

Research articleSubmitted: January 14th, 2020 - Accepted: March 15th, 2020 - Published: April 15th, 2020**DNA barcoding unveils the first record of *Andrena allosa* for Italy and unexpected genetic diversity in *Andrena praecox* (Hymenoptera: Andrenidae)**Maurizio CORNALBA¹, Paolo BIELLA^{2,*}, Andrea GALIMBERTI²¹ University of Pavia, Department of Mathematics - Via Ferrata 5, 27100 Pavia, Italy - maurizio.cornalba@unipv.it² University of Milano-Bicocca, Department of Biotechnology and Biosciences - Piazza della Scienza 2, 20126 Milano, Italy
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Abstract

DNA barcoding is well-known to support morphological species identification and it can be helpful for unveiling unexpected populations divergence patterns, especially in the context of the impacts on species posed by global change. In this note, we provided the first Italian record of the alpine mining bee *Andrena allosa* Warncke, 1975, confirmed with DNA barcoding. In addition, genetic identification of a specimen of *Andrena praecox* (Scopoli 1753) from western Italy pointed to an unexpected intraspecific genetic structuring at COI DNA barcoding region, with sequences from the Italian and the western sector of its global distribution differing 2.22% (p-dist) from populations of the eastern sector. Given the relevance of these records and of the genetic identity of bee populations from Italy, we argue that implementing molecular surveys in bee monitoring would surely contribute to the conservation of these important pollinators.

Keywords: Pollinators, fauna of Italy, molecular identification, mining bees, haplotype network, population structure.**Introduction**

Mountains generally exhibit higher levels of biodiversity than nearby lowlands (see for instance Viterbi et al. 2013), mainly due to their topographic and climatic heterogeneity (Körner 2000). On the other hand, the populations of mountain fauna and flora are very sensitive to environmental change, global warming, disturbance and abandonment of traditional activities (Assandri et al. 2017). These factors were included among those directly threatening the occurrence of some rare, endemic or declining bee species (Quaranta et al. 2019) and particularly of mountain endemics (see for instance Biella et al. 2017).

In this scenario, reaching quick and reliable species identifications seems particularly urgent. The integration of morphology with information from DNA markers (e.g., DNA barcoding) and other sources such as species distribution and life-history traits are often used in biology for describing the occurrences of populations and interactions with food resources, their evolutionary relationships, their taxonomy and conservation. When this approach is systematically applied to numerous species in different localities and if their DNA barcodes are publicly available, tools like DNA barcoding can be used also to confirm doubtful field observations (Bossert et al. 2016; Wauters et al. 2017).

In this study, we applied DNA barcoding to confirm the morphological identification of some bee specimens, freshly collected in the upper Val di Susa (Piedmont) and assigned to two species. The first of these is the mining bee *Andrena (Euandrena) allosa* Warncke, 1975, whose status was for a long time controversial, due in part to the uncertain delimitation of the taxon and the paucity of records (Gusenleitner & Schwarz 2002; Praz et al. 2019; Warncke 1975). The situation was clarified in Praz et al. (2019), where the complex of Alpine species close to *Andrena (Euandrena) bicolor* Fabricius, 1775 was revised from the point of view of genetics, morphology, phenology and floral preferences. In particular, it was shown that *A. allosa* is a valid species, easy to identify morphologically, that it has a particularly early phenology and that its females exhibit a marked preference for *Crocus* when collecting pollen. Our record of *Andrena allosa* appears to be the first reliable one for Italy. The second species is *Andrena (Andrena) praecox* (Scopoli, 1763), one of the earliest emerging bees, oligolectic on willow (*Salix* spp.) for pollen. This species is widely distributed in Europe, including the Alps, but is rarely reported from Italy. Quite unexpectedly, the DNA barcode sequence of our specimen turned out to be very similar to those of *A. praecox* specimens from the westernmost part of its range, but significantly divergent from the available ones from the rest of the distribution.

Methods

In order to substantiate the presence of *Andrena allosa* in Italy, in May 2019 we carried out a short, targeted search in the upper Val di Susa (Piedmont), not far from known French stations of the species, in an area chosen because of its accessibility and its presumed suitability as a habitat for *Andrena allosa*, based on the pattern of European occurrences and the phenological data in Praz et al. (2019). We located a large patch of *Crocus vernus* (L.) Hill, 1765 in full bloom, next to lingering snow, in a West-sloping pasture with scattered larch (*Larix decidua*) trees, situated at an elevation of approximately 1870 m. Several *Andrena* females were collecting *Crocus* pollen and a number of *Andrena* males were patrolling nearby. We collected 9 bee specimens, 6 females and 3 males, with a hand-net.

Two *Andrena* specimens, one male and one female, were stored in 95% ethanol for molecular identification. The remaining specimens were killed with ethyl acetate and pinned. All the specimens were morphologically identified to species using the keys and descriptions in (Schmid-Egger & Scheuchl (1997) and Praz et al. (2019).

To confirm species identification of the two samples, total DNA was extracted from one posterior leg, by using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy). DNA barcoding characterization of samples was conducted by comparing nucleotide sequence differences at the standard barcode region for metazoans (i.e., 658 bp at the 5' end of the mitochondrial COI, Hebert et al., 2003) following the same procedures described in Bellati et al. (2014). The presence of stop codons was verified for the obtained sequences by using the on-line tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI). The taxonomic status of the sampled bees was also tested by comparing the COI sequence with reference DNA barcodes stored in the Barcode of Life Database (BOLD System) using the Identification Engine tool (IDS) (http://www.boldsystems.org/index.php/IDS_IdentificationRequest; Species Level Barcode Records database), which returns unique species assignments based on 99% sequence similarity of the barcode sequence.

To compare previously published data to our samples, we assembled two separate COI datasets, one of *Andrena allosa* and one of *A. praecox*, by downloading from BOLD the COI sequences that were either directly named as *Andrena allosa* or *Andrena praecox* or matched totally (100%) sequences named as such. For each dataset, sequences were aligned using MAFFT online (<https://mafft.cbrc.jp/alignment/server/>) with default options. Due to different lengths of publicly available COI sequences, we trimmed the alignments to the same final length (of 582 and 631 bp, respectively). In order to verify the occurrence of a putative lineage including the Italian samples an unrooted minimum spanning network was obtained for each

bee species using the median-joining network algorithm (Bandelt et al., 1999) implemented in PopART (<http://popart.otago.ac.nz/howtocite.shtml> – default settings) (Leigh & Bryant 2015).

Results

The outcome of the morphological determinations of the collected specimens is as follows, where pMC stands for the personal collection of Maurizio Cornalba and MIB:ZPL stands for the collection of the ZooPlantLab of the University of Milano-Bicocca (<https://www.gbif.org/grscicoll/institution/995e2058-ed01-43dc-b642-1baecf26b391>):

- *Andrena allosa* Warncke, 1975. Italy: Piemonte, Sauze di Cesana (Torino), 44.9476 N, 6.8691 E, 1866 m, leg. & det. Maurizio Cornalba, 3 ♀♀, pMC (2), MIB:ZPL (1, MIB:ZPL:07785).
- *Andrena amieti* Praz, Müller & Genoud, 2019. Italy: Piemonte, Sauze di Cesana (Torino), 44.9476 N, 6.8691 E, 1866 m, leg. & det. Maurizio Cornalba, 2 ♀♀, pMC.
- *Andrena bicolor* Fabricius, 1775. Italy: Piemonte, Sauze di Cesana (Torino), 44.9476 N, 6.8691 E, 1866 m, leg. & det. Maurizio Cornalba, 1 ♀, pMC.
- *Andrena praecox* (Scopoli, 1763). Italy: Piemonte, Sauze di Cesana (Torino), 44.9476 N, 6.8691 E, 1866 m, leg. & det. Maurizio Cornalba, 3 ♂♂, pMC (2), MIB:ZPL (1, MIB:ZPL:07786).

All the females were collecting pollen of *Crocus vernus*, while two of the males were engaged in patrolling flights and the remaining one was resting inside a *Crocus* flower.

High quality DNA sequences were obtained for the two individuals analysed. The two barcode sequences contained no stop codons and were not biased by NUMT (*sensu* Bensasson et al. 2001) interference. The DNA barcoding identification, performed on one of the *A. allosa* females (GenBank accession number LR655818) and on one of the *A. praecox* males (GenBank accession number LR655819), unequivocally confirmed the morphological identifications. Specifically, when COI sequences obtained from the samples were used as query in GenBank-NCBI and BOLD-IDS tools, both systems returned a reliable match (i.e., similarity % > 99%) with reference DNA barcoding sequences of *A. allosa* and *A. praecox* respectively. The voucher specimens MIB:ZPL:07785 and MIB:ZPL:07786 are deposited in the MIB:ZPL collection.

The haplotype median-joining network of *A. allosa* showed that the sequences from France, Switzerland and Italy are separated by only one mutation step at the COI region (Fig. 1). In the case of *A. praecox*, the haplotype network is more structured than for *A. allosa*. As shown in Table 1, the COI sequences of *A. praecox* from the eastern distribution sector diverged 2.22 % in terms of p-distance

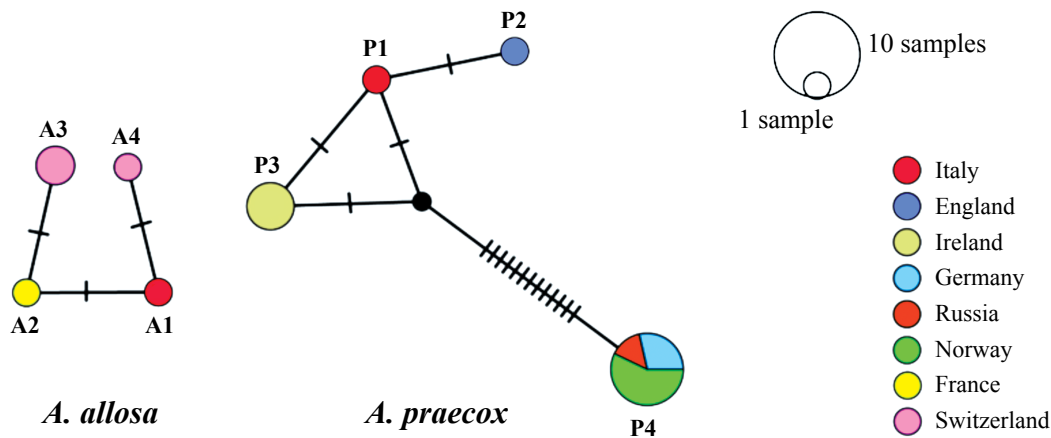


Fig. 1 – Median-joining network for the COI haplotypes identified from the *A. allosa* and *A. praecox* barcode sequences produced in this study and retrieved from publicly accessible data in BOLD. Each circle represents a haplotype and its size is proportional to haplotype frequency. Colours indicate different sampling countries (additional details are in Table 1). Small black traits represent possible median vectors, while dashes represent substitutions.

from the samples of the western distribution (namely England, Ireland, Italy, Fig. 1).

Discussion

In this study we confirmed the presence in Italy of the mining bee *Andrena allosa* Warncke, 1975, by combining morphological examination and DNA barcoding of freshly collected specimens, integrated with expert-based knowledge on the distribution and life-history traits of species.

Except for a couple of problematic records from Spain and the French Pyrenees, *A. allosa* appears to be restricted to the western Alps, where it is widely distributed in France and Switzerland from the Maritime Alps to the Berner Oberland (Praz et al. 2019). It is relatively common in this range (C. Praz, personal communication). Its occurrence in neighbouring parts of Italy was expected and has now been confirmed. In fact, it is conceivable, and even likely, that *A. allosa* may occur in much of the Italian western Alps, from the Ligurian Alps at least to the Aosta valley. It should be looked for in this area, but also in other parts of the Alps, as the eastern limits of its distribution are not known. *A. allosa* seems to be new for Italy, since the only previous Italian record of the species appears to be the result of a misidentification (Praz et al. 2019).

Together with *Andrena allosa* we found three other species of the same genus. One of them (*A. bicolor*) is a common and widespread Palaearctic species. A second one (*A. amieti*) has been described only very recently and has been found to be widely distributed in the mountains of Italy (Praz et al. 2019). Another interesting record are the males of *Andrena praecox*. There are very few records of this species from Italy, while it is more frequent and widespread in the rest of Europe. We are aware of a couple of old records from northern Italy, specifically from

Trentino (Bonelli 1971; Thomas 2019) and from Friuli Venezia Giulia (Zandigiacomo et al. 2012). In central Italy there are an old record from Lazio (map in Gusenleitner & Schwarz 2002) and a more recent one from Tuscany (Filippi & Strumia 2018). In the western Alps, the situation is even more puzzling, as several records are available from France but the species has been recorded only very recently from the E slopes of the western Alps in Italy (Terme di Valdieri, 1450 m, 26 May 2018, leg. and det. M. Aubert; Sauze di Cesana, this study, 2019). In particular, the latter two appear to be the first records of *Andrena praecox* from the Piemonte administrative region. In addition to its biogeographical relevance, the record of *A. praecox* in this study is remarkable because of the exceptionally high elevation at which our specimens were found. In fact, the upper limit of the distribution of the species in nearby Switzerland is given as 1500 m in Amiet et al. (2010) and as 1580 m in Müller (2019), with the majority of the records between 400 and 600 m. The reason why the male specimens we collected were patrolling at elevations about 300 meters above the known altitudinal range is unclear to us. A possible explanation could lie in the climatic peculiarity of the Susa Valley, which is markedly xeric (Manino et al. 2010). This might justify the establishment of populations at uncommon altitudes. Alternatively, or concurrently, a contribution of climate warming can be hypothesized, as already demonstrated in other alpine bee species (Biella et al. 2017; Ormosa et al. 2017).

The genetic identities of the two mining bees studied here showed different levels of divergence from specimens from other European countries. In the case of *A. allosa*, the genetic structure is very low, clearly due to the geographic proximity and the continuity in distribution within the regions of origin of the samples. As for *A. praecox*, the barcode sequence of our specimen clustered with sequences from the westernmost part of the species' dis-

Table 1 – Details of public COI sequences and their genetic distances (p-distance, %) from the Italian samples, for *Andrena praecox* and *A. allosa*.

Species	Distance to Italian sample	Distribution	Country	Locality	NCBI/BOLD code	Haplotype
<i>Andrena allosa</i>	Reference	Alps	Italy	Turin: Sauze di Cesana	LR655818	A1
<i>Andrena allosa</i>	0.172	Alps	France	Alpes-Maritimes: Moulinet	HYMAA059-18	A2
<i>Andrena allosa</i>	0.346	Alps	Switzerland	Valais: Leuk	FBAPD848-11	A3
<i>Andrena allosa</i>	0.346	Alps	Switzerland	Valais: Leuk	HYMAA022-18	A3
<i>Andrena allosa</i>	0.172	Alps	Switzerland	Valais: Saint-Martin	HYMAA020-18	A4
<i>Andrena praecox</i>	Reference	Alps	Italy	Turin: Sauze di Cesana	LR655819	P1
<i>Andrena praecox</i>	0.158	W Eur	England	London: Greenwich	BEEEE344-16	P2
<i>Andrena praecox</i>	0.158	W Eur	N. Ireland	Fermanagh: Monawilkin	IBBP004-10	P3
<i>Andrena praecox</i>	0.158	W Eur	Ireland	Leinster: Kilkenny	IBBP051-10	P3
<i>Andrena praecox</i>	0.158	W Eur	Ireland	Leinster: Kilkenny	IBBP052-10	P3
<i>Andrena praecox</i>	2.218	E Eur	Germany	Bavaria: Groebenzell	FBHAP549-09	P4
<i>Andrena praecox</i>	2.218	E Eur	Germany	Saxony-Anhalt: Bitterfeld-Wolfen	GBMIX800-14	P4
<i>Andrena praecox</i>	2.218	E Eur	Russia	Moscow Oblast: Abramtsevo	PCHEL357-12	P4
<i>Andrena praecox</i>	2.218	E Eur	Norway	Hedmark: Magnor	NOAPI183-14	P4
<i>Andrena praecox</i>	2.218	E Eur	Norway	Vestfold: Nord Maagero	NOAPI186-14	P4
<i>Andrena praecox</i>	2.218	E Eur	Norway	Vestfold: Larvik	NOAPI187-14	P4
<i>Andrena praecox</i>	2.218	E Eur	Norway	Telemark: Porsgrunn	NOAPI188-14	P4

tribution, while all sequences from this cluster diverged sharply ($\geq 2.22\%$) from all other available sequences, all coming from the eastern sector of the distribution. It would be important to ascertain whether this divergence is reflected or not in morphological differences, which up to now do not seem to be known. More generally, and apart from reflecting the geographic distance between the two groups of samples, our finding clearly points to a significant population structuring, which deserves to be investigated from the point of view of both morphology and genetics.

Conclusions

In this study, we presented key records of two mining bees by combining expert-based knowledge on the distribution of species, the morphological examination of specimens and their DNA-based identification. This procedure proved to be very helpful in the case of *A. allosa* for adding to the list of species known to occur in Italy, and in the case of *A. praecox* for showing the relevance of its genetic population structuring, with direct implications for the conservation of the two species.

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References

- Amiet F., Herrmann M., Müller A., Neumeyer R. 2010. Apidae 6. Centre suisse de cartographie de la faune, Neuchâtel.
- Assandri G., Bogliani G., Pedrini P., Brambilla M. 2017. Land-use and bird occurrence at the urban margins in the Italian Alps: implications for planning and conservation. *North-Western Journal of Zoology*, 13: 77–84.
- Bandelt H.-J., Forster P., Röhl A. 1999. Median-Joining Networks for Inferring Intraspecific Phylogenies. *Molecular Biology and Evolution*, 16: 37–48.
- Bellati A., Tiberti R., Cocca W., Galimberti A., Casiraghi M., Bogliani G., Galeotti P. 2014. A dark shell hiding great variability: a molecular insight into the evolution and conservation of melanic *Daphnia* populations in the Alps. *Zoological Journal of the Linnean Society*, 171: 697–715.
- Bensasson D., Zhang D.X., Hartl D.L., Hewitt G.M. 2001. Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution*, 16: 314–321.
- Biella P., Bogliani G., Cornalba M., Manino A., Neumayer J., Porporato M., Rasmont P., Milanese P. 2017. Distribution patterns of the cold adapted bumblebee *Bombus alpinus* in the Alps and hints of an uphill shift (Insecta: Hymenoptera: Apidae). *Journal of Insect Conservation*, 21: 357–366.
- Bonelli B. 1971. *Montagna viva. Il mondo degli Insetti in Val di Fiemme (Trentino)*. Reverdito, Trento.
- Bossert S., Gereben-Krenn B.-A., Neumayer J., Schneller B., Krenn H. 2016. The cryptic *Bombus lucorum* complex (Hymenoptera: Apidae) in Austria: Phylogeny, distribution, habitat usage and a climatic characterization based on COI sequence data. *Zoological Studies*, 55: 13.
- Filippi L., Strumia F. 2018. Gli Imenotteri Apoidei della Riserva Naturale di Monterufoli-Caselli (PI): un hotspot di

- biodiversità, in: Proceedings of “Codice Armonico 2018” Settimo Congresso di Scienze Naturali ed Ambiente Toscana. Edizioni ETS, Pisa, pp. 23–33.
- Gusenleitner F., Schwarz M. 2002. Weltweite Checkliste der Bienengattung *Andrena* mit Bemerkungen und Ergänzungen zu paläarktischen Arten (Hymenoptera, Apidae, Andreninae, *Andrena*). Entomofauna, Supplement 12: 1–1280.
- Hebert P.D.N., Cywinska A., Ball S.L., DeWaard J.R. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society London, B 270: 313–321.
- Körner C. 2000. Why are there global gradients in species richness? Mountains may hold the answer. Trends in Ecology & Evolution, 15: 513–514.
- Leigh J.W., Bryant D. 2015. PopART: Full-feature software for haplotype network construction. Methods in Ecology and Evolution 6, 1110–1116.
- Manino A., Patetta, A., Boglietti G., Porporato M. 2010. Bumble bees of the Susa Valley (Hymenoptera Apidae). Bulletin of Insectology, 63: 137–152.
- Müller A. 2019. *Andrena praecox* [WWW Document]. Les abeilles sauvages de Suisse. URL <https://species.infofauna.ch/groupe/1> (accessed 2019-6-25).
- Ormosa C., Torres F., De la Rúa P. 2017. Updated list of bumblebees (Hymenoptera: Apidae) from the Spanish Pyrenees with notes on their decline and conservation status. Zootaxa, 4237: 41–77.
- Praz C., Müller A., Genoud D. 2019. Hidden diversity in European bees: *Andrena amieti* sp. n., a new Alpine bee species related to *Andrena bicolor* (Fabricius, 1775) (Hymenoptera, Apoidea, Andrenidae). Alpine Entomology, 3: 11–38.
- Quaranta M., Cornalba M., Biella P., Comba M., Battistoni A., Rondinini C., Teofili C. 2019. Lista rossa IUCN delle api italiane minacciate (IUCN Red List of Italian threatened bees). Comitato Italiano IUCN e Ministero dell’Ambiente e della Tutela del Territorio e del Mare, Roma.
- Schmid-Egger C., Scheuchl E. 1997. Illustrierte Bestimmungstabellen der Wildbienen Deutschlands und Österreichs. Band III: Andrenidae. E. Scheuchl, Velden.
- Thomas J. 2019. Snow Entomological Museum Collection. Version 26.33. University of Kansas Biodiversity Institute. Occurrence dataset <https://doi.org/10.15468/fhntpy> accessed via GBIF.org on 2020-01-07. <https://www.gbif.org/occurrence/784989961>
- Viterbi R., Cerrato C., Bassano B., Bionda R., von Hardenberg A., Provenzale A., Bogliani G. 2013. Patterns of biodiversity in the northwestern Italian Alps: a multi-taxa approach. Community Ecology, 14: 18–30.
- Warncke K. 1975. Die Bienengattung *Andrena* F. in Iberien (Hym. Apidae). Teil A. Eos, 49 (1973): 293–314.
- Wauters L.A., Amori G., Aloise G., Gippoliti S., Agnelli P., Galimberti A., Casiraghi M., Preatoni D., Martinoli A. 2017. New endemic mammal species for Europe: *Sciurus meridionalis* (Rodentia, Sciuridae). Hystrix, 28: 1–8.
- Zandigiacomo P., Fortunato L., Barbattini R., Frilli F., Pagliano G., Quaranta M. 2012. Apoidea del Friuli Venezia Giulia e di territori confinanti. iii. Andrenidae. Gortania, 34: 101–136.