lucas species. In agreement with Larrousse (1923), we found *O. normandi* only in the subhumid and semiarid zones. Thus, the distribution of this endemic species seems to be restricted to the north of Tunisia above the 400 mm isohyets. By contrast, *O. erraticus* was mainly collected in burrows situated in the arid and Saharan zones (Table 1). *O. erraticus* occurs in southwestern ex-USSR, the Middle East, and the Mediterranean Basin (Morel 1965, Hoogstraal 1985, Manilla 1998). Shelters and hosts of this species are usually located in lowland, deserts, semideserts, and dry tracts in cultivated zones, but rarely in humid biotopes (Morel 1965, Hoogstraal 1985). In only one site (Jbel El Hnoucha), situated in the subhumid zone in northern Tunisia (site no. 1 in Table 1), *O. normandi* and *O. erraticus* were collected together in two burrows inhabited by *M. shawi*. However, all ticks from this site were free of *Borrelia* DNA and the *Borrelia* DNA detection was restricted to the *O. erraticus* collected in arid and Saharan zones. The most prevalent rodent species in Tunisia are *M. shawi* in the north and the center of the country and *P. obesus* in the center and the south (Bernard 1970, Fichet-Calvet et al. 2000). Indeed, these two species primarily inhabited the inspected burrows and our results suggest that they may be involved in the maintenance of *Ornithodoros* sp. in these areas and thus, possibly,
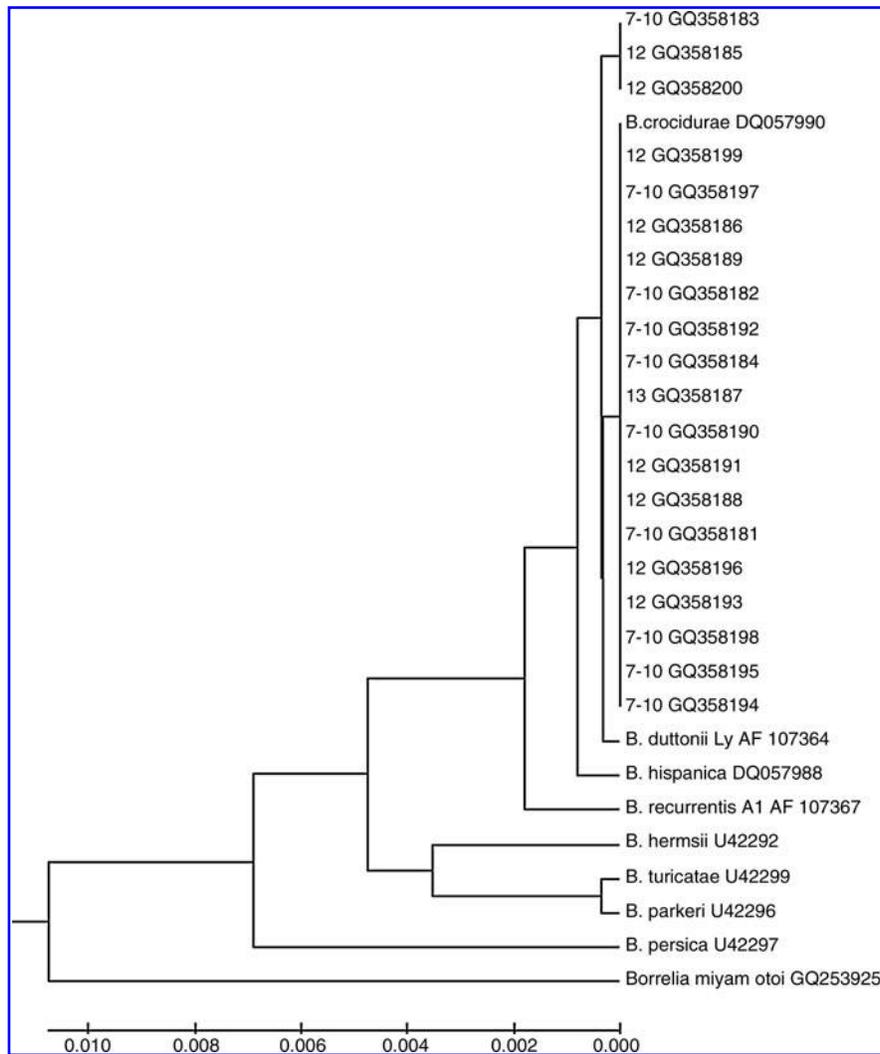
**FIG. 2.** Phylogenetic tree based on partial 16S rRNA sequences (1428 nt, position 43 to 1470) of 20 *Borrelia crocidurae* isolates from *O. erraticus* (small variety) collected in Tunisia. The tree was constructed by the unweighted pair group with mathematical average method, using a pairwise deletion procedure, and distances were calculated with the Jukes and Cantor method. Sequences are numbered beginning with the number of the site (Table 1) where the tick was collected (no distinction was made between sites 7, 8, 9, and 10) followed by the GenBank accession number.

in TBRF transmission. These results confirm the previous study that reported the detection of *Borrelia* sp. in 8% of blood samples collected from *P. obesus* captured in the central area of Tunisia (Fichet-Calvet et al. 2000).

In this study, we showed that sequencing the IGS region provided a high level of resolution for *B. crocidurae*, as for other species of relapsing fever *Borrelia*, with the exception of the complex *B. duttonii/B. recurrentis*, which cannot be separated by the IGS sequences, as previously shown (Scott et al. 2005, Sarih et al. 2009). *B. crocidurae* TBRF is common in West Africa where it is considered as a major cause of morbidity with a reported prevalence rate of 10% (Trape et al. 1996). In Senegal, Mauritania, and Mali, the average infection rate of the vector, *O. sonrai*, was 31% (Vial et al. 2006). In Tunisia, *B. crocidurae* relapsing fever has been endemic and may have been responsible for epidemic relapsing fever in North Africa in the wake of Word War II (Nicolle et al. 1932, Hoogstraal 1985). Surprisingly, for the

last few decades, no report on TBRF was recorded in this country. Here we showed that amplification of *B. crocidurae* DNA was successful from 15.1% of *O. erraticus* (small variety). The reported rate of *O. sonrai* infected by *B. crocidurae* in West Africa was higher than the one we estimated in *O. erraticus* in southern Tunisia (Vial et al. 2006). This difference may reflect either a difference in vector competence of each tick species or a variable abundance and infection of the mammalian reservoirs. Nevertheless, the *B. crocidurae* infection rate of ticks obtained in our study highlights the risk of TBRF transmission in southern Tunisia and our results confirm that *O. erraticus* (small variety) may be involved in *B. crocidurae* transmission in Tunisia and may infect humans. The human disease is probably underdiagnosed and neglected in Tunisia partly because of the low sensitivity of the direct microscopic analysis of blood samples, which is the only diagnostic test available in most clinical laboratories. In addition, transmission is expected to occur mainly in rural
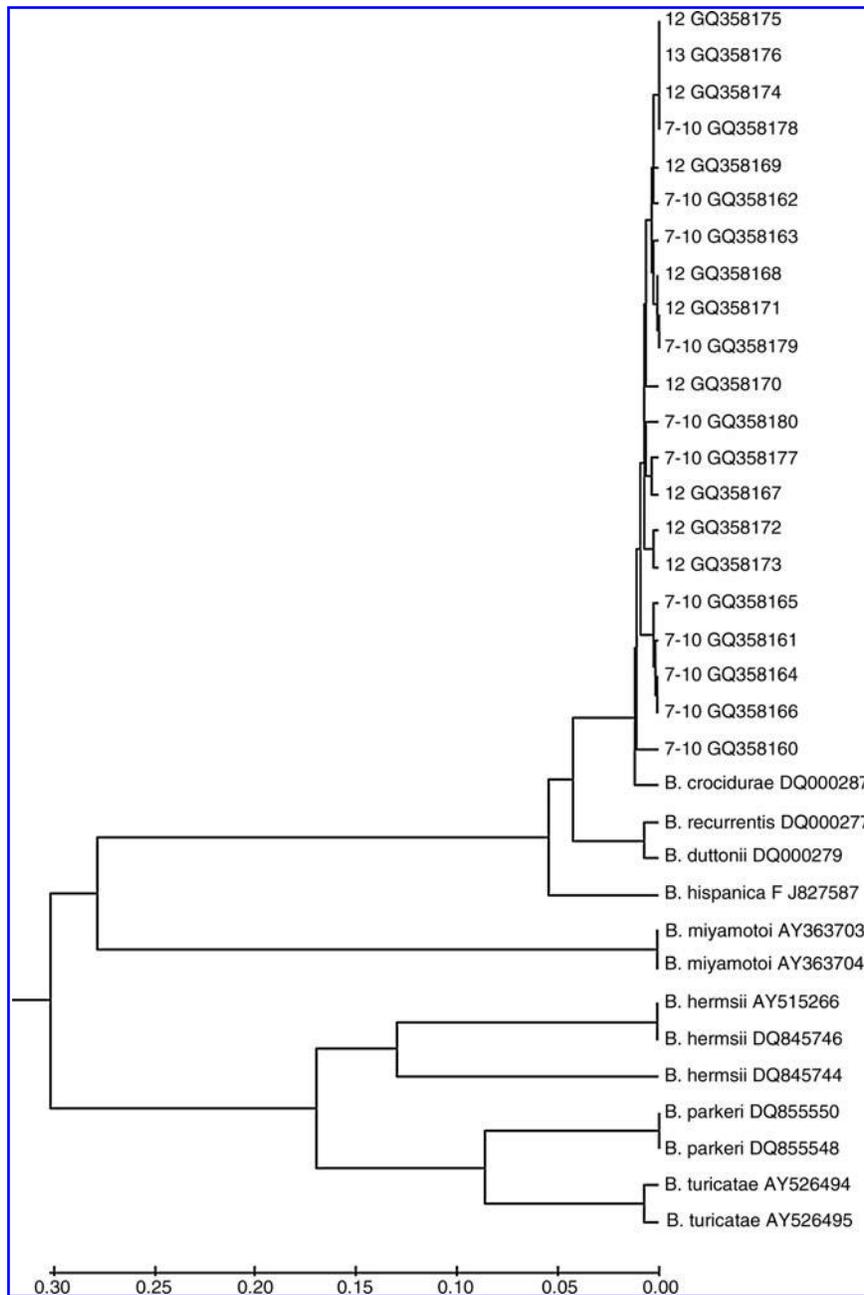
**FIG. 3.** Phylogenetic tree based on noncoding 16S–23S intergenic spacer sequences of 21 *B. crocidurae* isolates from *O. erraticus* (small variety) collected in Tunisia. The tree was constructed by the unweighted pair group with mathematical average method, using a pairwise deletion procedure, and distances were calculated with the Jukes and Cantor method. Sequences are numbered beginning with the number of the sampling site (Table 1) (no distinction was made between sites 7, 8, 9, and 10) followed by the GenBank accession number.

settings where human hosts have likely, if any, more contact with the tick vector. Rural community does not necessarily attend health facilities because of distance and also because of the mild severity of the *B. crocidurae* infection. In Morocco, TBRF due to *B. hispanica* still occurs and may be attributed in part to the vector behavior (Sarih et al. 2009). Indeed, *O. marocanus*, vector of *B. hispanica*, infests stables and pens of domestic animals, whereas *O. erraticus* lives in the immediate vicinity of rodent burrows, limiting contact with human hosts (Hoogstraal 1985).

Climate change (drought and temperature increase) may lead to the colonization of new areas in North Africa by *O. erraticus*, which acts as vector of *B. crocidurae*. Indeed, drought is reported as responsible for a considerable spread of tick-borne borreliosis in West Africa (Trape et al. 1996). Therefore, our results suggest the possibility of an underdiagnosis and neglected occurrence of TBRF in Tunisia and should lead to clinical studies to evaluate the incidence of TBRF in this country and to establish a surveillance of the emergence of this disease.

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## Disclosure Statement

No competing financial interests exist.

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