

RESEARCH ARTICLE

Discovering cryptic species in the *Aspiciliella intermutans* complex (Megasperaceae, Ascomycota) – First results using gene concatenation and coalescent-based species tree approaches

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Abstract

Taxonomic identifications in some groups of lichen-forming fungi have been challenge largely due to the scarcity of taxonomically relevant features and limitations of morphological and chemical characters traditionally used to distinguish closely related taxa. Delineating species boundaries in closely related species or species complexes often requires a range of multisource data sets and comprehensive analytical methods. Here we aim to examine species boundaries in a group of saxicolous lichen forming fungi, the *Aspiciliella intermutans* complex (*Megasperaceae*), widespread mainly in the Mediterranean. We gathered DNA sequences of the nuclear ribosomal internal transcribed spacer (nuITS), the nuclear large subunit (nuLSU), the mitochondrial small subunit (mtSSU) ribosomal DNA, and the DNA replication licensing factor MCM7 from 80 samples mostly from Iran, Caucasia, Greece and eastern Europe. We used a combination of phylogenetic strategies and a variety of empirical, sequence-based species delimitation approaches to infer species boundaries in this group. The latter included: the automatic barcode gap discovery (ABGD), the multispecies coalescent approach *BEAST and Bayesian Phylogenetics and Phylogeography (BPP) program. Different species delimitation scenarios were compared using Bayes factors species delimitation analysis. Furthermore, morphological, chemical, ecological and geographical features of the sampled specimens were examined. Our study uncovered cryptic species diversity in *A. intermutans* and showed that morphology-based taxonomy may be unreliable, underestimating species diversity in this group of lichens. We identified a total of six species-level lineages in the *A. intermutans* complex using inferences from multiple empirical operational criteria. We found little corroboration between morphological and ecological features with our proposed candidate species, while secondary metabolite data do not corroborate tree topology. The present study on the *A. intermutans* species-complex

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indicates that the genus *Aspiciliella*, as currently circumscribed, is more diverse in Eurasia than previously expected.

Introduction

The lichen-forming fungal family *Megasporaceae* Lumbsch, Feige & K. Schmitz is a monophyletic group, belonging to the Ascomycota [1]. Its representatives are characterized by their mostly crustose thallus, urceolate or lecanorine apothecia, world-wide distribution and predominantly saxicolous, muscicolous, but also terricolous and lignicolous habit [1–6]. Some members of *Megasporaceae* (e.g. *Aspicilia cinerea* (L.) Körb., *Circinaria caesiocinerea* (Nyl. ex Malbr.) A. Nordin, Savić & Tibell, known *C. calcarea* (L.) A. Nordin, Savić & Tibell, *C. contorta* (Hoffm.) A. Nordin, Savić & Tibell, *Lobothallia radiosa* (Hoffm.) Hafellner) are widely distributed and very common throughout Europe, and they significantly contribute to the saxicolous communities on various types of rocks. Nevertheless, the taxonomic concept of many species remains unclear and many lichenologists consider this family dauntingly difficult.

The currently accepted taxonomy of *Megasporaceae* includes seven genera: *Aspicilia* A. Massal., *Circinaria* Link, *Lobothallia* (Clauzade & Cl. Roux) Hafellner, *Megaspora* (Clauzade & Cl. Roux) Hafellner & V. Wirth, *Sagedia* Ach., *Teuvoa* Sohrabi & S. Leavitt, and *Aspiciliella* M. Choisy in Werner [7–8]. The genus *Aspiciliella* [9] was resurrected only recently based on three phylogenetic markers: ITS, mtSSU and nuLSU [5]. The study confirmed that *Aspiciliella intermutans* (Nyl.) M. Choisy in Werner and *A. cupreoglauca* (B. de Lesd.) Zakeri, Divakar & Otte, previously assigned to *Aspicilia* based on their general appearance, represent a distinct lineage (*Aspiciliella*) in *Megasporaceae* and form a monophyletic clade together with the newly described species *A. portosantana* Sipman & Zakeri [5]. Species of the genus are characterized by a rimose-areolate, partially continuous, K+ red thallus and a chlorococcoid photobiont. The apothecia are pale brown to dark grey or black, they are rarely surrounded by a distinct thalline margin and a proper exciple is always present, the epihymenium is green to olive-green to greenish-brown, N+ changing to light green. The exciple is hyaline. Hypothecium and subhymenium are colorless, I+ blue to rusty red. The hymenium is colourless, I+ blue to rusty red, the asci are 8-spored, of *Aspicilia*-type. The ascospores are ellipsoid, colourless, simple. The conidia are straight, 7–11 µm long [5,8]. *Lecanora intermutans* Nyl. was described from St. Laon (France) in 1872 [10]. Nylander [10] indicated in the original description that the species looks like *Aspicilia cinerea* but has larger ascospores 23–34 × 9–15 µm and smaller conidia 7–9 × 1 µm. Later, this species was transferred to the genus *Aspicilia* [11] and more recently to *Aspiciliella* [9,5]. *Aspiciliella intermutans* is widely distributed, especially in the Mediterranean Region. Collections are more abundant from Iran, Armenia, France and Greece. It is also very common in Italy [12] and the Iberian Peninsula [13]. In Central Europe, *A. intermutans* is rare and locally distributed. Its overall distribution reaches Great Britain [14], the Canary Islands [15], Tunisia (e.g. [16]), Syria [17], the Ural Mountains [18] and the U.S.A. [19], although not all of these occurrences were verified by DNA methods.

Our previous study has shown that *Aspiciliella intermutans* includes some well-supported clades that may represent a complex of unrecognized species [5]. This stimulated us to work more on this group to find out the phylogenetic relationships and to explore the possibility of cryptic speciation in this complex. Previous studies have shown the presence of cryptic species in numerous species complexes of mainly foliose and fruticose lichens (e.g. [20–25]). Recently, a growing number of methods for analyzing DNA sequence data in a coalescent-based framework is capable to critically evaluate species diversity in fungi [26]. The coalescent-based

species delimitation methods have otherwise not been used until now in *Megasporaceae* species, particularly not in studies of the *A. intermutans* complex. We used a combination of phylogenetic strategies to delimit species in the *Aspiciliella intermutans* complex applying coalescent-based approaches and other recently developed DNA-based methods.

Estimating a species tree and species delimitation using coalescent methods for closely related taxa have proven very useful and have been used for lichenized fungi in some species complexes, e.g. in *Parmotrema reticulatum* (Taylor) M. Choisy, *Parmotrema pseudoreticulatum* (Tav.) Hale, *Rhizoplaca melanophthalma* (DC.) Leuckert and *Cladia aggregata* (Sw.) Nyl. [20–25].

The objective of this study is to delimit species boundaries within the *Aspiciliella intermutans* complex by implementing single- and multi-locus phylogenetic analyses, and coalescent-based species delimitation methods.

Materials and methods

Specimen sampling and phenotypic study

We gathered 90 samples from Asia and Europe; they were mostly collected by the authors in Iran, Armenia and the Czech Republic. Specimens were also obtained from the following herbaria: B, GZU, PRA, PRC, PL, CR and GLM.

Locations of samples:

1. **Armenia**, Aragatsotn, Byurakan, Mts. Aragats, H20 road to lake Kari, after diversion to Amberd castle, alt. 2470 m, 40°24'54"N, 44°14'51"E, Z. Zakeri 16.06.2015 (GLM 40741).
2. **Armenia**, Aragatsotn, Byurakan, Mts. Aragats, H20 road, before diversion to Amberd castle, alt. 2200 m, 40°23'55"N, 44°15'07"E, Z. Zakeri 16.06.2015 (GLM 40763, 40562, 40745).
3. **Armenia**, Aragatsotn, Byurakan, Mts. Aragats, road connecting M3 to H20, alt. 1800 m, 40°24'48"N, 44°22'03"E, Z. Zakeri 21.06.2015 (GLM 40475, 40480, 40469, 40487, 40494).
4. **Armenia**, Ararat, Vedi, Urtsadzor, Khosrov Forest State Reserve, alt. 1390 m, 39°59'07"N, 44°53'51"E, Z. Zakeri 17.06.2015 (GLM 40503, 40499).
5. **Armenia**, Ararat, Vedi, Urtsadzor, Khosrov Forest State Reserve, alt. 1600 m, 40°00'42"N, 44°54'41"E, Z. Zakeri 17.06.2015 (GLM 40519).
6. **Armenia**, Shirak province, Artik, northern slope of Mt. Aragats, SE of Mets Mantash village, W of reservoir, alt. 2465 m, 40°33'17"N 44°6'34"E, basalt, S. Harutyunyan & H. Mayrhofer 2006 (GZU 280743).
7. **Armenia**, Vayots Dzor, Areni, Amaghu valley along Noravank Monastery road, alt. 1265 m, 39°41'54"N, 45°12'35"E, Z. Zakeri 23.06.2015 (GLM 39711).
8. **Armenia**, Vayots Dzor, Shatin, Yeghegis, alt. 1530 m, 39°52'03"N, 45°20'38"E, Z. Zakeri 23.06.2015 (GLM 39702).
9. **Armenia**, Vayots Dzor, Shatin, Yeghegis, alt. 1600 m, 39°52'14"N, 45°23'26"E, Z. Zakeri 23.06.2015 (GLM 39729, 39727).
10. **Armenia**, Vayots' Dzor province, area c. 13 km E of Vayk, near Artavan, c. 1.4 km SW of village, forest along rivulet, forest and forest edge, alt. 1940 m 39°39'6"N 45°36'20", E, M. Oganesian 2009 (GZU 28656).
11. **Azerbaijan**, in finibus Talysorum in regione montuosa zuvand vocata prope vicum Mistan in rupibus, alt. 1900 m, ca. 38°38'39.3"N, 48°26'07.7"E, V. Otte 2013 (GLM 38734, 38706).

12. **Bulgaria**, Eastern Rodopi Mts., Kadžali, Momčilgrad, Bregovo, in the valley of Varbica river ca 2 km E of the village, alt. 300 m, 41°28'N, 25°25'E, on rather acid volcanic rock, J. Vondrák 2004 (PRA/JV2151).
13. **Czech Republic**, Central Bohemia, Český kras, Černošice, Karlík: a diabase rocky-hill just N of the village, alt. 250 m, 49°56'17.4"N, 14°15'42.2"E, Z. Palice 2011 (PRA/ZP14789).
14. **Czech Republic**, Central Bohemia, Křivoklátsko, Králův Dvůr, Trubínský vrch Nature Monument, alt. 330–360 m, 49°56'39"N 13°59'46"E, J. Malíček 2010 & V. Lenzová 2013 (JM2640, PRC/VL25).
15. **Czech Republic**, Central Bohemia, Praha, PR Prokopské údolí: Albrechtův vrch Nature Monument, 50°12'56.4"N, 14°16'43.4"E, S exposed diabase rock, V. Lenzová 2015 (PRC/VL255).
16. **Czech Republic**, Central Bohemia, Praha, PR Prokopské údolí: Hemrovy skály, 50°2'34.6"N, 14°21'13.5"E, S exposed diabase rock, V. Lenzová 2015 (PRC/VL253).
17. **Czech Republic**, Central Bohemia, Praha, Řeporyje, 50°1'52.7"N, 14°18'53.3"E, diabase, V. Lenzová 2014 (PRM784504, PRC/VL254).
18. **France**, Bretagne, Loire-Atlantique, Frossay, chemin des Carris, sur rocher de gneiss, alt. 5m, 47°17'35.06"N, 1°52'23.06"W, J.Y. Monnat 2011 (CR 26741).
19. **France**, Languedoc-Roussillon, Pyrénées-Orientales, Nohédes, à proximité immédiate de la réserve naturelle de Nohédes, 170 m á ONO de Cortal, en bordure d'une lande à *Cytisus purgans*, près de la piste, sur petites parois de schiste non calcaire, alt. 1030 m, 42°37'49.44"N, 2°16'10.56"E, C. Roux 2009 (CR 25602).
20. **France**, Provence-Alpes-Côte d'Azur: Var, Évenos, tuj sub jak S de la kastelo de ÉVENOS, sur rokbloko el bazalto, alt. 350 m, 44°18'57.96"N, 6°37'53.04"E, M. Bertrand & C. Roux 2010 (UPS L-591195/CR 25790).
21. **Greece**, Cyclades Archipelago. Milos island, W part, Angathia Bay, alt. 0–360 m, ca. 24°20.7' E, 36°43.65' N, H. Sipman & Th. Raus 2012 (B 59827, 59969, 59825).
22. **Greece**, North Aegean Region, Lesbos: ca. 3 km E of Filia along road to Kalloni, alt. 2–700 m, 26°09.55' E, 39°15.29' N, H. Sipman & Th. Raus 2016 (B 62695, 61847, 61933, 62681, 62663, 62614, 62252, 61911, 62653, 62271, 62704, 62439, 62105).
23. **Iran**, Ardebil, Sarein, alt. 3044 m, around 38°12'21.2"N, 47°52'38.6"E, Z. Zakeri 06.2014 (GLM 40760, 49966).
24. **Iran**, Ardebil, Sarein, alt. 1898 m, ca. 38°8'35.0"N, 48°0'96"E, Z. Zakeri 06.2014 (GLM 49960, 49959, 49965, 49269, 49269).
25. **Iran**, Golestan, Azadshahr, alt. 1802 m, ca. 36°50'08"N, 55°21'39.19"E, Z. Zakeri 08.2014 (GLM 50003).
26. **Iran**, Khorasan Razavi, Quchan, alt. 1880–1992 m, ca. 36°54'53.1"N, 58°07'11.6"E, Z. Zakeri 2015 (GLM 49964, 49961, 49962, 49963).
27. **Iran**, West Azerbaijan, Urmia, alt. 1305 m, ca. 37°50'37.5"N, 45°34'47.5"E, Z. Zakeri 06.2014 (GLM 49270, 49271, 49967, 49267).
28. **Italy**, Sardinia: Monte Limbara Sud, Azienda Foresta Demaniale, an Abzweigung Richtung „Su Nodu Nieddu“, alt. 650 m, 40°46'N, 9°11'E, auf Granit, N. Nöske 1999 (B 368).

29. **Romania**, Munții Măcin Mts; Brăila, Măcin, on top of hill with former quarry ca 1 km SW from Mt Cheia, 45°14'N, 28°08'E, granitoid rock, J. Šoun 2005 (hb. Šoun 82).
30. **Spain**, Prov. Zamora. SW of Zamora, towards Pereruala, Arroyo del Zape, alt. 630 m, 41°28.3'N, 5°47.2'W, conglomerate rock outcrops in steppe-like area, W-slope, H. Sipman 2000 (B 45379).
31. **Ukraine**, Mikolajivska oblast, Pervomaisk, pasture near village Liushniuvate, alt. 80 m, 48°10'15,30"N 30°26'55,17"E, on nutrient-rich granite boulder, J. Vondrák 2006 (PRA/JV5204).
32. **Ukraine**, Pervomaysk, Blagodatnoe, in valley of brook running to Yuzhniy Bug, on granite outcrop in steppe slope, J. Vondrák (PRA/JV7340).

Molecular data of specimens tentatively referred as *Aspiciliella intermutans* were analyzed using 66 ITS, 51 MCM7, 61 nuLSU and 73 mtSSU sequences as they have been shown to be useful to resolve phylogenetic relations in this group of lichenized fungi ([e.g. [1,5,6,27]). Five samples of *Aspiciliella cupreoglauca* were selected as outgroup, since this species is closely related to *Aspiciliella intermutans* [5].

Morphology and anatomy of the specimens were observed, using a Leica M165 C stereomicroscope, and a Leica DM 2500 P compound light microscope connected to a Leica MC 190 HD digital camera. Detailed observations of thallus anatomy, asci, ascospores and conidia were made on hand-cut sections of thallus, apothecia and pycnidia mounted in tap water.

Chemistry of the specimens was investigated by thin layer chromatography (TLC), using solvent systems B and C, and by spot tests, using KOH, C and I, applied directly on the lichen thalli [28].

DNA extractions, PCR amplifications and sequencing

Total DNA was extracted from freshly collected material according to Park et al. [29] but with some modifications as described in Zakeri et al. [5,6].

The primer pair ITS1F [30] and ITS4 [31] was used for the PCR amplifications of the ITS region. For PCR amplification of the nuLSU region the primers LR0R, LR7 and LR5 [32], for the mtSSU region the primers mrSSU1 and mrSSU3R [33], and for the MCM7 region the primers Mcm7-1348rev [34] and X_mcm7_F [35] were used. PCR amplifications were performed in a 12.5 µL volume containing 2 µL undiluted DNA, 0.5 µL of each primer (10 mM), 6.4 µL of sterile water, 1 µL dNTP (2 mM), 1 µL s-buffer, 1 µL MgCl₂, 0.1 µL Taq-polymerase (Peqlab). Primer details and PCR conditions are summarized in Table 1. Thermal cycling

Table 1. Primers used for PCR amplification and sequencing of the nuclear ribosomal internal transcribed spacer 1, 5.8S and internal transcribed spacer 2 (ITS) region, the nuclear large subunit (nuLSU), the mitochondrial small subunit (mtSSU) ribosomal DNA and the DNA replication licensing factor MCM7.

Marker	Primer name	Forward primer sequence	Annealing Temperature (°C)	Reference
ITS	ITS1F:	5'-CTTGGTCATTAGAGGAAGTAA-3'	54°C,	(Gardes & Bruns 1993)
	ITS4:	5'-TCCTCCGCTTATTGATATGC-3'	54°C,	(White et al. 1990)
nuLSU	LR0R	5'-ACC CGC TGA ACT TAA GC-3'	64°C	(Vilgalys & Hester 1990)
	LR7	5'-TAC TAC CAC CAA GAT CT-3'	64°C	(Vilgalys & Hester 1990)
	LR5	5'-ATCCTGAGGGAAACTTC-3'	64°C	(Vilgalys & Hester 1990)
MCM7	Mcm7-1348rev	5'-GAYTTDGCACICCCIGGRTCCWCCC AT-3'	58°C	(Schmitt et al. 2009)
	X_mcm7_F	5'-CGT ACA CYT GTG ATC GAT GTG-3'	58°C	(Leavitt 2010)
mtSSU	mtSSU1	5'-AGCAGTGAGGAATATTGGTC-3'	59°C	(Zoller et al. 1999)
	mtSSU3R	5'-ATGTGGCACGTCTATAGCCC-3'	59°C	(Zoller et al. 1999)

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parameters were initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 secs at 95°C, 30 secs at 54°C for amplifying ITS, 64°C for nuLSU, 59°C for mtSSU, 58°C for MCM7, and 1 min at 72°C, a final extension step of 3 min at 72°C was added, after which the samples were kept at 4°C. The amplification products were visualized by electrophoresis on 1% agarose gels and stained with ethidium bromide, and were purified by adding 2 µL ExoSAP (Exonuclease 1-shrimp alkaline phosphatase, USB) to 5 µL of the PCR products, followed by a heat treatment of 15 min at 37°C and 15 min at 80°C. Both DNA strands of the PCR product were sequenced on an ABI 3730 by the Bik-F Laboratory Center in Frankfurt am Main.

Sequence alignment

Sequence fragments generated for this study were assembled and edited using BioEdit v.7.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Sequence identity was confirmed using the mega-BLAST search function in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were aligned by the program MAFFT v.7 [36] on a web server (<https://mafft.cbrc.jp/alignment/server/>), implementing the E-INS-I alignment algorithm for nuLSU and G-INS-I alignment algorithm for all other markers, ‘200PAM/K = 2’ scoring matrix and with an offset value of 0.0, with the remaining parameters set to default values. Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) was used to delimit and exclude ambiguously aligned positions, applying settings allowing for smaller final blocks, gap position within the final blocks and less strict flanking position [37].

Phylogenetic analyses

Maximum likelihood (ML) analyses were performed for the single loci separately and for the concatenated dataset using the online version of IQ-TREE [38–40] with simultaneous inference of the optimal partitioning scheme and substitution models for each data partition. For concatenated dataset, a partition of seven subsets has been proposed (Group I intron, ITS1, 5.8S rDNA, ITS2, mtSSU, nuLSU and MCM7). Conflicts among the single gene trees were assessed using the tree topology and following the criterion of nodes bootstrap support equal or above 95% in one but below this value in the other tree for no conflict. No conflicts were found and thus all the datasets were concatenated. Branch support was estimated with the ultrafast bootstrap algorithm [41] based on 1000 bootstrap replicates and using a maximum of 1000 iterations and a minimum correlation coefficient of 0.99 as a stopping rule.

The ITS PCR product obtained was around 800 bp. The larger sizes of ITS were due to the presence of insertions of about 200 bp identified as Group I intron [42] at the 3’ end of the SSU rDNA. Group I intron were present in all the samples of *Aspiciliella intermutans* complex.

We used the Markov Chain Monte Carlo approach implemented in MrBayes v.3.2.2 [43] to infer phylogenetic trees applying the partitioning scheme inferred with IQ-TREE and slightly simplified substitution models, because most of the models inferred by IQ-TREE are not implemented in MrBayes. See Table 2 for details on locus partitions and substitution models. Two independent runs, each with four heated Metropolis–Coupled MCMC chains (“temperature” 0.2) were initiated and run for 5,000,000 generations, with tree and parameter sampling

Table 2. Details on locus partitions and substitution models.

	Group I intron	ITS1	5.8S rDNA	ITS2	nuLSU	MCM7	mtSSU
No. taxa	61	69	69	69	65	51	77
Position	1–183	184–414	415–553	554–725	726–1798	1799–2305	2306–3205
Substitution model	TIM2e+G4	TIM2e+G4	TVMe+I+G4	K2P+I+G4	TPM3+I+G4	TNe+I	F81+I+G4

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every 100th generations. Convergence among the parameters of the both runs was assessed in Tracer v.1.6 following the criterion of effective sample size (ESS) values above 200. The consensus tree was generated after discarding the initial 25% trees.

Species delimitation analyses

In order to infer the most likely species numbers in our *Aspiciliella intermutans* dataset, we chose a method for single-locus DNA-based species delimitation, the automatic barcode gap discovery [44], and two coalescent-based models *BEAST [45] and BPP v.3.2 [46] to analyze the four loci concatenated dataset.

Our exploratory analyses showed that the ITS region is very variable in the *A. intermutans* group and the nuLSU is very conserved (results not shown) and thus they were not used for the final single-locus species delimitation analyses.

ABGD is an automatic procedure that sorts the sequences into hypothetical species based on the barcode gap. This method automatically finds the distance where the barcode gap is located [44]. The ABGD method was carried out for the mtSSU and MCM7 datasets using the Web interface at <http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>. Default parameters were chosen using Kimura 2-parameter (K2P) distances that correct for transition rate bias (relative to transversions) in the substitution process. The default for the minimum relative gap width was set to different values between 0.1 and 0.15.

Since the results of different single-locus species delimitation analyses were not congruent, we used the main monophyletic groups obtained from concatenated dataset analysis in IQ tree, as species scenarios for subsequent analyses. We tested a total of four different species delimitation scenarios: with four (A1+A2+A3+A4, B, C and D), five (A1+A2+A3, A4, B, C and D), six (A1+A2, A3, A4, B, C and D) and nine species (A1, A2, A3, A4, B, C, D1, D2 and D3) (Fig 1).

We used the coalescent-based hierarchical Bayesian model *BEAST implemented in BEAST v.1.8.0 to estimate species tree following our proposed species delimitation scenarios. *BEAST incorporates the coalescent process and the uncertainty associated with gene trees and nucleotide substitution model parameters and estimates the species tree directly from the sequence data [47].

Species assignments are required *a priori* for *BEAST analyses. To designate species in *BEAST, we used four different scenarios (4, 5, 6 and 9 species) for assigning individuals to a 'candidate species', based on well supported groups in the phylogenetic tree obtained from the concatenated dataset. We used lognormal distributions for the relaxed uncorrelated rates for all loci, we selected a Yule process and gamma-distributed population sizes for the species-tree prior and a piecewise linear population size model with a constant root. Default values were used for remaining priors. Two independent Markov chain Monte Carlo (MCMC) analyses were run for a total of 100 million generations, sampling every 1000th generations and excluding the 12500 trees as burn-in. We assessed the MCMC convergence and determined burn-ins by examining ESS values and likelihood plots in the program Tracer version 1.6. The posterior probabilities of nodes were computed from the sampled trees (excluding burn-in samples) using TreeAnnotator 1.8.0 [47].

We used the multispecies coalescent model implemented in the program BP&P v.3.2 [46] to estimate the posterior probability for each species scenario (4, 5, 6 and 9 species). BP&P incorporates coalescent theory and phylogenetic uncertainty into parameter estimation; and the posterior distribution for species delimitation models is sampled using a reversible-jump Markov Chain Monte Carlo (rjMCMC) method. We used the unguided species delimitation algorithm [48]. This algorithm explores different species delimitations and different species

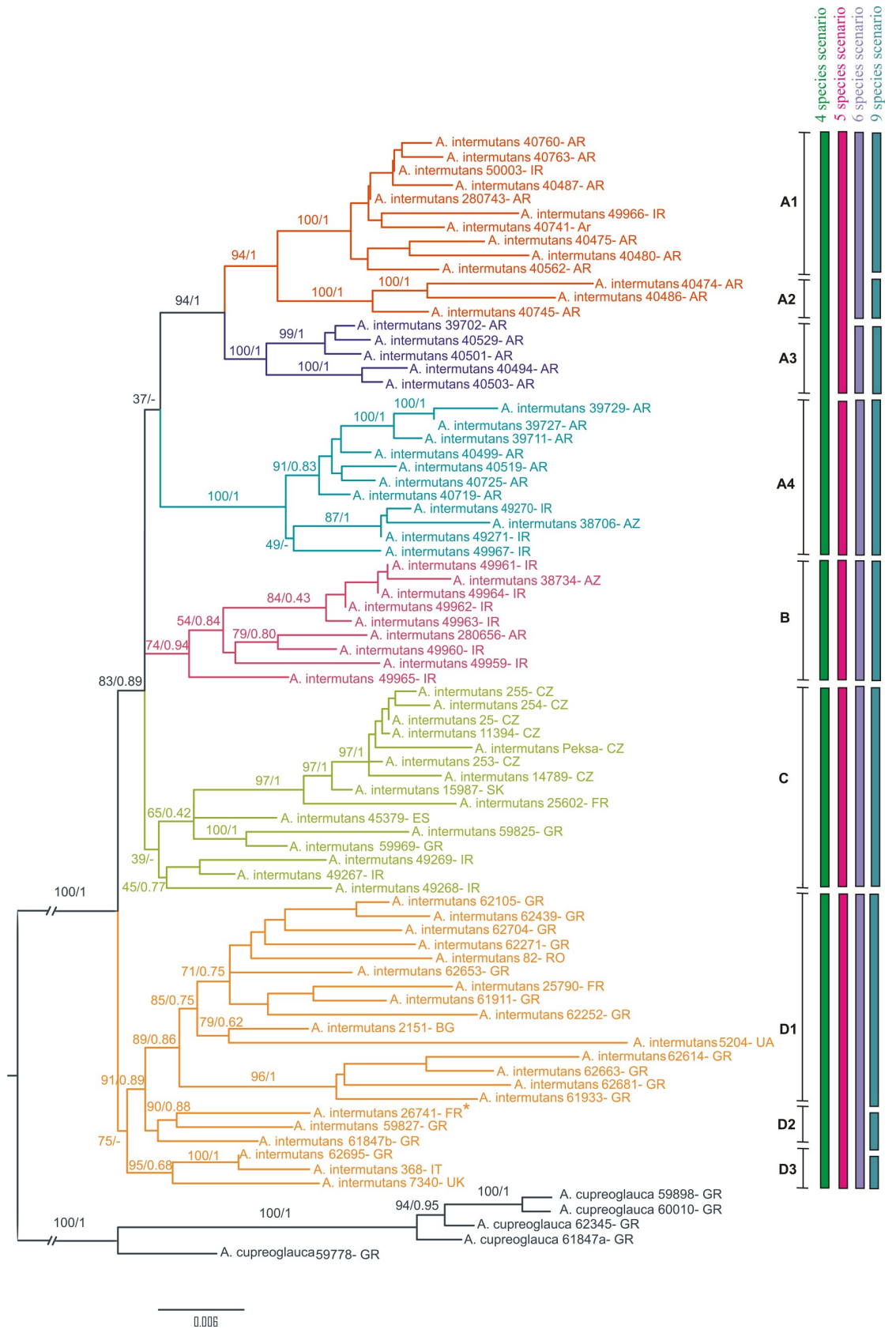


Fig 1. Concatenated gene tree. Maximum likelihood tree (IQ tree analysis) showing phylogenetic relationships among *Aspiciliella intermutans* candidate species based on the ITS, nrLSU, MCM7 and mtSSU concatenated dataset. Bootstrap values and Bayesian posterior probabilities (BS/PP) are shown above their respective branches. The six candidate species within the *A. intermutans* complex obtained in the species delimitation analysis based on BP&P and Bayes factor are highlighted by the different color. * = Sample collected at 160 km from the type locality.

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phylogenies, with fixed specimen assignments to populations. The program attempts to merge populations into one species and uses the nearest neighbour interchange (NNI) or subtree pruning and regrafting (SPR) algorithms to change the species tree topology [46]. We performed analyses using algorithms 0 and 1 using three prior theta (θ : is a parameter that is proportional to the product of the population size and the mutation rate) assigned to gamma distributions of G (2, 10), G (2, 100) and G (2, 1000), combined with root age (τ_0) assigned G (2, 2000). Rates were allowed to vary among loci (locus rate = 1), and the analyses were set for automatic fine-tune adjustments. Each reversible-jump Monte Carlo (rjMCMC) analysis was run for 100,000 generations, sampling each generation and specified a burn-in of the first 8 000 generations. Each analysis was run twice to confirm consistency between runs. Speciation probabilities greater than 0.95 were considered as supported species delimitations.

Since different species delimitation analyses supported different scenarios for the *Aspiciliella intermutans* complex, the most likely hypothesis of species boundaries was assessed using Bayes factor delimitation (BFD) test [49]. Bayes factor uses a Bayesian coalescent-based reconstruction of species trees to compare the different species delimitation models, calculating the marginal likelihood and does not require a guide tree. We calculated marginal likelihood estimates (MLEs) for four species delimitation scenarios (4, 5, 6 and 9) species. For each scenario, we reconstructed a species tree using *BEAST v.1.8.0. The analyses were run with the same substitution models used in MrBayes, a birth-death model for the species tree prior; population size model set to piecewise linear and constant root. The MCMC chain was run for 20,000,000 generations, sampled every 1000th, and the first 25% of each run was discarded as burn-in. MLEs were estimated using path sampling (PS) and stepping-stone sampling (SS) in *BEAST, with 100 path steps, a chain length of 100,000 generations and likelihoods saved every 100th generations. The Bayes factor was calculated as $2 \times$ (marginal likelihood of the best model (model1)—marginal likelihood of alternative model (model2)). Positive values of Bayes factor support the best model against alternative models.

Results

Phylogenetic analyses

We generated a seven-locus data matrix consisting of 3205 aligned nucleotide positions. The matrix of the data sets had 183, 231, 139, 172, 1073, 507 and 900 unambiguously aligned nucleotide positions for Group I intron, ITS1, 5.8s, ITS2, nuLSU, MCM7 and mtSSU respectively. Table 2 summarized the best-fitting models of evolution for each locus. The multilocus data set was based on 79 individuals. A total of 218 new DNA sequences (ITS, nuLSU, mtSSU and MCM7) were generated for this study and were aligned with sequences obtained from GenBank (Table 3). The partitioned ML analysis of the concatenated data matrix yielded the optimal tree with Ln likelihood value = -12042.961 The mean LnL value of the two parallel runs of the Bayesian analysis for the seven combined loci was -12512.24 with a standard deviation of ± 2.17 .

Since the topologies of the trees estimated from Bayesian methods and ML did not have any well-supported conflict, only ML topologies from IQ tree are shown with bootstrap and posterior probability values indicated on the nodes (Fig 1). In the concatenated tree topology,

Table 3. Voucher specimens and NCBI GenBank accession numbers of the ITS1-5.8S-ITS2, nrLSU, mtSSU and MCM7 sequences used in the phylogenetic analyses. New sequences are in bold.

Taxon	Country, Province, Voucher	nrLSU	mtSSU	ITS	MCM7
<i>A. cupreoglauca</i>	Greece, North Aegean, <i>Sipman & Raus 61847</i> (B)	KY576954	KY576930	KY618843	---
<i>A. cupreoglauca</i>	Greece, North Aegean, <i>Sipman & Raus 62345</i> (B)	KY576955	KY576931	KY618844	---
<i>A. cupreoglauca</i>	Greece, Cyclades Archipelago, <i>Sipman & Raus 59778</i> (B)	MH290743	MH349000	MH290788	---
<i>A. cupreoglauca</i>	Greece, Cyclades Archipelago, <i>Sipman 59898</i> (B)	MH248842	MH248890	---	---
<i>A. cupreoglauca</i>	Greece, Cyclades Archipelago, <i>Sipman 60010</i> (B)	MH248843	MH248891	MH255584	---
<i>A. intermutans</i>	Iran, West Azerbaijan, <i>Zakeri 49267</i> (GLM)	KY576020	KY576019	KY596018	---
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49268</i> (GLM)	KY576943	KY576919	KY596005	---
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49269</i> (GLM)	KY576949	KY576925	KY596013	---
<i>A. intermutans</i>	Iran, West Azerbaijan, <i>Zakeri 49270</i> (GLM)	KY576950	KY576926	KY596014	---
<i>A. intermutans</i>	Iran, West Azerbaijan, <i>Zakeri 49271</i> (GLM)	KY576951	KY576927	KY596015	---
<i>A. intermutans</i>	Armenia, Vayots Dzor, <i>Zakeri 39727</i> (GLM)	KY576941	KY576917	KY596006	---
<i>A. intermutans</i>	Armenia, Vayots Dzor, <i>Zakeri 39729</i> (GLM)	KY576942	KY576918	KY596007	---
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40474</i> (GLM)	KY576944	KY576920	KY596008	---
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40494</i> (GLM)	KY576945	KY576921	KY596009	---
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40501</i> (GLM)	KY576946	KY576922	KY596010	---
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40503</i> (GLM)	KY576947	KY576923	KY596011	---
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40719</i> (GLM)	KY576948	KY576924	KY596012	---
<i>A. intermutans</i>	Azerbaijan, Lerik, <i>Otte 38706</i> (GLM)	KY576952	KY576928	KY596016	---
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 61911</i> (B)	KY576958	KY576935	KY618848	---
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62681</i> (B)	KY576960	KY576937	KY618850	---
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62695</i> (B)	MH257199	MH257235	MH210647	MH257270
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 61847</i> (B)	MH257198	MH257234	MH210648	MH257261
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62653</i> (B)	MH257200	MH257236	MH210649	MH257262
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62704</i> (B)	MH257201	MH257237	MH210650	MH257263
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62252</i> (B)	MH257202	MH257238	MH210651	MH257264
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62271</i> (B)	MH257203	MH257239	MH210652	MH257271
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62105</i> (B)	MH257204	MH257240	MH210653	MH257265
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62439</i> (B)	MH257205	MH257241	MH210654	MH257272
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62614</i> (B)	MH257206	MH257242	MH210655	MH257273
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 62663</i> (B)	MH257207	MH257243	MH210656	MH257274
<i>A. intermutans</i>	Greece, North Aegean, <i>Sipman & Raus 61933</i> (B)	MH257208	MH257244	MH210657	MH257266
<i>A. intermutans</i>	Armenia, Vayots Dzor, <i>Zakeri 39702</i> (GLM)	---	MH257209	MH210658	MH257267
<i>A. intermutans</i>	Armenia, Vayots Dzor, <i>Zakeri 39711</i> (GLM)	---	MH257210	MH210659	MH257245
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40475</i> (GLM)	MH257184	MH257211	MH210660	MH257246
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40480</i> (GLM)	MH257185	MH257212	MH210661	MH257268
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40486</i> (GLM)	MH257186	MH257213	MH210662	---
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40487</i> (GLM)	MH257187	MH257214	MH210663	MH257247
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40499</i> (GLM)	MH257188	MH257215	MH210664	MH257248
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40519</i> (GLM)	---	MH257216	MH210665	---
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40529</i> (GLM)	MH257189	MH257217	MH210666	---
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40562</i> (GLM)	MH257190	MH257218	MH210667	MH257249
<i>A. intermutans</i>	Armenia, Ararat, <i>Zakeri 40725</i> (GLM)	---	MH257219	MH210668	---
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40741</i> (GLM)	MH257191	MH257220	MH210669	MH257250
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40745</i> (GLM)	MH257192	MH257221	MH210670	---
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 40760</i> (GLM)	---	MH257222	MH210671	MH257269
<i>A. intermutans</i>	Armenia, Aragatsotn, <i>Zakeri 40763</i> (GLM)	MH257193	MH257223	MH210672	MH257251

(Continued)

Table 3. (Continued)

Taxon	Country, Province, Voucher	nrLSU	mtSSU	ITS	MCM7
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49965</i> (GLM)	MH257194	MH257224	MH210673	MH257252
<i>A. intermutans</i>	Iran, West Azerbaijan, <i>Zakeri 49967</i> (GLM)	---	MH257225	MH210674	MH257253
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49960</i> (GLM)	MH257195	MH257226	MH210675	MH257254
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49959</i> (GLM)	MH257196	MH257227	MH210676	---
<i>A. intermutans</i>	Iran, Ardebil, <i>Zakeri 49966</i> (GLM)	---	MH257228	MH210677	MH257256
<i>A. intermutans</i>	Iran, Khorasan Razavi, <i>Zakeri 49964</i> (GLM)	---	MH257229	MH210678	MH257257
<i>A. intermutans</i>	Iran, Khorasan Razavi, <i>Zakeri 49961</i> (GLM)	---	MH257230	MH210679	MH257258
<i>A. intermutans</i>	Azerbaijan, Lerik, <i>Otte 38734</i> (GLM)	MH257197	MH257231	MH210680	---
<i>A. intermutans</i>	Iran, Khorasan Razavi, <i>Zakeri 49963</i> (GLM)	---	MH257232	MH210681	MH257259
<i>A. intermutans</i>	Iran, Khorasan Razavi, <i>Zakeri 49962</i> (GLM)	---	MH257233	---	MH257260
<i>A. intermutans</i>	Iran, Golestan, <i>Zakeri 50003</i> (GLM)	---	---	---	MH257255
<i>A. intermutans</i>	Romania, Măcin Mts, <i>J. Šoun 82</i> (hb. Šoun)	MH248846	MH248875	---	---
<i>A. intermutans</i>	Slovakia, Cerová vrchovina, <i>Z. Palice 15987</i> (PRA)	MH248857	MH248871	MH255575	MH293579
<i>A. intermutans</i>	France, Provence-Alpes-Côte d'Azur, <i>C. Roux 25790</i> (CR)	MH248863	MH248869	MH255576	MH293580
<i>A. intermutans</i>	France, Languedoc-Roussillon, <i>C. Roux 25602</i> (CR)	MH248849	MH248870	---	MH293588
<i>A. intermutans</i>	France, Bretagne, <i>C. Roux 26741</i> (CR)	MH248847	MH248874	MH255573	MH293576
<i>A. intermutans</i>	Armenia, Vayots Dzor province, <i>M. Oganessian</i> (GZU280656)	MH248845	MH248877	MH255572	MH293590
<i>A. intermutans</i>	Armenia, Shirak province, <i>S. Harutyunyan & H. Mayrhofer</i> (GZU280743)	MH248858	MH248878	---	MH293591
<i>A. intermutans</i>	Spain, Prov. Zamora, <i>H. Sipman 45379</i> (B)	MH248844	MH248868	---	MH293582
<i>A. intermutans</i>	Italy, Sardinia, <i>N.M. Nöske 368</i> (B)	MH248862	MH248879	---	MH293583
<i>A. intermutans</i>	Greece, Cyclades Archipelago, <i>H. Sipman 59825</i> (B)	MH248859	MH248882	MH255571	MH293570
<i>A. intermutans</i>	Greece, Cyclades Archipelago, <i>H. Sipman 59827</i> (B)	MH248860	MH248881	MH255569	MH293571
<i>A. intermutans</i>	Greece, Cyclades Archipelago, <i>H. Sipman 59969</i> (B)	MH248861	MH248873	MH255570	MH293572
<i>A. intermutans</i>	Czech Republic, Otavická skála, <i>O. Peksa</i> (PL)	MH248850	MH248889	---	MH293592
<i>A. intermutans</i>	Ukraine, Pervomaysk, <i>J. Vondrák 7340</i> (PRA)	MH248848	MH248876	MH255567	MH293573
<i>A. intermutans</i>	Ukraine, Mikolajivska oblast, <i>J. Vondrák 5204</i> (PRA)	---	MH248880	MH255577	MH293574
<i>A. intermutans</i>	Bulgaria, Eastern Rodopi Mts, <i>J. Vondrák 2151</i> (PRA)	MH248865	MH248872	MH255568	MH293575
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>V. Lenzová 255</i> (PRC)	MH248854	MH248885	MH255579	MH293593
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>V. Lenzová 254</i> (PRC)	MH248851	MH248886	MH255578	MH293589
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>V. Lenzová 253</i> (PRC)	MH248856	MH248883	MH255582	MH293587
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>V. Lenzová 25</i> (PRC)	MH248852	MH248887	MH255581	MH293577
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>Z. Palice 14789</i> (PRA)	MH248853	MH248884	MH255583	MH293569
<i>A. intermutans</i>	Czech Republic, Central Bohemia, <i>Z. Palice 11394</i> (PRA)	MH248855	MH248888	MH255580	MH293578

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specimens of *A. intermutans* were recovered in several distinct clades (named hereafter A, B, C and D), in some cases with low support especially in Bayesian analyses (Fig 1). In all the groups, subclades with a strong support were also found.

Species delimitation analyses. Our exploratory analyses showed that the single gene tree were not congruent. Further, ITS region was resulted too variable and the nuLSU too conserved in the *A. intermutans* group (results not shown). The monophyletic groups B, C, D1, D2 and D3 obtained in the concatenated tree (Fig 1) were not reciprocally monophyletic in the single gene trees. The clades A1, A2, A3 and A4 were recovered in mtSSU gene tree and A3 in MCM7 gene tree (see S1 Fig and S2 Fig).

ABGD analyses applied to MCM7 and mtSSU dataset detected 12 and 9 candidate species respectively. The results of these analyses are shown in supporting information.

The results of the multispecies coalescent species delimitation method BP&P with different algorithms 0 and 1 are summarized in Tables 4 and 5. The two algorithms in BP&P

Table 4. Species delimitations (excluding outgroup) and their posterior probabilities.

Species scenarios	Ancestral population sizes (theta)					
	Algorithm 0			Algorithm 1		
	G (2,10)	G (2-100)	G (2,1000)	G (2,10)	G (2,100)	G (2,1000)
4-species	0.99	0.99	1.00	0.99	1.00	0.96
5-species	0.99	1.00	1.00	0.99	0.99	1.00
6-species	0.99	1.00	1.00	0.99	1.00	1.00
9-species	0.38	0.67	0.82	0.59	0.71	0.95

Prior distributions on ancestral population sizes (theta) assigned to different gamma distributions (G) combined with root age (tau) assigned to G (2, 2000).

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consistently distinguished 4–6 independent evolutionary lineages. While the 4, 5 and 6 species topologies had a speciation probability of >0.96 threshold in the runs with algorithm 1 and 0 (values ranged between 0.96–1.0), the nine species topology was only supported (0.95) with algorithm 1 and a prior distribution on ancestral population size (theta) with a gamma distribution of G (2, 1000).

The Bayes factor delimitation results are provided in Table 6. BFD test supports the 6-species delimitation scenario model as the best scenario over other models tested, i.e. four, five and nine species delimitation scenarios. The 6-species scenario corresponds to the well supported clades: A1+A2, A3, A4, B, C and D. Clades C and D were not strongly supported in the concatenated gene tree. However, these were supported by the *BEAST species tree (Fig 2).

Table 5. Delimited species and their posterior probabilities.

Species scenarios		Algorithm 0			Algorithm 1		
		G (2,10)	G (2,100)	G (2,1000)	G (2,10)	G (2,100)	G (2,1000)
4-species	A	1.00	1.00	1.00	1.00	1.00	1.00
	B	0.99	0.99	1.00	0.99	1.00	0.99
	C	0.99	0.99	1.00	0.99	1.00	0.99
	D	0.99	1.00	1.00	1.00	1.00	1.00
	outgroup	0.99	1.00	1.00	1.00	1.00	1.00
	D+outgroup	0.0001	-	-	-	-	-
	B+C	0.00006	0.00001	-	0.0019	-	0.03
5-species	A1+A2+A3	1.00	1.00	1.00	1.00	1.00	1.00
	A4	1.00	1.00	1.00	1.00	1.00	1.00
	B	0.99	1.00	1.00	0.99	0.99	1.00
	C	0.99	1.00	1.00	0.99	0.99	1.00
	D	1.00	1.00	1.00	1.00	1.00	1.00
	outgroup	1.00	1.00	1.00	1.00	1.00	1.00
	B+C	0.0011	-	-	0.0023	0.00007	-
6-species	A1+A2	1.00	1.00	1.00	1.00	1.00	1.00
	A3	0.99	1.00	1.00	0.99	1.00	1.00
	A4	0.99	1.00	1.00	0.99	1.00	1.00
	B	0.99	1.00	1.00	0.99	1.00	1.00
	C	0.99	1.00	1.00	0.99	1.00	1.00
	D	1.00	1.00	1.00	1.00	1.00	1.00
	outgroup	1.00	1.00	1.00	1.00	1.00	1.00
	B+C	0.0016	-	-	0.0018	-	-
A4+C	0.0008	-	-	0.0009	-	-	

(Continued)

Table 5. (Continued)

Species scenarios		Algorithm 0			Algorithm 1		
		G (2,10)	G (2,100)	G (2,1000)	G (2,10)	G (2,100)	G (2,1000)
9-species	A1	1.00	1.00	0.99	0.99	0.99	1.00
	A2	0.97	0.99	0.99	0.96	0.99	0.99
	A3	1.00	0.99	1.00	0.99	0.99	1.00
	A4	0.98	0.99	0.99	0.98	0.99	1.00
	B	0.92	0.99	1.00	0.98	0.99	1.00
	C	0.65	0.99	0.99	0.95	0.99	0.97
	D1	0.99	0.77	0.89	0.97	0.88	1.00
	D2	0.68	0.67	0.83	0.63	0.71	0.95
	D3	0.40	0.89	0.96	0.62	0.83	0.95
	outgroup	1.00	0.99	0.99	1.00	0.99	0.99
	D1+D2	-	0.22	0.13	0.01	0.11	-
	D2+D3	0.20	0.09	0.03	0.29	0.16	0.02
	C+D3	0.29	0.001	0.0005	0.01	0.005	0.0009
	A3+A2	-	0.0006	-	0.0007	0.00005	-
	A4+A2	0.01	0.0005	0.0003	0.01	0.0009	-
	B+D3	0.006	0.0003	-	0.01	0.0003	-
	D1+B	0.003	0.0003	-	0.004	-	-
	C+D2+B	0.05	0.0002	0.00003	0.03	0.001	0.01
	C+D2	-	0.0001	0.0009	-	0.002	0.001
	C+B	0.001	0.0001	-	-	0.005	-
	A3+D3	-	0.00008	-	-	-	-
	B+D2	0.03	0.00007	0.0009	0.002	0.002	-
D2+ outgroup	-	0.00003	-	-	0.00003	0.0005	
A2+D2	-	-	-	-	-	0.0004	

Prior distributions on ancestral population sizes (theta) assigned to different gamma distributions (G) combined with root age (tau) assigned to G (2, 2000).

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Phenotypical and chemical examination of *Aspiciliella intermutans* samples

Chemistry. Two chemotypes were detected by TLC: 1) with norstictic acid and traces of connorstictic acid; 2) with norstictic, stictic and traces of cryptostictic acids. Similar results of a chemical analysis of *A. intermutans* from Greece were published by Sipman & Raus [50]. The second chemotype was only detected in some samples of the clade D, and the first chemotype was found in all clades. The two chemotypes detected inside the *A. intermutans* complex do not discriminate putative lineages.

Table 6. Marginal likelihood and Bayes factor values for alternative species delimitation scenarios.

Model	Marginal Likelihood estimates (MLE)	MLE 6 species (model 1) -MLE i species (model 2)	2lnBF = 2 (MLE 6 species -MLE i species)
9 species	-13865,84647	129,0528333	258,1056665
6 species	-13736,79364	N/A	
5 species	-13765,60161	28,80797094	57,61594187
4 species	-13756,53862	19,74498099	39,48996197

Marginal-likelihood estimates and Bayes factor testing results (2lnBF) BF = 2 x (model1-model2); the model receiving the best marginal-likelihood score for each estimation method is indicated by a 2lnBF; score = N/A, and its associated marginal likelihood is in bold.

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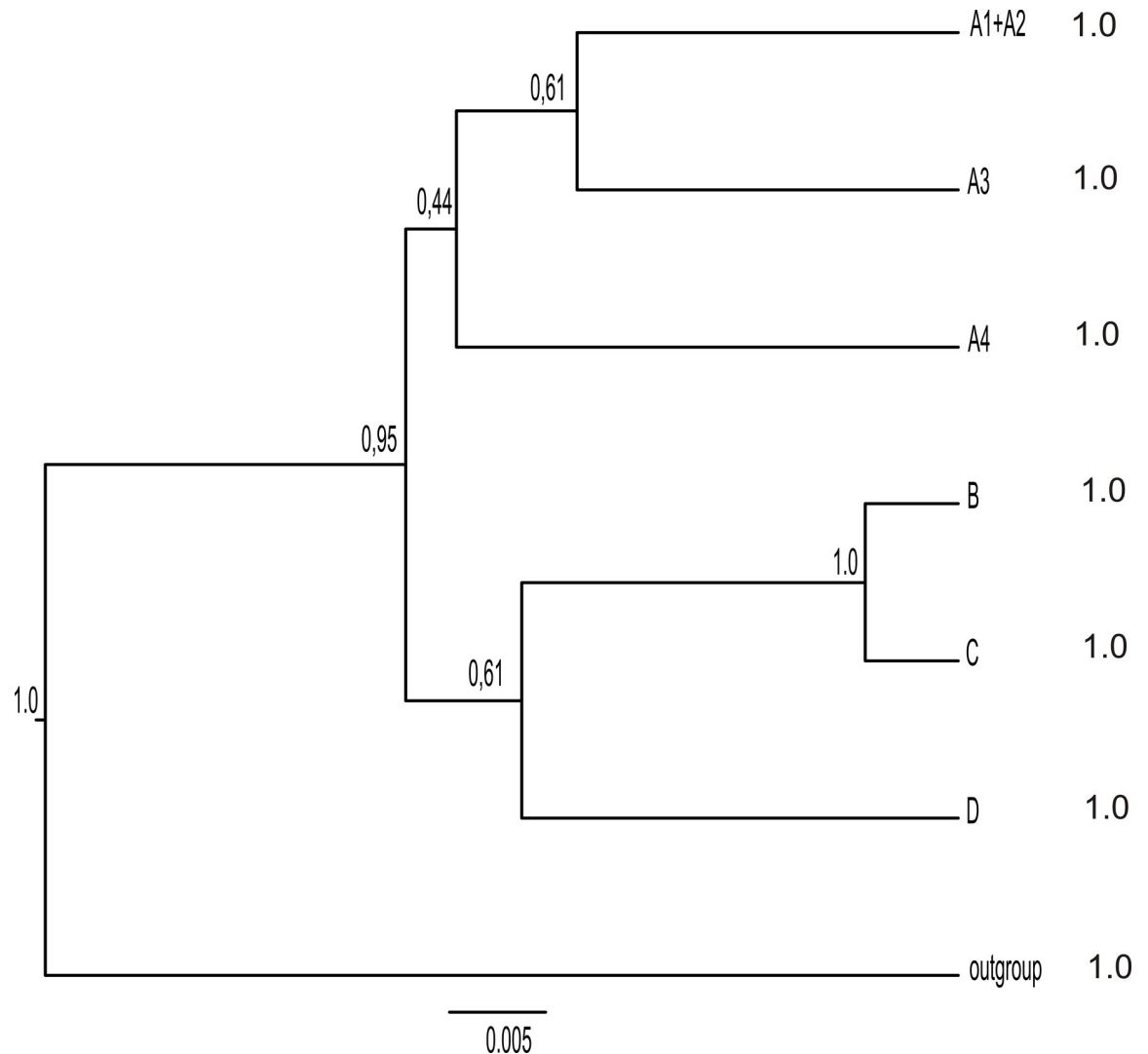


Fig 2. Species tree. Consensus *BEAST species tree of the *Aspiciliella intermutans* complex for six species scenario. Posterior probabilities at nodes indicate the support in the *BEAST analyses. The posterior probability of each delimited species calculated by BP&P is indicated in front of each putative species.

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Morphology. No distinct morphological features were observed to segregate candidate species, except for the areoles color and form. Clades A1+A2 and B are characterized by a thick thallus and larger areoles with a brown to red brown color, in contrast to the thinner thallus and smaller areoles with a gray to light brown color of the samples in clades A3 and A4. However, in samples from different parts of Europe, grouped in clades C and D, all these characters were totally intermixed. Although, the variability in the thallus form and color of the *A. intermutans* complex was considerable; these appeared more or less homogeneous in the samples from Iran and Armenia. Consequently, we tend to not consider them taxonomically relevant in this group.

Substrate preferences and distribution

The species of the *Aspiciliella intermutans* complex occur on siliceous or volcanic rocks in open habitats. The species grow on substrates with mesic (e.g. basalt) to low pH (e.g. andesite,

granite and quartzite rocks). They often grow on exposed, more or less horizontal faces of outcrops. The clades C and D appear to have broad distributions, collected from geographically different regions; clades A1, A2, A3, A4 and B consisted only of specimens collected in western north Iran, Armenia and Azerbaijan.

Discussion

The frequent occurrence of cryptic species has been shown in macrolichen species complexes, examples include the *Cladia* aggregate [25], *Dictyonema glabratum* (Spreng.) D. Hawksw. [51], *Letharia columbiana* (Nutt.) J. W. Thomson [52], *Parmelia saxatilis* (L.) Ach. [53], *Parmotrema reticulatum* (Del-Prado et al. 2016), *Pectenaria atlantica* (Degel.) P. M. Jørg., L. Lindblom, Wedin & S. Ekman [54] and *Rhizoplaca melanophthalea* [22]. However, this phenomenon has poorly been described in microlichen species complexes (see e.g. *Graphis scripta* (L.) Ach.) [55]. This could most probably be due to less attention paid to microlichens or the thought that microlichen species usually encompass differential features, especially ascomata.

Our study uncovers cryptic species diversity in the widespread Mediterranean microlichen species, *Aspiciliella intermutans*, and adds another example of species complexes to the microlichens. We show that *A. intermutans* as currently described is monophyletic but include several cryptic lineages. Our multispecies coalescent species delimitation approaches and BFD test supported the occurrence of at least six candidate species in the *A. intermutans* complex. Our results are in the accordance with a previous study showing different genetic clusters within the *A. intermutans* group [5].

The results of phylogenetic analyses of single locus datasets were incongruent in *A. intermutans* group, suggesting incomplete lineage sorting in the loci or ongoing gene flow or long generation times. These dramatically increase the amount of time required for a species to be recognized under a strict monophyly criterion [56]. The phylogenetic species delimitation criteria such as reciprocal monophyly has been widely used to recognize species however, this criterion may fail to accurately delimit species boundaries in recently diverged species [57]. Multispecies coalescent-based approaches offer a statistic framework for testing species boundary in group with recent diversification histories and does not require species to be reciprocally monophyletic in single gene trees [58,59].

Since the results of single locus datasets were inconclusive, a multilocus data set from *A. intermutans* samples was analyzed in multispecies coalescent-based approaches. Mitochondrial SSU tree topology recovered most lineages identified in the combined analyses, suggesting that a strong phylogenetic signal from mtSSU may have been dominating the analysis of the concatenated genes. The distinctiveness of the different monophyletic clades in the concatenated phylogenetic tree is clear but phylogenetic analyses alone are insufficient (lack of a strong support in some clades) to draw conclusions on species boundaries in this complex. Therefore, we used a combination of methods to address the species delimitation problems in this complex.

Although the species delimitation analyses indicated the presence of different species lineages in the *A. intermutans* complex, we followed a conservative approach as suggested by *BEAST and BP&P, which is sensitive enough to detect evolutionarily distinct lineages at very shallow timescales [60]. Based on all of the available evidence, including the multispecies coalescent-based species delimitation inferences *BEAST, BP&P, and Bayes Factor test, we could objectively circumscribe six candidate species within the *A. intermutans* complex.

We also examined chemistry, morphology, substrate preferences and geographic distribution of the samples, in order to correlate with tree topology. Generally, we found little

Table 7. The phenotypic and geographic features corresponding to the different clades in the *Aspiciliella intermutans* complex recognized by BP&P and Bayes factor analyses on the concatenated phylogenetic tree.

Clades	Geographical region	No. of samples (74)	Altitude (m)	Substrate	Thallus form	Thallus color	Chemotypes
A1 +A2	Armenia, Northwestern and North of Iran	13	1800–3044	Mostly basaltic and andesite rocks	Large and irregular areoles (0.3–2 mm)	Gray-brown to light-brown with pale marginal lines	1
A3	Armenia	5	1390–1850	Granite	Small and regular (0.3–1 mm)	Gray and pruinose	1
A4	Armenia, Northwestern Iran and Azerbaijan	11	1300–1600 (one sample 1900 m)	Andesite, quartz-andesite, granites	Small and regular (0.3–1 mm)	Gray and pruinose	1
B	Armenia, Northwestern and Northeastern Iran and Azerbaijan	9	1885–1992	Mostly quartz-lamprophyre and granites	Large and irregular areoles (0.3–2 mm)	Gray-brown to light-brown with pale marginal lines	1
C	Czech Republic, Slovakia, Greece, France, Spain and Northwestern Iran	15	0–1898	Mostly basalts and andesite	0.3–1.5 mm	Gray to dark-gray	1
D	Romania, Ukraine, Bulgaria, Italy, France and Greece (mostly)	21	2–760	Mostly granite	No distinctive features	No distinctive features	1 and 2

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corroboration between morphological and ecological characters with our candidate species, and also secondary metabolite data provided only a very limited support for the putative species. However, that is a case as in other cryptic lineages of lichenized-fungi. The presence of cryptic species in lichen-forming fungi without any recognizable phenotypical characterization of the clades has been demonstrated in some studies [61].

We did not try to extract DNA of the *A. intermutans* type material from St. Laon [10], because the material was too old to obtain a sequence. However, we obtained DNA from an *A. intermutans* sample (France, Bretagne, *C. Roux 26741*) collected 160 km northwest of St. Laon, and the sample is grouped in the clade D together with samples from other parts of Europe. We suggest the candidate species D (clade D) as the putative species for *A. intermutans* s. str. and all other clades as putative different cryptic species for *A. intermutans* complex.

We know that all published taxa with the characteristics of *Aspiciliella* should eventually be compared with the species distinguishable in this group in order to recognize any names with priority or synonyms. However, this should be the last step after the taxa have been convincingly identified and circumscribed in a manner that those older type specimens can be doubtlessly affiliated to them. Currently, our study suggests that it is not possible to delimit such taxa convincingly, and therefore it is not possible for us on the moment to decide about valid taxonomic names.

Characters of the putative species in the *Aspiciliella intermutans* complex

The phenotypic and geographic features corresponding to the different clades are summarized in Table 7.

Clades A1+A2 were recovered as a well-supported lineage (BS = 94/PP = 0.1), sister to the clade A3 with strong nodal support (BS = 94/PP = 0.1) they show some differences in ecological and morphological patterns. The samples of clade A1 developed a gray-brown to light-brown thallus with pale marginal lines and large (0.3–2 mm) and irregular areoles; some areoles come over others and show a superimposed structure. They are commonly found at medium to higher elevation (1800–3044 m) in the North and North-West of Iran and in Armenia.

Clade A3 was recovered as a well-supported lineage (BS = 100/PP = 0.1). The samples develop a gray thallus, which is light brown with pale marginal lines at the edge of the thallus

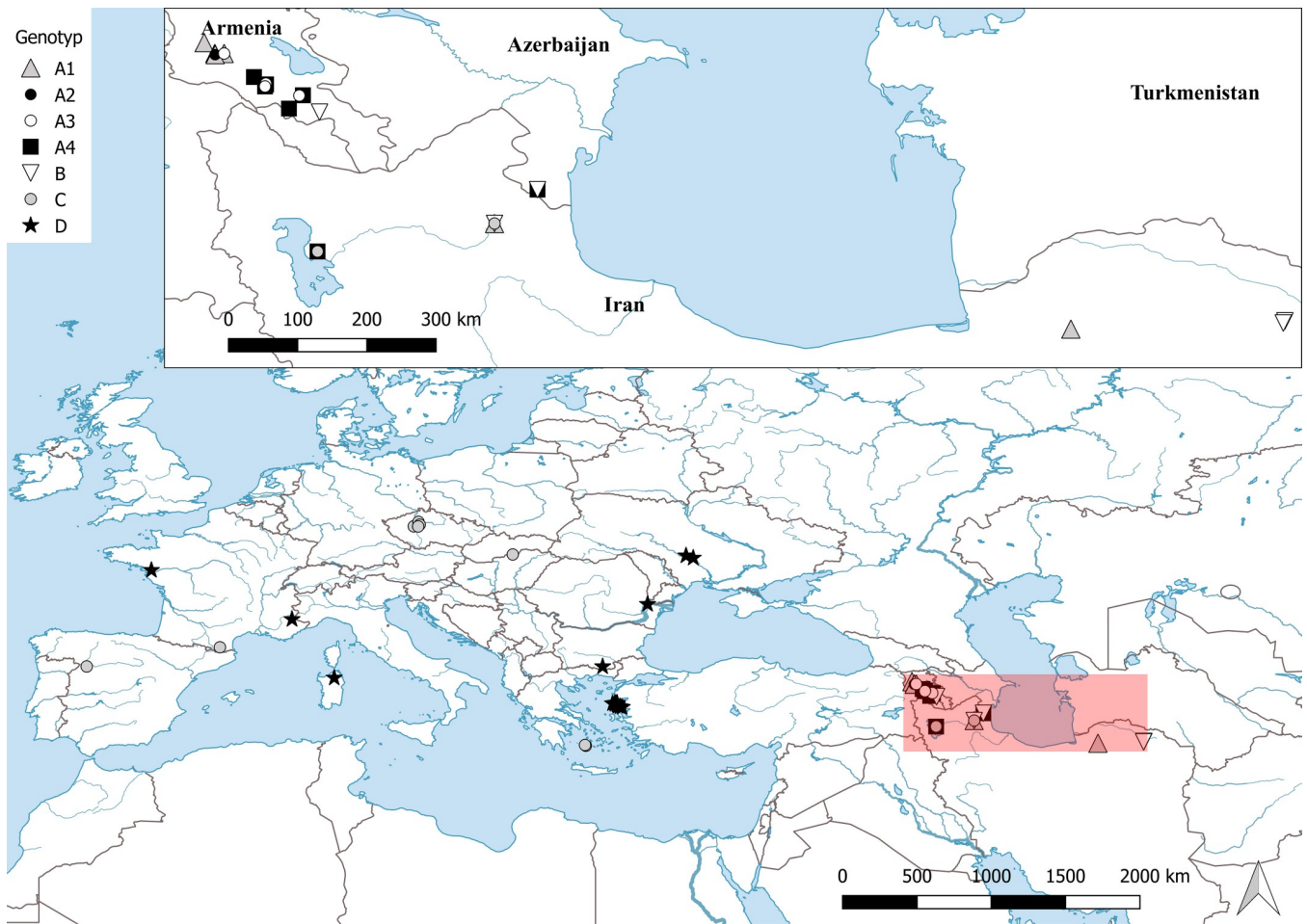


Fig 3. Geographical distribution. Geographical distribution of the *Aspiciliella intermutans* complex examined samples in this study. Symbols indicate the putative species.

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and in the middle totally gray and pruinose; areoles are smaller (0.3–1 mm) than samples in A1+A2 clades and they do not show an irregular structure. They are commonly found at lower to medium elevation (1390–1850 m) in Armenia.

Clade A4 was recovered with a high nodal support (BS = 100/PP = 0.1) and includes samples morphologically similar to clade A3, but prefers substrates with lower pH. The samples were collected from Azerbaijan, Armenia and Northwest of Iran, at medium elevation (1300–1900 m).

Clade B was recovered with a low nodal support (BS = 74/PP = 0.89) and includes samples from Azerbaijan, Armenia, the Northwest and Northeast of Iran, at medium to high elevation (1885–1992 m). The samples are morphologically similar to those in clade A1, but prefer substrates with higher pH.

Clade C was recovered with a low nodal support (BS = 39) and it occurs mostly on basaltic rocks at low to medium elevations, 0–1030 m. It has been collected from the Czech Republic, Slovakia, Greece, France and Spain. It has been recorded also on andesite rocks, in medium to high elevations (1305–1898 m) in northwestern of Iran.

Clade D was recovered with a medium support (PP = 75) and it is sister to clade A-C with the remaining *Aspiciliella intermutans* samples (BS = 83/PP = 0.89). The samples have a wide

morphological variation and they are from Romania, Ukraine, Bulgaria, Italy, France and mostly from Greece (North Aegean Region), at low elevations (2–760 m), mostly on granite rocks. Some samples in this clade show a chemotype with norstictic acid, stictic acid and a trace of cryptostictic acid, observed exclusively inside this clade

Conclusions

Our study of the *Aspiciliella intermutans* species complex indicates that the genus *Aspiciliella* and the *A. intermutans* complex are more diverse in Eurasia than previously expected. Further, this study highlights the occurrence of cryptic species in microlichens. Our sampling was more intensive in Armenia, Iran, Greece and the eastern part of Europe however the samples from other distribution ranges of this species complex were either scarce or absent. While four of the six species-level lineages within the *A. intermutans* complex were restricted to Armenia and Iran, only two broadly distributed lineages were found in samples from Greece and also other parts of Europe (Fig 3). Based on our results we suggest that the Caucasian region could be the main biogeographical region for speciation in this complex.

Ultimately, rigorous sampling in other regions, such as Western and Central Europe, the Caucasian region, NE Iran, Turkmenistan Anatoly, Central Asia mountains (and also North America), will be needed to more accurately assess the distribution patterns of lineages within this group. Understanding the general geographic distribution of the candidate species identified in this study requires robust data from a broader geographic sampling, which may or may not correlate to additional lineages within the *A. intermutans* species-complex.

The authors plan a detailed revision of the *A. intermutans* species-complex in the near future, with more taxonomic, morphological and geographical sampling to characterize boundaries between the candidate species.

Supporting information

S1 Fig. mtSSU ML tree. ML phylogenetic tree (IQ tree analysis) of *Aspiciliella intermutans* complex from mtSSU sequences. Bootstrap values are shown above their respective branches. Our six candidate species within the *A. intermutans* complex based on BP&P and Bayes factor on the concatenated phylogenetic tree are highlighted by different colors as in Fig 1. The result of ABGD analyses for the mtSSU-single locus are shown in the picture.
(TIF)

S2 Fig. MCM7 ML tree. ML phylogenetic tree (IQ tree analysis) of *Aspiciliella intermutans* complex from MCM7 sequences. Bootstrap values are shown above their respective branches. Our six candidate species within the *A. intermutans* complex based on BP&P and Bayes factor on the concatenated phylogenetic tree are highlighted by different colors as in Fig 1. The result of ABGD analyses for the MCM7-single locus are shown in the picture.
(TIF)

S1 Table. Geographical distribution data.
(ODS)

S1 File. MCM7 sequence alignment.
(TXT)

S2 File. mtSSU sequence alignment.
(TXT)

S3 File. Sequence alignment of Concatenated gene tree.
(TXT)

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