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Survey for the Pathogenic Chytrid Fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans* on the Caucasian Salamander in Northeastern Turkey

Abdullah Altunişik[1], *Serkan Gül, Sümeyye Altunişik, and Ayşenur EmiNoğlu*

Department of Biology, Faculty of Arts and Sciences, Recep Tayyip Erdoğan University, Zihni Derin Campus, Atatürk Street, 53100, Rize, Turkey
[1]Corresponding author, e-mail: abdullah.altunisik@erdogan.edu.tr

Abstract.—**Chytridiomycosis is an infectious disease of amphibians caused by either of two aquatic fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). In this study, we carried out *Bd* and *Bsal* screening to determine the presence or absence of these fungal pathogens in seven populations of Caucasian Salamanders (*Mertensiella caucasica*), which is categorized as Vulnerable on the Red List of the International Union for Conservation of Nature and whose distribution is limited to the countries of Georgia and Turkey. We collected 70 skin swabs from seven populations in northern Turkey, and analysed them using Real-time PCR (quantitative PCR, qPCR). We did not detect the presence of either fungal pathogen or signs of morbidity or mortality in any of the populations sampled. Moreover, we classified the quality of the streams occupied by the species using criteria established by the Ministry of Agriculture and Forestry of the Republic of Turkey for inland surface waters. We suggest that the apparent absence of both pathogens is related to the habitat selectivity of the species because Caucasian Salamanders inhabit waters that fall into similar categories of quality criteria parameters, including dissolved oxygen, nitrate, and pH. We hope that this preliminary data will contribute to effective protection management of this vulnerable species in the future.**

Key Words.—biodiversity; chytridiomycosis; conservation; habitat quality; fungal disease; *Mertensiella caucasica*

Introduction

Over the last three decades, declines in amphibian populations have occurred rapidly throughout the world (Daszak et al. 2003). Six main factors (commercial use, the introduction of predatory exotic species, habitat change, pollutants, climate change, and infectious diseases) stand out as potential drivers of these declines (Collins et al. 2009). Infectious diseases are among the factors that have had the most negative impact on amphibian populations in recent years (https://amphibiaweb.org). According to the latest records, 38 species of amphibians live in Turkey. Five of these species are listed as Endangered (EN), one of which is categorized as Critically Endangered (CR; https://amphibiaweb.org). In addition, 73% of the populations of the species are decreasing, 20% of them are stable, 3.3% of them are increasing, and the status of 3.3% of them is unknown (International Union for the Conservation of Nature [IUCN] 2021).

Chytridiomycosis is an infectious disease observed in amphibians, and is caused by two aquatic fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*; Daszak et al. 2003; Martel et al. 2014). These pathogens have spread across the world and, along with habitat degradation, have become one of the major threats to amphibian biodiversity (Scheele et al. 2019). In relation to its negative effects on biodiversity, chytridiomycosis may be the worst infectious disease in recorded history (Skerratt et al. 2007; Stegen et al. 2017). To protect biodiversity, research has focused recently on surveys of these fungal pathogens to track the spread of chytridiomycosis (Reeves et al. 2017; Blaustein et al. 2018; Sabino-Pinto et al. 2018; Adams et al. 2020; Diaz et al. 2020). Berger et al. (1998) initially described chytridiomycosis as a disease responsible for amphibian deaths, and the pathogen *Bd* causing this disease was identified a year later (Longcore et al. 1999). Initially, a single species, *Bd,* was known to cause this disease. The increasing deaths of Common Fire Salamanders (*Salamandra salamandra*) in the Netherlands (Spitzen-Van Der Sluijs et al. 2013) led to the discovery of a second species of pathogenic fungus, *Bsal* (Martel et al. 2013).

Bsal differs morphological and genetically from *Bd* and grows optimally at lower environmental temperatures (Martel et al. 2013). For example, although *Bsal* grows at temperatures as low as 5° C, with optimal growth between 10° C and 15° C and death at ≥ 25° C (Martel et al. 2013), *Bd* grew at temperatures as high as 28° C (Longcore et al. 1999), with optimal growth between 17° C and 23° C and death at ≥ 29° C or below 0° C (Sonn et al. 2017). Although optimum growth occurs at higher temperatures in culture, *Bd* has a higher prevalence in natural environments at high altitudes and in the winter season (Woodhams and Alford 2005; Sapsford et al. 2013). Given that *Bsal* grows better at a lower temperature *in vitro*, high altitude locations with a cold climate are primary areas for surveillance (Parrott et al. 2017). Moreover, environmental temperature (Blooi et al. 2015; Beukema et al. 2021) affects immune defenses and susceptibility to *Bsal* (Carter et al. 2021).

Caucasian Salamanders (*Mertensiella caucasica*) are distributed from the western part of the Lesser Caucasus Mountains to southwest Georgia and northeast Turkey. Turkey has a variety of climatic conditions because of its irregular topography. The Black Sea region, which encompasses the habitat of *M. caucasica*, has an oceanic climate (Köppen climate classification: Cfb); therefore, it is humid and wet (winter 7° C, summer 23° C; Şensoy et al. 2008). In Turkey, *M. caucasica* inhabits appropriate habitat in Ordu, Giresun, Gümüşhane, Trabzon, Rize, and Artvin provinces (Baran et al. 2012), and this species is considered Vulnerable (VU) by the IUCN (Kaya et al. 2009). One of the main threats for *M. caucasica* is habitat change caused by alteration of the environment by humans. Forest degradation (tree cutting), transporting timber in streams used by salamanders for breeding, and habitat destruction from livestock are all documented causes of population declines (https://amphibiaweb.org). This species avoids human contact in general, and its populations do not thrive in anthropogenically modified environments.

There has been little research on *Bd* in Turkey (Göçmen et al. 2013; Erişmiş et al. 2014, 2019; Erişmiş 2019) and none on *Bsal*, despite both the potential for suitable conditions and the presence of threatened species, including salamanders. Erişmiş et al. (2014) tested 228 individuals of seven amphibian species, including Marsh Frogs (*Pelophylax ridibundus*), Oriental Tree Frogs (*Hyla orientalis*), Eurasian Green Toads (*Bufotes variabilis*), Beyşehir Frogs (*Pelophylax caralitanus*), Syrian Spadefoots (*Pelobates syriacus*), Southern Crested Newts (*Triturus karelinii*), and Iranian Long-legged Frogs (*Rana macrocnemis*) for *Bd*, and they and reported the presence of this pathogen in four of these species, all anurans (*e.g.*, *P. ridibundus*, *H. orientalis*, *B. variabilis*, and *P. caralitanus*). In the Eastern Black Sea Region of Turkey, Erişmiş (2019) found *Bd* in 13 of 62 individuals of six species, including *P. ridibundus,* Common Toads (*Bufo bufo*), Caucasian Toads (*B. verrucosissimus*), Caucasian Parsley Frogs (*Pelodytes caucasicus*), Northern Banded Newts (*Ommatotriton ophryticus*), and *B.variabilis*. Furthermore, in the Lake District of southwest Turkey, Erişmiş et al. (2019) detected *Bd* in endemic Beyşehir Frogs (*Pelophylax caralitanus*); however, Göçmen et al. (2013) did not found *Bd* in terrestrial Turkish salamanders in the genus *Lyciasalamandra* from southwest Turkey despite the occurrence of this pathogen in syntopic Levant Water Frogs (*Pelophylax bedriagae*). We surveyed for the presence of the pathogens that can cause chytridiomycosis on Caucasian Salamanders (*Mertensiella caucasica*), and we classify the quality of the habitats in which the species lives.

Materials and Methods

Studied species.—The Caucasian Salamander is a habitat-selective species that especially prefers Mediterranean bush forests and mixed forests with Oriental Beech (*Fagus orientalis*), boxwood (*Buxus* sp.), and coniferous trees (Nordmann Fir, *Abies nordmanniana*; and Caucasian Spruce, *Picea orientalis)*. This species avoids habitats modified for human use (Kaya et al. 2009), and it is found typically near waterfalls in streams at elevations of 500–2,800 m in Turkey (Özeti and Yılmaz 1994) and 50–2,400 m in Georgia (Tarkhnishvili and Kaya 2009). Individuals tend to avoid large and rapid currents and primarily inhabit smaller tributaries of rivers that do not exceed 100–150 cm width and 20–30 cm depth during spring flow. These streams are mostly shaded by a canopy formed by dense trees and herbaceous vegetation, including Ostrich Fern (*Mateuccia strutiopteris*; Fig. 1).

Field sampling.—We sampled live, wild Caucasian Salamanders from seven populations (10 individuals for each habitat) that encompassed the distribution of the species in Turkey (Fig. 2), and that represented different ecological conditions. We captured 70 salamanders by dip net or hand between May 2018 and September 2018 and used sterile nitrile gloves to place each of them in a separate plastic container. We swabbed five times on both sides of the venter, inner thighs, pelvic area, and the medial surface and between toes on of both manus and pes (Marquis et al. 2019) with a dry, sterile cotton swab (MW113; Medical Wire and Equipment Co. Ltd., Corsham, UK). At the end of sampling, we released animals to their own habitats, and stored collected samples at -80° C until analysis. We analyzed water chemistry at each site to guage quality of aquatic habitat and to provide context for examining pathogen occurrence. We measured environmental temperature, water temperature, pH, dissolved oxygen, and conductivity with a portable multimeter device (model HQ40D; Hach Company, Loveland, Colorado, USA). Furthermore, we measured phosphate ion, ammonium nitrogen, nitrate, and nitrite using a spectral photometer in the water chemistry analysis laboratory of Recep Tayyip Erdogan University. If we could not analyze the water samples on the same day, we froze them until the day of analysis.

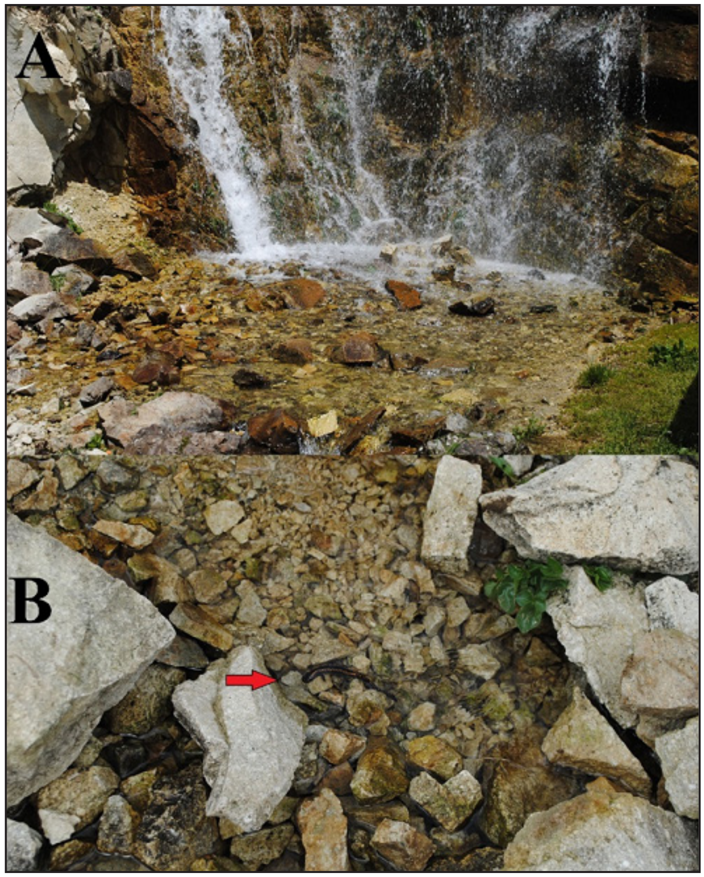

Figure 1. (A) Habitat of the Caucasian Salamander (*Mertensiella caucasica*) in Güngören, Turkey, and (B) a salamander (red arrow) in the habitat. (Photographed by Serkan Gül).

***Quantification of* Bd *and* Bsal *infection*.**—We analyzed the samples for presense of *Bd* and *Bsal* by a protocol of duplex real-time polymerase chain reaction (qPCR; Blooi et al. 2013). We obtained cultures (*Bd*: JEL423, *Bsal*: AMFP13/1 type strain) from Dr. An Martel (Ghent University, Belgium). We prepared 0.1 to 1,000 zoospore equivalents of serial dilution series for the *Bd* and *Bsal* standard curves for each plate. We performed duplex real-time polymerase chain reaction (qPCR) with a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) using previously described methods (Blooi et al. 2013). We included positive, negative, and no-template controls in each PCR run.

***Statistical analyses*.**—We categorized swabs as *Bd* or *Bsal* positive at ≥ 1 genomic equivalent (GE) of zoospores and as *Bd*- or *Bsal*-negative at < 1 GE (Kriger et al. 2006; Hyatt et al. 2007; Savage et al. 2011; Erişmiş et al. 2014). We computed the prevalence of infection for both pathogens by dividing the number of infected samples by the total specimens. We used the Chi-square Test to identify differences between water quality parameters. We processed all statistical tests with the SPSS 22 (IBM, Armonk, New York, USA) and Excel (Microsoft, Redmond, Washington, USA) at $\alpha = 0.05$. To evaluate water quality of streams inhabited by Caucasian Salamanders, we used quality criteria by classes of inland surface water resources of the Ministry of Agriculture and Forestry of the Republic of Turkey (Altunışık et al. 2021; https://www.resmigazete.gov.tr/eskiler/2015/04/20150415-18.htm), which recognizes four classes of water quality: Class I. High-quality water; Class II. Slightly polluted water; Class III. Contaminated water; and Class IV. Heavily contaminated water that can be used only after improvement.

Results

We did not detect *Bd* or *Bsal* on any of the 70 salamanders sampled from the seven populations of Caucasian Salamanders in northern Turkey; furthermore, we did not note any lesions on the skin of any salamanders (Fig. 3; Appendix Table). Based on water quality criteria, aquatic habitats at Dereli and Kocadal were Class I, those at Çambaşı Güngören and Güvenköy were Class III, while the aquatic habitats at Kayabaşı and Camili habitats were Class IV (Appendix

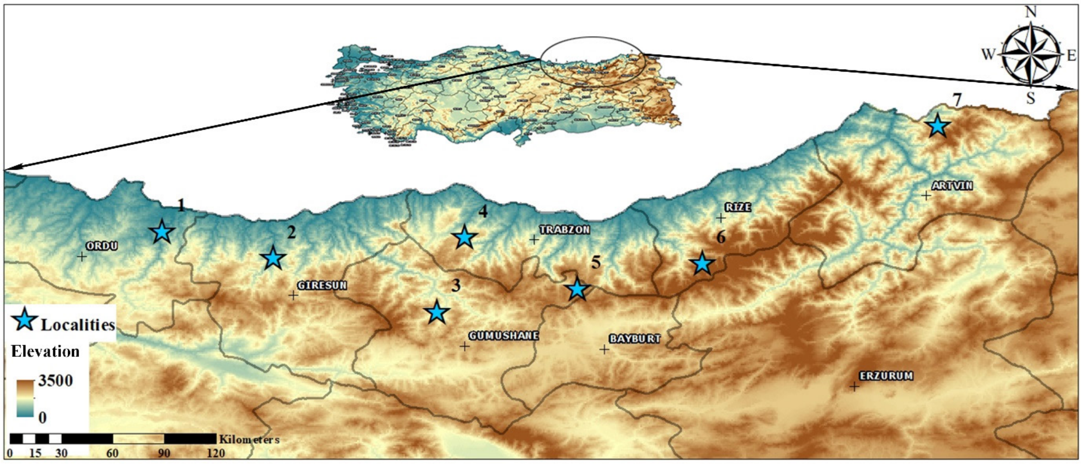

Figure 2. Locations where Caucasian Salamanders (*Mertensiella caucasica*) were sampled for *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) in northern Turkey: (1) Çambaşı. Kabadüz/Ordu, (2) Dereli/Giresun, (3) Kocadal village. Torul/Gümüşhane), (4) Kayabaşı. Akçaabat/Trabzon, (5) Güngören village. Gümüşhane, (6) Güvenköy. İkizdere/Rize, (7) Camili. Borçka/Artvin.

Table). Based on dissolved oxygen, most habitats were Class II, the exception was the habitat at Camili. Based on nitrate concentration, Dereli habitat was Class I, Güngören habitat was Class III and the other five habitats (Çambaşı, Kayabaşı, Kocadal, Güvenköy, Camili) were Class II (Appendix Table).

Figure 3. (A) Dorsal and (B) ventral view of a Caucasian Salamander (*Mertensiella caucasica*) from Güvenköy, Turkey. (Photographed by Abdullah Altunışık).

Discussion

Salamanders inhabiting Turkey have not yet suffered from infection by the fungal pathogens *Bd* and *Bsal* (Göçmen et al. 2013; Erişmiş 2019, our data). The lack of *Bd* and *Bsal* occurrence on 70 Caucasian Salamanders from seven populations in the Black Sea region of northern Turkey adds to the work of Erişmiş (2019) who did not find *Bd* in three individuals. Similarly, Göçmen et al. (2013) did not detect *Bd* in nine endemic Turkish salamanders (belonging to genus *Lyciasalamandra*) from southwest Turkey. We suggest that the relative isolation and lack of human disturbance of preferred habitat is associated with the low occurrence of these fungal pathogens in salamanders. Quality of aquatic habitat and timing of sampling period possibly affects our ability to detect the pathogens, particularly if their prevalence is low.

Nitrogen fertilizers (commonly nitrate or ammonium compounds) are used in large quantities in most agricultural areas; consequently, many habitats used by amphibians are contaminated with nitrogen (Marco and Ortiz-Santaliestra 2009). Nitrate concentrations > 3 mg N/L indicate anthropogenic pollution (Madison and Brunet 1985) and are commonly observed in ponds and small streams in agricultural and urban areas (Marco and Ortiz-Santaliestra 2009). Among the aquatic habitats we analyzed, we do not think that nitrogen-based pollution exists because nitrogen levels in all locations are < 3 mg/L. Although Kocadal and Güngören populations have higher nitrate, ammonium, and sulfate values compared to other populations, this difference is not statistically significant. According to the water quality classification, almost all habitats are in the same category in terms of dissolved oxygen, which means that these salamanders can live in habitats with a certain oxygen level that may be related to the habitat selectivity of the species. The ion exchange rate between amphibians and their surroundings is influenced by several environmental factors (Wells 2007). In amphibian larvae, low pH (high acidity) impairs osmoregulation by reducing sodium ion loss from the skin and active sodium ion absorption (Freda and Dunson 1985; Altunışık et al. 2021). According to the stream quality analysis, although the pH values of Dereli and Kocadal habitats are lower than the others, this does not appear to have influenced the prevalence rate. Temperature (water and air temperatures) is likely to be important, as it promotes a heightened impact and persistence at low host density (Dazsak et al. 2003; Carter et al. 2021). In habitats we studied, air temperatures ranged between 17° C and 28° C, while water temperatures ranged between 12.4° C and 22.5° C. Permanent streams inhabited by Caucasian Salamanders have lower water temperature values than air temperature. We sampled primarily in May and July, when *M. caucasica* are active in every habitat and our sampling dates potentially affect our ability to detect the fungal pathogens. There is much variation between the temperature at which *Bd* grows best *in vitro* and the temperature at which it grows best *in vivo* (Sonn et al. 2017). The outcomes of our study may have been influenced by the exposure of salamanders to varying ambient temperatures and environmental conditions in native habitats, as opposed to the laboratory environments. We acknowledge that our data do not demonstrate that *Bd* and *Bsal* are absent from the site for the species, just that if they are present, they are at relatively low prevalences.

Martel et al. (2014), O'Hanlon (2018), and Schelee et al. (2019) indicate the pathogens originated in Central Asia, and their spread occurs through human influence, including animal trade. In light of the information in the literature and based on our data, the low prevalence of *Bsal* and *Bd* in Caucasian Salamanders may be associated with the relatively low human population and limited habitat modification in the region. We hope that the information given here about the apparent lack of chytridiomycosis will contribute to effective protection management of a species with a conservation status of Vulnerable.

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Abdullah Altunışık received his B.Sc. in Biology from Harran University, Turkey. Abdullah obtained a Ph.D. from the University of Recep Tayyip Erdoğan, Rize, Turkey, in 2015, and since 2017 he has been an Associate Professor at the same university. His research interests are biology and ecology of amphibians and reptiles. (Photographed by Sümeyye Altunışık).

Serkan Gül is an Associate Professor at Recep Tayyip Erdoğan University (Rize, Turkey). His main areas of interest are the phylogeny, ecology, and morphology of amphibians and reptiles in Turkey. Serkan has been focusing recently on ecological niche modeling, and he has published many articles in this area. He is now conducting several research projects using amphibians and reptiles as the test subjects. (Photographed by Kamil Candan).

Sümeyye Altunışık received her B.Sc. and M.Sc. in Biology from Karadeniz Technical University, Trabzon, and Recep Tayyip Erdoğan University, Rize, Turkey, respectively. Her postgraduate thesis was The Investigation of The Presence of Chytridiomycosis in *Mertensiella caucasica* (Waga. 1876) Populations in Rize and Gümüşhane Provinces. Her main research interest is ecology and disease of amphibians. (Photographed by Abdullah Altunışık).

Ayşenur Eminoğlu received a B.S. in Biology from Selçuk University, Konya, Turkey, an M.S. in Molecular Biology from Recep Tayyip Erdogan University, Rize, Turkey, and a Ph.D. in Molecular Biology in 2018 from Karadeniz Technical University, Trabzon, Turkey. Her research interest is mainly on biofuel production in anaerobic bacteria by metabolic engineering and production of some valuable chemicals via genetic and metabolic engineering in bacteria. Currently, she is a Postdoctoral Researcher in Recep Tayyip Erdogan University in the Department of Molecular Biology. (Photographed by Ali Murat Altıparmak).

Appendix Table. Chemical parameters of aquatic habitats used by populations of Caucasian Salamanders (*Mertensiella caucasica*) in northeastern Turkey. Abbreviaitons are n = number of individuals, CI = confidence interval, ET = environmental temperature, WT = water temperature, DO = dissolved oxygen, and C = Conductivity. Four Classes or water quality are recognized: Class I. High-quality water: (1) Surface waters with high potential to be drinking water; (2) Water usable for recreational purposes, including those that require body contact, such as swimming; (3) Water that can be used for trout production; (4) Water that can be used for animal production and farm needs. Class II. Slightly polluted water: (1) Surface waters that have the potential to be drinking water; (2) Water that can be used for recreational purposes; (3) Water that can be used for fish production other than trout; (4) Irrigation water, provided that it meets the irrigation water quality criteria determined by the current legislation. Class III. Contaminated water; Water and industrial water that can be used for aquaculture after appropriate treatment, excluding facilities that require qualified water such as food and textiles. Class IV. Heavily contaminated water. These are surface waters are of lower quality than the quality parameters given for Class III and can be used by improving them to the upper-quality class.

Locality (elevation in meters)	Sampling date	n	Prevalence (95% CI)	ET (°C)	WT (°C)	pH	DO (mg/L)	C. (**µs**/cm)	Nitrate (mg/L)	Nitrite (mg/L)	Sulfate (mg/L)	Amonium (mg/L)
Çambaşı/Ordu (1,672)	7 September 2018	10	0.00 (0.00, 0.31)	28	22.5	8.80	6.58	63.8	0.6	0.006	5	0.73
Dereli/Giresun (1,170)	10 July 2018	10	0.00 (0.00, 0.31)	24	18.8	8.11	7.25	262.0	0.5	0.001	17	0.68
Kayabaşı/ Trabzon (1,699)	10 July 2018	10	0.00 (0.00, 0.31)	25	17.0	9.17	7.17	196.1	0.8	0.015	38	0.82
Güngören/ Bayburt (1,770)	18 May 2018	10	0.00 (0.00, 0.31)	27	12.4	8.64	7.97	162.9	1.2	0.020	51	1.10
Kocadal/ Gümüşhane (1,929)	17 September 2018	10	0.00 (0.00, 0.31)	17	15.9	8.04	6.53	96.0	2.1	0.006	60	1.21
Güvenköy/ Rize (1,894)	11 July 2018	10	0.00 (0.00, 0.31)	26	22.0	8.75	7.11	78.0	0.8	0.006	50	0.93
Camili/Artvin (532)	12 July 2018	10	0.00 (0.00, 0.31)	27	18.0	9.05	9.09	91.6	0.7	0.009	10	0.82