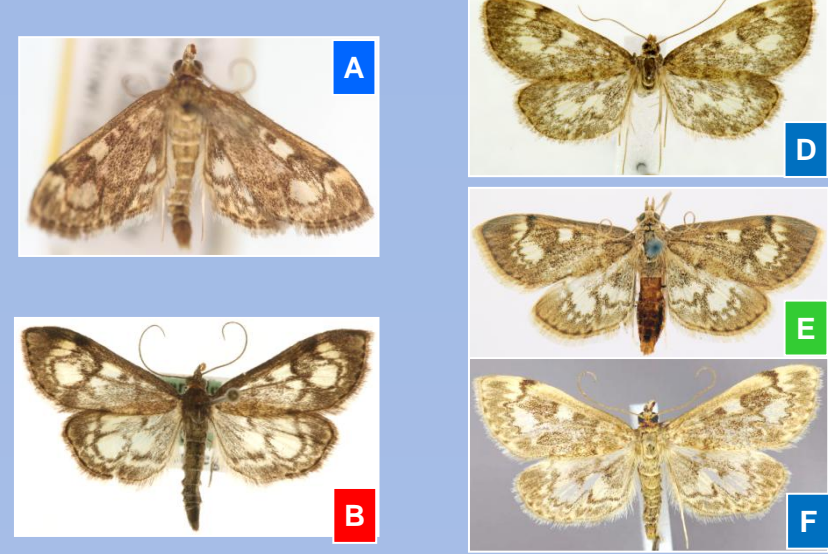# Integrative taxonomy: DNA barcoding and morphological studies reveal three cryptic species of *Anania* (Lepidoptera: Crambidae: Pyraustinae) in North America, all distinct from their European counterpart

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Who is real A. coronata?

## INTRODUCTION

*Anania coronata* (Hufnagel, 1767) is a Holarctic species of Pyraustinae that ranges throughout Europe, in Asia and Japan, and across North America (Munroe 1976, Spiedel 1996, Sinev 2008). Munroe (1954, 1976) treated North American populations as a subspecies, *A. coronata tertialis* (Guenée, 1854).

Two other North American taxa have long been placed in synonymy with *A. coronata tertialis*: *Botys plectilis* Grote and Robinson, 1867, and *Botys syringicola* Packard, 1870 (Dyar 1903, McDunnough 1938, Munroe 1976, Hodges et al. 1983). Munroe (1976) noted that more than one species or subspecies might be recognized under this taxon on the account of its wide Holarctic distribution and morphological variation, but he did not analyze it. From an examination of North American specimens in the Muséum d'Histoire Naturelle in Paris, Leraut (2005) raised both *E. tertialis* and *B. plectilis* to full species, distinct from the nominal *coronata* and from each other. However, Leraut did not examine any type specimens and relied entirely on the identifications of specimens in the Paris Museum, which were unverified. He left *syringicola* as a synonym of *tertialis*.

Barcode data revealed that specimens of *A. coronata* from North America and Europe separated into four sequence clusters. This study examines the geographic distribution of these four lineages and the morphological divergence among them. We compared phenotypic characters and COI sequences to illuminate species differences among North American and European samples, and clarify the nomenclature.

## METHODS

### Taxon sampling

Eighty-nine specimens of *A. coronata* from sites in Europe and North America (**Figure 1**) were analyzed as well as 20 specimens belonging to three congeneric taxa, *A. quebecensis* (Munroe, 1954), *A. perlucidalis* (Hübner, 1809), *A. stachydalis* (Germar, 1822).
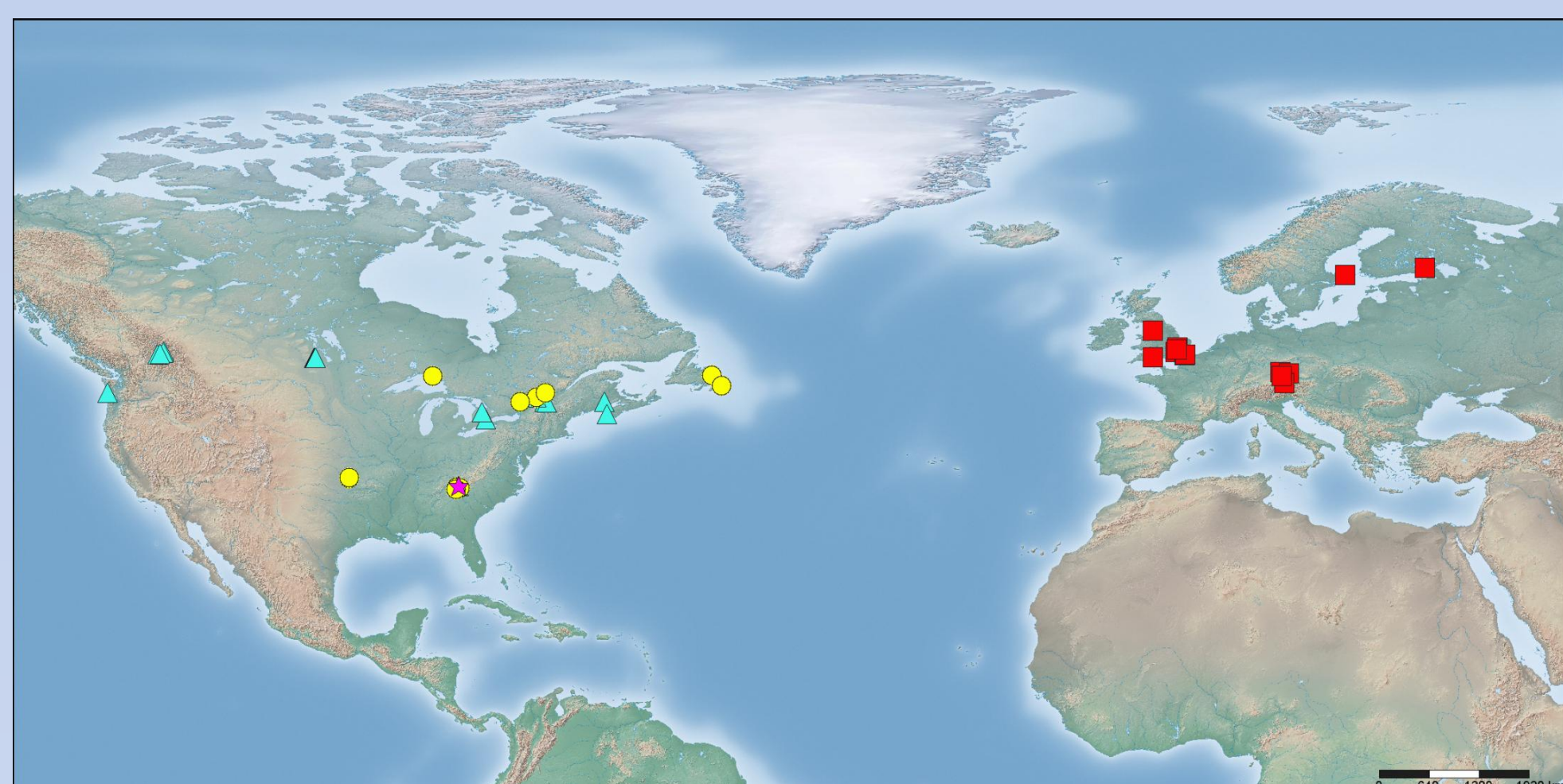


Fig.1 Distribution of collection localities of analyzed specimens of '*A. coronata*' in this study.

### Morphological characters and morphometric analysis

Eight female genitalic characters and 14 male genitalic characters were compared among individuals in each of the four different lineages of the *A. coronata* complex (7 traits for females and 11 characters for males were morphometrically analysed) (**Figure 2**). In total, 12 females and 23 males were examined morphologically, including the female holotype of '*B. plectilis*' and a female paralectotype of '*E. tertialis*'.

Statistical comparisons of female and male genitalia were performed using Statistica 8.0 for Windows (Statsoft, Inc., 1999). Principal components analysis (PCA) was performed to summarize patterns of variation in female and male genitalia. Discriminant function analysis (DFA) was used to determine the morphological variables that best discriminate individuals of the four barcode lineages.

### DNA extraction and PCR amplification

Barcode records were obtained from each of the 109 specimens by extracting DNA from a single leg. All samples were processed at the Canadian Centre for DNA Barcoding (CCDB) using a silica-based 96-well extraction automation protocol for DNA extraction (Ivanova et al. 2006). For two ancient type specimens, we used whole abdomen for DNA extraction before removing the genitalia for dissection (Knölke et al. 2005). COI sequences were assembled from shorter amplicons by using the primer combinations for ancient type specimens (Rodolphe Rougerie & Sean Prosse, pers. comm).

### Molecular and phylogenetic analysis

We selected *A. leuschneri*, A. *stachydalis* and *A. quebecensis*, which are morphologically and genetically the most similar, as the primary outgroup to root the trees. The number of haplotypes was

calculated with DnaSP 5.10 (Rozas et al. 2003). Phylogenies were inferred using Maximum likelihood (ML) and Bayesian Inference (BI), using PhyML v.3.0 (Guindon and Gascuel 2010) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), respectively
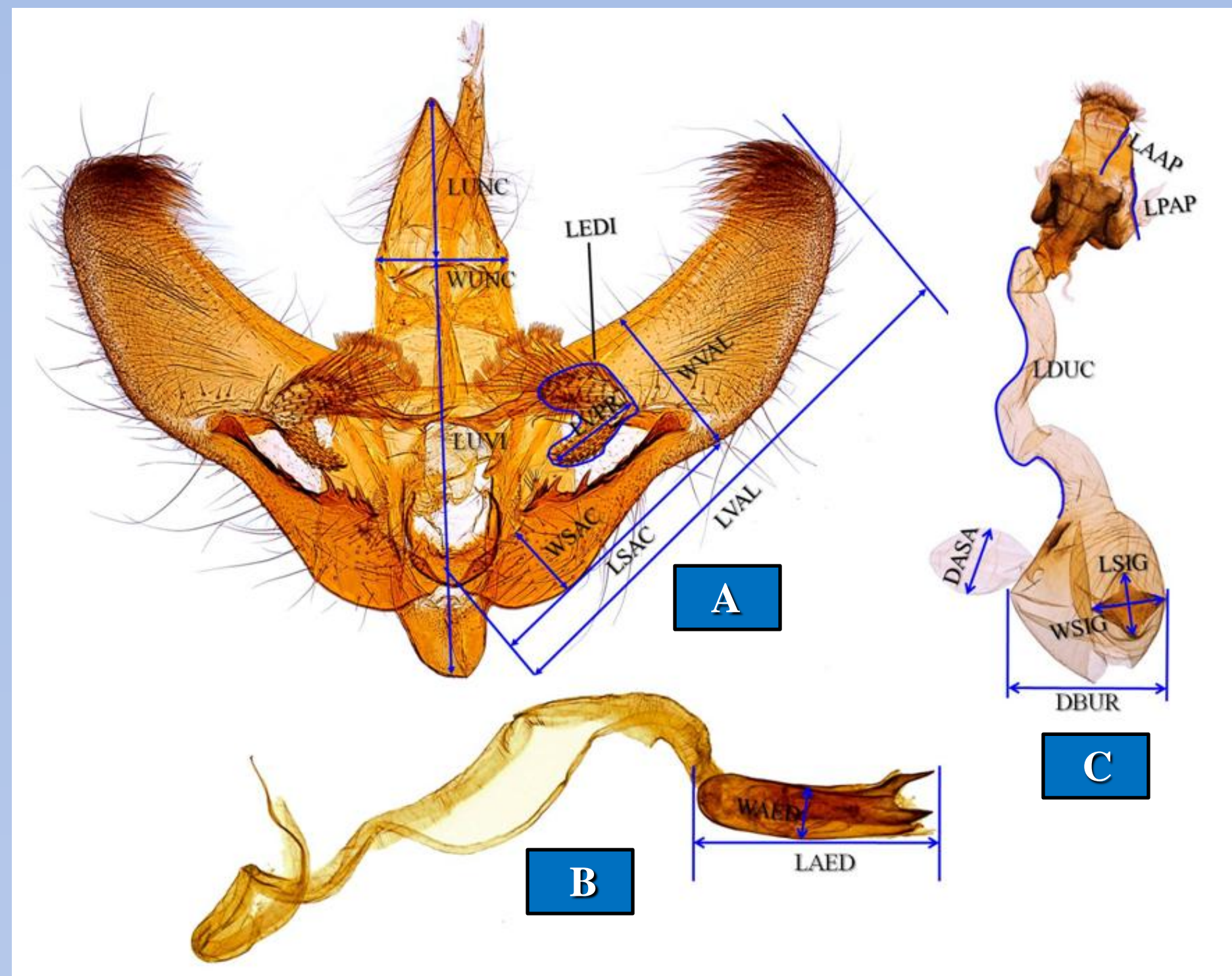


Fig. 2 Morphological traits measured in the present study. A, Male genitalia; B, Aedoeagus; C, Female genitalia. LUNC, length of uncus; WUNC, width of uncus; LUTV, length from uncus to vinculum; LVAL, length of valve; LSAC, length of sacculus; WVAL, width of valve; WSAC, width of sacculus; PEDI, perimeter of editum; LVPR, length of ventral process of editum; LAED, length of aedoeagus; WAED, width of aedoeagus; LAAP, length of anterior apophyses; LPAP, Length of posterior apophyses; LDUC, length of ductus; LSIG, length of signum; WSIG, width of signum; DBUR, Diameter of bursa; DASA, diameter of accessory sac.

## RESULTS

### DNA barcoding analysis

The 109 COI sequences ranged in length from 307 - 658 bp (mean length = 644 bp) and variation was detected at 109 sites (16.6%). Within the *A. coronata* group, four distinct and well-supported clades were observed, namely NEA, TN, EU, and NE, which constituted four putative species (**Figure 3, A**). Divergence between any pair of *A. coronata* lineages regardless of geographic area exceeded 2%. In general, divergence levels of COI within each lineage were low, whereas high divergences were observed between lineages.

### The assignment of mini-barcodes from 130-year-old type specimen

A 130-bp fragment of COI barcode region was successfully recovered from the 130-year-old type of '*plectilis*' using a novel primer sets for PCR amplication. To test the assignment of the mini-barcode from that old type, we included it in an overall NJ analysis with all 109 ingroup and outgroup sequences. **Figure 3, A** shows that the mini-barcodes of the type *B. plectilis* clustered in NEA lineages.
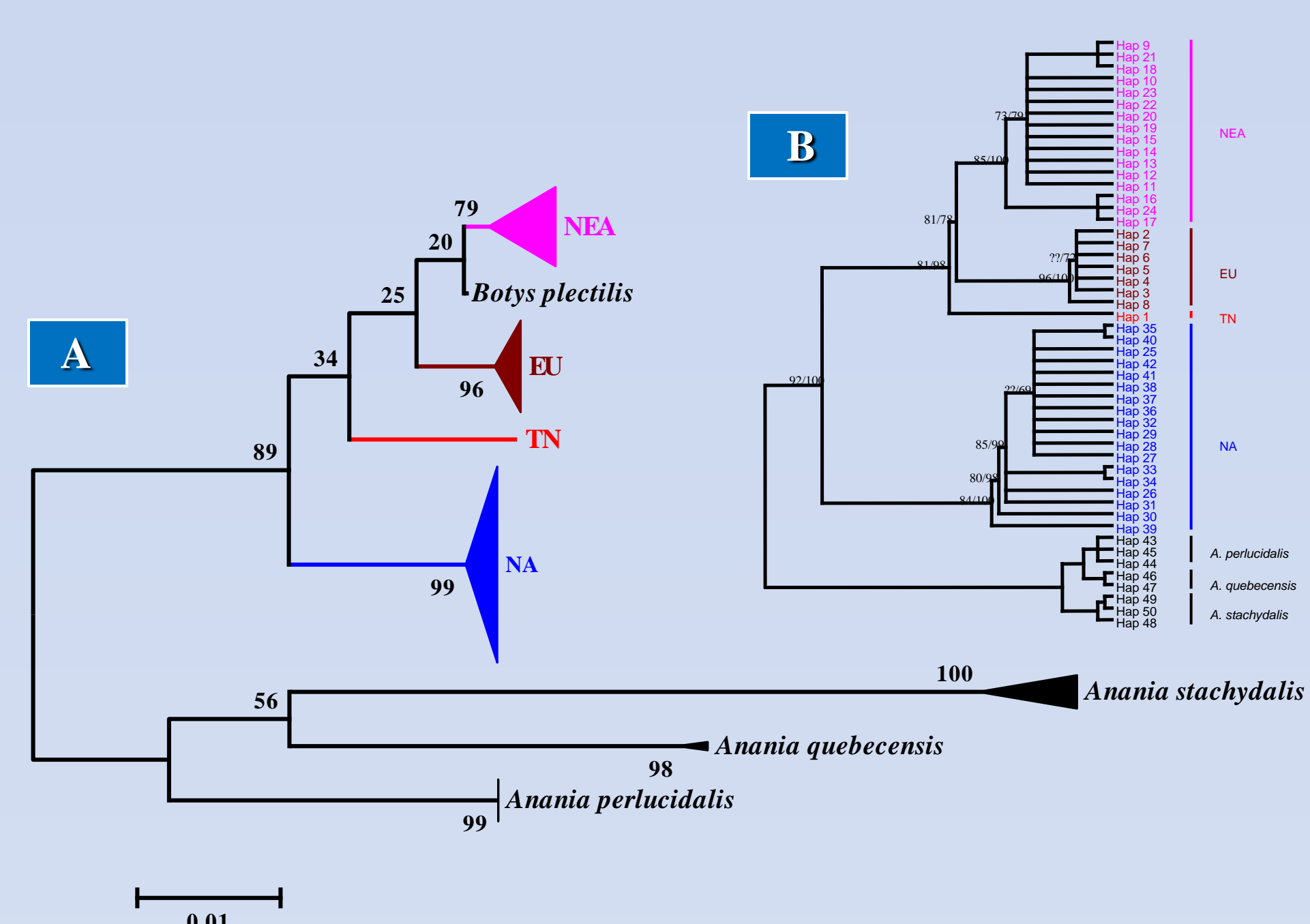


Fig. 3. A, Neighbor-joining tree (K2P) for COI sequences including *A. coronata* species complex, rooted with *A. perlucidalis*, *A. quebecensis* and *A. stachydalis* as outgroup. The depth of each branch shows divergence within lineages; B, Majority rule consensus trees based on Bayesian (MB) phylogenetic analyses of COI of 50 haplotypes for this study. The node support: bootstrap ML/Bayesian posterior probabilities. Single values on the MB tree correspond to Bayesian posterior probabilities. Question marks representing the bootstrap values less than 50.

### Phylogenetic analysis of COI gene

Of 109 barcodes sequenced for COI, we detected 50 distinct haplotypes, comprising 42 haplotypes from four lineages of ingroup *A. coronata* and 8 haplotypes from outgroup species represented by *A. perlucidalis*, *A. quebecensis*, *A. stachydalis*. The best-fit model of nucleotide substitution selected by jMODELTEST 0.1.1 was TPM2uf+I with a relative AIC weight

of 0.1747. ML and BI analyses recovered the same typology, and all haplotypes were assigned to the same main clades. In ML and BI analyses, the main clades were well-supported reciprocal monophyletic groups with high bootstrap support and posterior probabilities (**Figure 3, B**).

### Morphological characters and morphometric analysis

Comparative analyses of qualitative morphological characters show four distinct groups that are congruent with the four genetic lineages of the *coronata* group. The results show that the first two components of PCA together explain 77.92 % and 64.14 % of the total variance for the female and male respectively. This analysis distinguishes almost completely lineages NEA, TN, NA and EU from each other (**Figure 4 A, B**). In DFA, the most significant diagnostic variables for adult males were LUNC (F = 10.9, P < 0.004), LSAC (F = 6.4, P < 0.022). For female, there were no significant diagnostic variables after DFA, but combining those variables enables to distinguish specimens from the four separate groups. Moreover, the first roots of canonical analysis yielded similar results, separating the lineages in four groups (**Figure 4 C, D**).
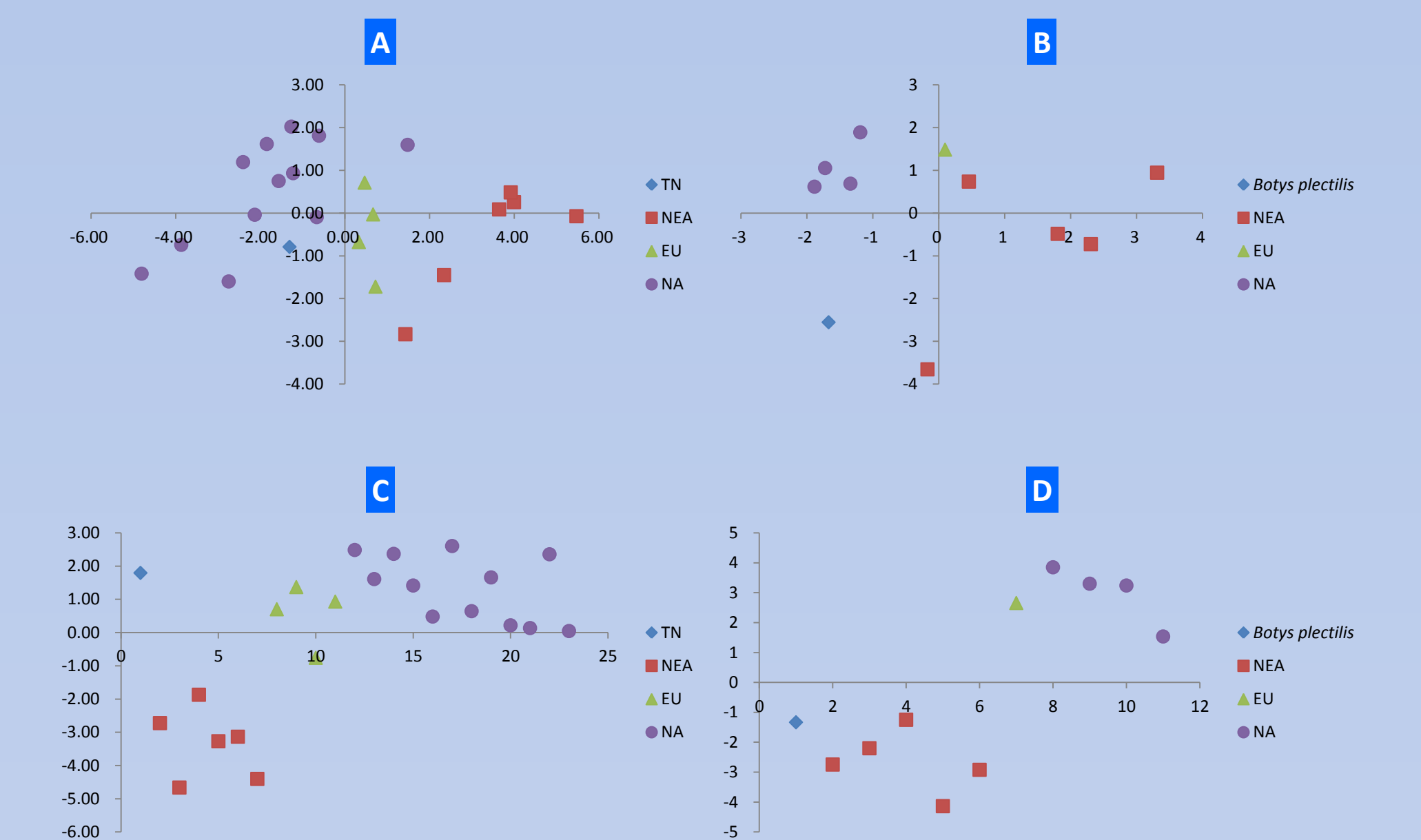


Fig. 4 Principal component analysis (PCA) scatter plot comparing variation of the first two principal components for all morphological characters analyzed (A, males; B, females) and scatter plot of the canonical measures calculated after the discriminant function analysis (DFA) for the morphometric data along the frist roots (C, males; D, females). Four female genital variables (DASA, WSIG, LAAP, DBUR) and six male genital variables (LSAC, LUNC, PEDI, WUNC, WVAL, LVAL) have their highest loadings on the first component (P>0.75).

## CONCLUSION

Both molecular and morphological evidence establish that '*Anania coronata*' is actually a complex of four species. *Anania coronata* is restricted to Europe, while three species are present in North America - *Anania tertialis* (Guenée, 1854) comb. nov., *Anania plectilis* (Grote & Robinson, 1867) comb. nov. and *Anania tennesseensis* sp. n..

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Adults of '*Anania coronata*' species complexes.
A, *Anania tennesseensis* sp. n.;
B, *Anania coronata*;
C, holotype of '*Ebulea tertialis*' in USNM;
D, *Anania tertialis* com. nov.;
E, holotype of '*Botys plectilis*' in AMNH;
F, *Anania plectilis* com. nov.

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