Phylogenetic relationships of the tribe Toxotrypanini (Diptera: Tephritidae) based on molecular characters

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A R T I C L E   I N F O

Article history:
Received 12 January 2017
Revised 3 May 2017
Accepted 11 May 2017
Available online 21 May 2017

Keywords:
Anastrepha
Toxotrypana
Systematics
Molecular phylogeny
Monophyly
Species group

A B S T R A C T

Current hypotheses of relationship among the species of the fruit fly genera Anastrepha and Toxotrypana are tested using sequence data from six DNA regions: the mitochondrial regions 16S, CAD, and COI, and the nuclear regions EF1a, PER, and PGD. DNA sequences were obtained from 146 species of Anastrepha, representing 19 of the 21 species groups as well as five of the six clades of the robusta group, and four species of Toxotrypana in addition to species of Hexachaeta, Pseudophorella, Alujamyia, and 13 other tephritid genera used as outgroups. The results indicate that Hexachaeta is more closely related to the Molynocella group than to Toxotrypana and Anastrepha, and it is removed from the tribe Toxotrypanini. The group Anastrepha + Toxotrypana and the genus Toxotrypana are strongly supported as monophyletic, consistent with previous studies, but Toxotrypana arises within Anastrepha, confirming that Anastrepha as currently defined is paraphyletic. The placement of Toxotrypana within Anastrepha is clearly defined for the first time with high support, as the sister group to the cryptostrephina clade of the robusta group of Anastrepha. Within Anastrepha, the daciiformis, dentata, leptozona, raveni, and striata species groups are highly supported clades. The serpentina group is recognized with lower support, and the fraterculus and pseudoparallela groups are supported with minor alterations. The robusta group is resolved as polyphyletic, but four of the six species clades within it are recovered monophyletic (one clade is not represented and another is represented by one species). The punctata and pananensis groups are resolved together in a clade. At least some species of the mucronata group are related, however this group requires further study. The benjimini, grandis, and spatulata groups appear to be polyphyletic. Relationships among the species groups are generally poorly resolved, with the following exceptions: (1) the lineage including Toxotrypana, the cryptostrephina clade, and the tripancata group; (2) the sister group relationship of the daciiformis + dentata groups; (3) a clade comprising the punctata and pananensis groups; and (4) the large clade comprising the pseudoparallela + spatulata + ramosa + grandis + serpentina + striata + fraterculus groups.

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1. Introduction

The true fruit flies (Tephritidae) comprise one of the most diverse families of the Order Diptera, with more than 4900 described species (Norrbom, unpubl. data). This family includes
major pests of many fruit and vegetable crops and is the most agriculturally important family of flies (White and Elson-Harris, 1992; Norrbom, 2010).

With more than 300 species, Anastrepha Schiner and Toxotrypana Gerstaecker form the largest clade of fruit flies in the New World (Norrbom et al., 1999a, 1999b; 2015; Norrbom, 2004a; Norrbom and Korytkowski, 2009, 2011, 2012). Anastrepha includes nearly 300 described species and Toxotrypana includes seven, but new species are currently being described (Norrbom et al., 2012, 2014, 2015) and numerous additional undescribed species of both genera are known (Norrbom, unpublished data). These genera also include the most important pest fruit fly species in the Neotropics, such as the Mexican fruit fly (Anastrepha ludens (Loew)), the West Indian fruit fly (Anastrepha obliqua (Macquart)), the South American fruit fly (Anastrepha fraterculus complex), and the papaya fruit fly (Toxotrypana curvicauda Gerstaecker), impacting major commercial and subsistence crops such as mango, citrus, guava, papaya and many others (Norrbom, 2004b).

Despite their importance to agriculture and the great diversity of these flies, the phylogenetic relationships within the Anastrepha/Toxotrypana clade are poorly understood. Together these two genera form a well-defined monophyletic group supported by both morphological (Norrbom et al., 1999b) and molecular studies (Han and McPheron, 1997; McPheron et al., 1999; Segura et al., 2006; Han and Ro, 2009). Based on previous studies, Toxotrypana also is clearly monophyletic, but Anastrepha may be paraphyletic; the exact relationships among Toxotrypana and the various species groups of Anastrepha are uncertain (McPheron et al., 1999b; Norrbom et al., 1999b; Barr et al., 2005). Both genera are classified in the tribes Toxotrypanini (subfamily Trypetinae), which also includes Hexachaeta Loew (Norrbom et al., 1999b).

Anastrepha has been divided into various species groups based on morphological characters (Norrbom and Kim, 1988; Norrbom et al., 1999b, 2012; Norrbom and Korytkowski, 2009). Norrbom et al. (2012) recognized 21 species groups, one of which (the robusta group) included six clades (Norrbom and Korytkowski et al. (2012) recognized 21 species groups, one of which (the robusta group) included six clades (Norrbom and Korytkowski et al., 2012) and Norrbom et al. (2015) recognized the mitochondrial protein-coding gene cytochrome oxidase subunit I (COI), and Segura et al. (2006), using mitochondrial DNA encompassing the cyt b, tRNASer and ND1 genes, studied six species of Anastrepha and Toxotrypana. Ruiz et al. (2007a, 2007b) surveyed the relationships among 12 species based on sequences of the sex-determination nuclear genes doublesex (dsx) and transformer (tra). And in a study focused on A. obliqua (Macquart), Scally et al. (2016) analyzed relationships among ten species, mostly in the fraterculus group, based on seven nuclear and two mitochondrial loci. Although these preliminary investigations are important contributions, their limited dimensions in terms of taxa and characters and low support values for most clades provide few competing hypotheses of relationship.

The relationships of Hexachaeta, the third genus in the Toxotrypanini, and those of the Toxotrypanini with other tribes of Trypetinae are poorly understood. Hexachaeta includes 28 described and at least 8 undescribed species ranging from southern Texas (USA) to northern Argentina and the Greater Antilles (Jamaica) (Hernández-Ortiz, 2006). Hernández-Ortiz (2006) analyzed the phylogenetic relationships within Hexachaeta based on morphological characters. He divided it into two subgenera, each with two species groups: Hexachaeta s. str., including the colombiana and eximia species groups; and Costamyia Hernández-Ortiz, including the amabilis and socialis species groups. Hancock (1994) followed Foote (1980) and suggested to keep this genus in the tribe Trypetini. Korneyev (1994) placed Hexachaeta in the monotypic tribe Hexachaetini of the subfamily Xarmutinae, which is not currently recognized. Korneyev (1999) later included the Australasian genera Alincocalistomyia Hardy and Callistomyia Bezzi in the Hexachaetini within the subfamily Trypetinae. Based on analysis of 16S sequences, Han and McPheron (1997) hypothesized Hexachaeta as the possible sister group of Anastrepha + Toxotrypana, and on this basis Norrbom et al. (1999a, 1999b) included Hexachaeta in the Toxotrypanini. Norrbom (2004) performed a morphological phylogenetic analysis of the Molyncocelia group, which includes the genera Alujamyia Norrbom, Molyncocelia Giglio-Tos, and Pseudophorella Lima. In his work, Norrbom (2006) suggested that those genera may be closely related to those previously included in the Hexachaetini and Toxotrypanini and/or the Adramini.

The aim of the present work is threefold: first, to infer the phylogenetic relationships of the Toxotrypanini, including Anastrepha, Toxotrypana and Hexachaeta, using molecular characters; second, to test the monophyly of Anastrepha or its paraphyly with respect to Toxotrypana; and last, to test the monophyly of and to infer the relationships among the species groups and subclades of Anastrepha. To accomplish these objectives, six DNA regions were sequenced and analyzed: part of the mitochondrial 16S rRNA gene, the 5′-region of the carbamoylphosphate synthase (CPS) domain of the nuclear rudimentary gene (CAD) (aminoacids 54–405); the entire mitochondrial protein-coding gene cytochrome c oxidase subunit I (COI); part of the nuclear protein-coding gene elongation factor 1-alpha (EF1a); part of the nuclear protein-coding gene period (PER); and part of the nuclear protein-coding gene 6-phosphogluconate dehydrogenase or pgd (PGD).

2. Material and methods
2.1. Taxonomic sampling
A full list of included taxa is provided in Table 1. Voucherized specimens are identified with unique specimen identifier numbers following lab and/or institutional convention. For instance, USNM specimens have USNMENT codes. Some specimens have multiple identifiers or laboratory codes because the voucher specimen is deposited in one institution, but DNA was extracted at another laboratory. Codes starting with 0 and a letter in third position pertain to specimens whose DNA extractions were done at the California Department of Food and Agriculture (CDFA); Codes starting with PHK or with 6 digit numbers starting with 0 and a letter in third position pertain to specimens whose DNA extractions were done at the California Department of Food and Agriculture (CDFA); Codes starting with PSU originated in the McPheron laboratory at Pennsylvania State University; additional DNA regions were sequenced in the APHIS laboratory (Edinburgh) from extractions of these specimens. Codes starting with a V or TBI pertain to the APHIS lab. To avoid confusion but to maintain traceability of the specimens, unique identifier codes for individual...
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<th>Identifier/Depository collection</th>
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<th>PCG</th>
<th>EF-1a</th>
<th>PERIOD</th>
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<td>KY428226</td>
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<td>KY428106</td>
<td>USA: Florida, Miami-Dade Co., Kendall, USDA Subtropical Agricultural Research Center, 25°38'35.9&quot;N 80°17'38.9&quot;W, 19. May.2004, P. H. Kerr; male; det. A.L. Norrbom</td>
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<td>AF152051</td>
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<td></td>
<td></td>
<td>KY428121</td>
<td>COSTA RICA: Guanacaste, near Liberia, 12. Mar.2003, Sandad Vegetal, McPhail trap; female; det. A. L. Norrbom</td>
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| Anastrepha anna Norrbom                    | previously fraterculus, here as unknown | USNMENT00744776//USNM | KY428390           | KY428390           | KY428088 |     |       |       | KY428497 | PERU: Cusco, Estación Biológica Villa Carmen, trail 4, next to airplane, 12.89341°S 71.40557°W, 543 m, emerged 23–25.Feb.2013 reared from fruit of

Table 1: Taxon sampling used in the molecular analysis listed in alphabetic order, including GenBank accession numbers. All GenBank accession numbers starting with KY42 denote new sequences used for the first time in the present study. Composite taxa are indicated in bold.


Peru: Tingo Maria area, [no date], G. Egoavil, M003; female; det. A. L. Norrbom.


Puerto Rico: Mayagüez: S of Maricao, Road 120, km. 14.6, 18.17°N 66.975°W; McPhail trap 120-09 in mango, 29 March 2007, I. M. Perez & E. O. Rivera; male; det. A. L. Norrbom.

Puerto Rico: Rd. 120, km. 18, trap 120-10, 6–20 March 2006, Ramirez & Perez; ALN-2006-58; male; det. A. L. Norrbom.


Guatemala: Taxisco, 1994. (Barr et al., 2005)


Brazil: Minas Gerais, Arceburgo, 1991. (Barr et al., 2005)

Panama: Panamá, Parque Nacional Chagres, Cerro Jefe, 9°12'38.8"N 79°22'37.3"W, 814 m., trap II 9UA2, 13 Nov. 2009, E. J. Rodriguez; male; det. C. A. Korytkowski.

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<th>Taxon</th>
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<th>Identifier/Depository collection</th>
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<th>COI 3' (2195–3014)</th>
<th>16 S</th>
<th>PGD</th>
<th>EF-1a</th>
<th>PERIOD</th>
<th>CAD (54–410)</th>
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<tr>
<td>Anastrepha bezzii Lima</td>
<td>mucronota</td>
<td>USNM00745006/USNM</td>
<td>KY428254</td>
<td>KY428254</td>
<td>KY427975</td>
<td>KY428600</td>
<td>KY428426</td>
<td>KY428519</td>
<td>KY428125</td>
<td>PANAMA: Parque Nacional Chagres, Cerro Azul, Escuela, 7'9&quot;8.3&quot;N 79°23'29.8&quot;W, Escuela, 323 m, trap III 3UM 1, 8-Apr.2010, E. J. Rodríguez; female; det. E. J. Rodríguez</td>
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<td>PSU-971210-02/-</td>
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<td>BRAZIL: São Paulo, Louveira, from lab colony of J. S. Morgante and A. Malavasi, Dept. de Biol., USP, São Paulo, 1990, G. J. Steck. (McPheron et al., 1999; Barr et al., 2005)</td>
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<td>KY428571</td>
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<td>PERU: Cusco, Echarate, Urusayhxa, May.2012, T. Guevara; holotype female; det. A. L. Norrbom</td>
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<td>KY428520</td>
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Table 1 (continued)
Anastrepha cocorae hastata
Norrbom & Korytkowski

hastata
USNMENT00671689/USNM
KY428258 KY428258 KY427979 KY428603 KY428430 KY428522


Anastrepha cocorae Norrbom & Korytkowski

hastata
USNMENT00745012/USNM
KY428259 KY428259 KY427980 KY428604 KY428431 KY428523 KY428127


Anastrepha compressa Stone

fraterculus
USNMENT00745041/USNM
KY428260 KY428260 KY427981 KY428605 KY428432 KY428524 KY428128

PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3"N 79°21'54.2"W, 747 m, trap I 9 BM1, 22. Oct.2009, E. J. Rodríguez; female; det. C. A. Korytkowski

Anastrepha concava Greene

previously robusta, here as unknown
USNMENT00212059 (08A300)/USNM
KY428076


Anastrepha congesta Aldrich

robusta, cryptostrepha clade
V080807-9 (06X653)/–
KY428334 KY428334 AF152060
AY532495

MEXICO: Veracruz, Los Tuxtlas, ex Tabernaemontana alba, 1994, M. Aluja et al. (McPheron et al., 1999; Barr et al., 2005)

Anastrepha consobrina (Loew)
pseudoparallela
USNMENT00875731 (05V053)/–
KY428043


Anastrepha cordata Carrejo & González

fraterculus
USNMENT00875648 (07Y424)/–
KY428261

VENEZUELA: Palmichal, 1.May.1993, K. Katiyar (McPheron et al., 1999; Barr et al., 2005)

Anastrepha coronilli Carrejo & González

fraterculus
USNMENT00875648 (07Y424)/–
KY428261

VENEZUELA: Palmichal, 1.May.1993, K. Katiyar (McPheron et al., 1999; Barr et al., 2005)

Anastrepha crebra Stone

mucronata
05CA008/–
KY428335 KY428335


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Anastrepha cryptostepha Hendel

robusta, cryptostrepha clade
USNMENT00744886/USNM
KY428448 KY428634


Anastrepha cryptostrephoides Norrbom & Korytkowski

robusta, cryptostrepha clade
USNMENT00744414/USNM
KY428262 KY428262 KY427982 KY428433 KY428525 KY428130


Anastrepha curitis pseudoparallela, Norrbom & Korytkowski

robusta, cryptostrepha clade
USNMENT00744414/USNM
KY428262 KY428262 KY427982 KY428433 KY428525 KY428130

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<th>PGD</th>
<th>EF-1a</th>
<th>PERIOD</th>
<th>CAO (54–410)</th>
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Anastrepha fraterculus (Wiedemann), Brazil-1 morph

Anastrepha fraterculus (Wiedemann), Peruvian morph
fraterculus USNMENT00745234/USNM KY428270 KY428270 KY427989 KY428608 KY428439 KY428531 KY428135 PERU: Huanuco, Huallaga Central, Tingo María, Naranjillo, trap McPhail, 01.02.01.01.0, [no date]; female; det. A. L. Norrbom

Anastrepha furcata Lima robusta, robusta clade

Anastrepha furcata Lima robusta, robusta clade

Anastrepha fuscata Lima robusta, robusta clade


Anastrepha grandicula Norrbom grandis USNMENT00671776/SENASA KY428275 KY428275 PERU: Junín, Chanchamayo, Pichanaki, Perené, 2009, G. Gonzalez I.; male; det. A. L. Norrbom


Anastrepha hadracantha Norrbom & Korytkowski mucronata, megaranthis clade


Anastrepha haplacantha Norrbom & Korytkowski mucronata, megaranthis clade

Anastrepha hyperacantha Norrbom & Korytkowski mucronata, megaranthis clade

Anastrepha integra (Loew) mucronata USNMENT00744578/USNM KY428343 KY428343 KY428049
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<td>KY428139</td>
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Anastrepha acresia  
(Walker) **serpentina**  05CA015/CSCA  KY428349 KY428349 KY428053 KY428173

Anastrepha pallens  
Coquillet **daciformis**  USNM00875768 (2004PHK037; V080807-21)/USNM  KY428350 KY428350 KY428054 KY428174

Anastrepha pallida  
Norrbom **psuedoparallela, pallidipennis complex**  USNM00212767 (06X809)/USNM  KY428351 KY428351 KY428055

Anastrepha pallida  
Greene **pseudoparallela, pallidipennis complex**  PSU-960919-03/– KY428352 AF152079 KY428521

Anastrepha panamensis  
Greene **panamensis**  USNM00745039/USNM  KY428303 KY428303 KY428019 KY428621 KY428460 KY428553 KY428154

Anastrepha paradentata  
Norrbom & Korytkowski **dentata**  USNM00213728 (05V848)/USNM  KY428401 KY428401 KY428098 KY428213

Anastrepha partita  
Norrbom & Korytkowski **robusta, nigra clade**  USNM00744911/USNM  KY428385 KY428385 KY428492 KY428197

Anastrepha passiflorae  
Greene **psuedoparallela**  USNM00212673 (06X810)/USNM  KY428353 KY428353 KY428056 KY428217

Anastrepha pastranai  
Blanchard **pseudoparallela**  USNM00875767 (05V739)/USNM  KY428354 KY428354 KY428057 KY428177

Anastrepha pickeli  
Lima **spatulata**  USNM00744133/USNM  KY428306 KY428306 KY428020 KY428461 KY428554 KY428156

Anastrepha psidivora  
Norrbom **unknown**  USNM00744569/USNM  KY428361 KY428361 KY428062 KY428477 KY428570 KY428179

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<th>Taxon</th>
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<th>CAD (54–410)</th>
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Anastrepha serpentina (Wiedemann)  

**serpentina**  

**USNM**00745219/USNM  

KY428311 KY428311 KY428025 KY428465  

L. Norrbom  

PERU: Huánuco, Huallaga central, Tingo María, Naranjillo, 01.02.01.010, N. Nolazco; female; det. A. L. Norrbom  

(AY532525)  

VENEZUELA: Aragua, Maracay, ex. Maniliuru zaputo, 1988, G. J. Steck. (Barr et al., 2005)

---

Anastrepha shannoni Stone  

**grandis**  

**USNM**00744020/USNM  

KY428312 KY428312 KY428026 KY428466  


---

Anastrepha similis Greene  

**mucronota**  

**USNM**00671979/USNM  

KY428313 KY428313 KY428027  


---

Anastrepha sororcula Zucchi  

**fraterculus**  

**USNM**00671589/USNM  

KY428314 KY428314 KY428028 KY428467  

BOLIVIA: Cochabamba, Cono Sur, Mizque, 17°56’38.5”S 65°21’49”W, 2044 m, McPhail trap 09 in guayaba, 29.Feb.2008, Rojas; female; det. A. L. Norrbom  

---

Anastrepha sp.1  

[dentata group]  

**dentata**  

**USNM**00745051/USNM  

KY428296 KY428296 KY428012  

PANAMA: Panamá, Parque Nacional Chagres, Cerro Jefe, 9°12’38.8”N 79’22’37.3”W, 814 m, trap II 19 UP2, 23. Apr.2010, E. J. Rodriguez; female; det. E. J. Rodriguez  

---

Anastrepha sp.2  

[dentata group]  

**dentata**  

**USNM**00745042/USNM  

KY428307 KY428307 KY428622 KY428462  


---

Anastrepha sp.3  

[dentata group]  

**dentata**  

**USNM**00745053/USNM  

KY428337 KY428337 KY428045 KY428474  


---

Anastrepha sp.4  

**mucronota**  

**USNM**00745052/USNM  

KY428255 KY428255 KY427976 KY428427  


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Anastrepha sp. [BOL-02]  

**unknown**  

**USNM**00744992/USNM  

KY428374 KY428374 KY428074 KY428481  


---

Anastrepha sp. [nr. limae Stone & anduzei Stone]  

**pseudoparallela**  

**USNM**00212023/USNM  

KY428283 KY428283 KY428001 KY428446 KY428537 KY428140  


---

Anastrepha sp. [nr. margarita Caraballo]  

**panamensis**  

**USNM**00744115/USNM  

KY428299 KY428299 KY428015 KY428456 KY428549 KY428150  


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<th>PERIOD</th>
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<td>BOLIVIA: Cochabamba, Chimoro, 16°51’14”S 65°6’1”W, 196 m, McPhail trap 10 in banano, 11:14 Apr.2008, Salinas; female; det. A. L. Norrbom</td>
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<td>PERU: Madre de Dios, Estación Biológica Villa Carmen, trail 13, at trail fork below junction with trail 1, 12.8701’S 71.4261’W, 661 m, trap VC-ML-34, 23 Feb.–1 Mar.2013, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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Table 1 (continued)
Anastrepha striata Schiner

- **Anastrepha striata**
  - Striated fruit fly
  - Known from various localities in Central and South America, including Panama, Peru, and Brazil.
  - Known to feed on a wide range of fruits, including *Psidium guajava*.
  - Specimens have been reared from *Psidium guajava* in Cusco, Peru.

Anastrepha subramosa Stone

- **Anastrepha subramosa**
  - Known for its role in seed dispersal.
  - Found in Panama and Peru.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha superflua Stone

- **Anastrepha superflua**
  - Has been recorded in Panama, Peru, and Brazil.
  - Known for its role in seed dispersal.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha suspensa (Loew)

- **Anastrepha suspensa**
  - Found in various localities in Central and South America.
  - Known for its role in seed dispersal.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha sylvicola Knab

- **Anastrepha sylvicola**
  - Known from Panama and Peru.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha tecta Zucchi

- **Anastrepha tecta**
  - Found in various localities in Central and South America.
  - Known for its role in seed dispersal.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha tehuacana Norrbom

- **Anastrepha tehuacana**
  - Known from Mexico.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha tumida Stone

- **Anastrepha tumida**
  - Known for its role in seed dispersal.
  - Found in various localities in Central and South America.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha woodleyi Norrbom & Korytkowski

- **Anastrepha woodleyi**
  - Known for its role in seed dispersal.
  - Found in various localities in Central and South America.
  - Feeds on a variety of fruits, including *Psidium guajava*.

Anastrepha zenildae Zucchi

- **Anastrepha zenildae**
  - Found in various localities in Central and South America.
  - Known for its role in seed dispersal.
  - Feeds on a variety of fruits, including *Psidium guajava*.
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<td>KY428165</td>
<td>Carmen, garden area, 12°53'42&quot;S 71°24'10&quot;W, 534 m, trap VC-ML-21 in guava tree on canal, 6–11 Jan.2013, T. Förster; female; det. A. L. Norrbom (identification tentative)</td>
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<td>An. zeteki paraensis USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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<td>KY428589</td>
<td>KY428409</td>
<td>KY428506</td>
<td>KY428102</td>
<td>An. zeteki paraensis USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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<td><em>Ceratitis capitata</em></td>
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<td>KY428231</td>
<td>KY427954</td>
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<td>KY428107</td>
<td>Ceratitis capitata (Panama) USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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<td><em>Campiglossa genalis</em></td>
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<td>KY427951</td>
<td></td>
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<td>KY428108</td>
<td>Campiglossa genalis (Panama) USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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<td>KY428107</td>
<td>Dioxyina picciola (Panama) USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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<td><em>Hexachaeta eximia</em></td>
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<td>KY427962</td>
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<td>Hexachaeta amabilis (Panama) USNMENT00745014/USNM KY428322 KY428322 KY428036 KY428629 KY428472 KY428564 KY428165 PANAMA: Panamá, Parque Nacional Chagres, Doña Julia, 9°13'42.3&quot;N 79°21'54.2&quot;W, 747 m, trap II UL2, 08. Apr.2010, E. J. Rodriguez; female; det. A. L. Norrbom</td>
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**Table 1 (continued)**
**Hexachaeta fallax** Lima *socialis* PSU-2-8-95-16/– KY428224 AF152089 AY532534 male; det. by A. L. Norrbom

**Hexachaeta seabrai** Lima *eximia* USNMENT00213020 (05V985)/USNM KY428223 KY428223 KY427948

**Hexachaeta sp.1** [amabilis group] *amabilis* USNMENT00671715/USNM KY428238 KY428238 KY427961 KY428416

**Hexachaeta sp.2** [amabilis group] *amabilis* USNMENT00213511/USNM KY428240 KY428240 KY427963

**Hexachaeta sp.** [socialis group] *socialis* USNMENT00671659/USNM KY428233 KY428233 KY427956 KY428413 KY428509 KY428112 USA: New Jersey, Cape May Co., Town Bank, 38°59′28.5″N 74°57′24.1″W, emerged 23 Aug 2009 reared ex fruit of *Ilex* sp. collected 24 Jul 2009, A. L. Norrbom; male; det. A. L. Norrbom


**Toxotrypana australis** Blanchard AY532484 ARGENTINA: Tucumán, Burruyacu, Taruca Pampa, Finca San Augustine, McPhail trap in citrus orchard, 14 May. 1992

**Toxotrypana curvicauda** AY532484 ARGENTINA: Tucumán, Burruyacu, Taruca Pampa, Finca San Augustine, McPhail trap in citrus orchard, 14 May. 1992

**Tachinisca cyaneiventris** Kertész 2004PHK223/CSCA KY428221 KY428221 KY427946

**Toxotrypana australis** Blanchard

**Toxotrypana curvicauda**
<table>
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<tr>
<th>Taxon</th>
<th>Species group</th>
<th>Identifier/Depository collection</th>
<th>COI 5' (1490–2198)</th>
<th>COI 3' (2195–3014)</th>
<th>16 S</th>
<th>PGD</th>
<th>EF-1a</th>
<th>PERIOD</th>
<th>CAD (54–410)</th>
<th>Specimen data</th>
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<tbody>
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<td>KY427967</td>
<td>KY428595</td>
<td>KY428420</td>
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<td>KY428118</td>
<td>PERU: Huánuco, Tingo Maria, Pozo Azul, 9°11’1”S 75°57’58”W, 721 m, SENASA trap, 1.2.21.11, 21 Apr. 2010, D. Damas; female; det. A. L. Norrbom</td>
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<td>KY428097</td>
<td>KY428650</td>
<td>KY428504</td>
<td></td>
<td>KY428211</td>
<td>BOLIVIA: Cochabamba, Aiguile, 18°19’34”S 65°13’43”W, 04-06-03-06, 2080 m, McPhail trap, 22 Jan. 2009; female; det. A. L. Norrbom</td>
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<tr>
<td>Xanthaciura insecta (Loew)</td>
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<td>KY428104</td>
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<td>USA: Florida, Miami-Dade Co., Kendall, USDA Subtropical Agricultural Research Center, 25°38’35.9”N 80°17’38.9”W, 19. May 2004, P. H. Kerr; male; det. P. H. Kerr</td>
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<tr>
<td>Zonosemata electa (Say)</td>
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<td>USNMENT00671936/USNM</td>
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<td>KY428234</td>
<td>KY427957</td>
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<td>USA: South Carolina, Cherokee, 35°12’58”N 81°7’53”W, farm, 24. May 2010, D. Piner; female; det. A. L. Norrbom</td>
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</tbody>
</table>
specimens are listed in the third column of Table 1 together with the depository institution, and other related codes and numbers are listed in the ‘Specimen data’ column. For example, sample codes starting with ALN are in this column. Voucher specimens are deposited in the following institutions as listed in Table 1:

APHIS – Center for Plant Health Science and Technology Mission Laboratory, USDA-APHIS, Edinburg, USA
BARC – Beltsville Agricultural Research Center, United States Department of Agriculture, Beltsville, USA
CSCA – California State Collection of Arthropods, California Department of Food and Agriculture, Sacramento, USA
MEUP – Museo de Entomología, Universidad de Panama, Panama
MHNJP – Museo de Historia Natural «Javier Prado», Universidad Nacional Mayor de San Marcos, Lima, Peru
SENASA – Servicio Nacional de Sanidad Agraria, La Molina, Lima, Peru
USNM – National Museum of Natural History, Smithsonian Institution, Washington D.C., USA

The examined material was collected from a variety of sources in the New World. Specimen data and species group designation for each species are indicated in Table 1, as well as the GenBank® (National Center for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD, 20894 USA) accession numbers for all the sequences.

A total of 146 Anastrepha species, representing 19 of the 21 species groups as well as five of the six clades of the robusta group, were included in the present analysis; this included 17 species without an assigned group or clade. Only the

“touchdown” program: initial denaturation for 2 min at 92 °C, 12 touchdown cycles from 58 °C to 46 °C (10 s at 92 °C, 10 s at 58–46 °C, 1.5 min at 72 °C), 27 cycles at 10 s at 92 °C, 10 s at 45 °C, 1.5 min at 72 °C, and a final extension for 7 min at 72 °C. Primers for PCR and DNA sequencing are listed in Table 2. Four new primers were designed for Anastrepha species to amplify the COI gene, as well as one primer for PGD; all of them listed in Table 2 with the prefix AnCo followed by a number corresponding to the 3–most base of the primer compared with the genome of Drosophila yakuba Burla (Clary and Wolstenholme, 1985), and followed by F or R for forward or reverse primers, respectively. No introns were amplified for any gene.

PCR products were cleaned for sequencing using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) or gel purification using the QIAquick PCR Purification Kit® (Qiagen, Valencia, CA, USA). Sequencing reactions were carried out with the Big Dye® Terminator version 3.1 Cycle Sequencing kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and fractionated on an ABI PRISM® 3100 Genetic Analyzer or ABI 3730xl DNA Analyzer. Contigs were assembled for each gene region with the software package Geneious version R6 (Biomatters, Auckland, New Zealand). Newly generated sequences (this project) are listed in Table 1 with GenBank accession numbers starting with with KY42.

2.3. Sequence alignment

The protein-coding genes COI, EF1a, and PGD were aligned manually and no gap was needed. The alignment of CAD was straightforward, but gaps were needed as the sequence of Anastrepha panamensis Green is three nucleotides longer than the rest. In the alignment of PER, we found two gaps: the first one is 12 nucleotides long and is due to the longer sequences of this gene for the outgroup species, and a second gap of 12 nucleotides to accommodate the longer sequence of Euphranta lemniscata (Ender}

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ties (PP) were calculated using a 50% majority-rule consensus tree check convergence and acceptable mixing. The initial 1500 trees and Rambaut, 2007; Rambaut and Drummond, 2007) was used to trees every 5000 generations. The program Tracer 1.5 (Drummond 2001; Ronquist and Huelsenbeck, 2003) was performed from 1000 replicates using the same independent models in Garli. The likelihood score for the best maximum likelihood tree was −87576.370208 (Figs. 1–3). The 50% majority-rule consensus tree resulted from MrBayes analysis was consistent and concordant with the ML tree and only the Bayesian posterior probabilities are shown from the Bayesian inference together with the bootstrap support values in Figs. 2 and 3. The present analysis was not designed to study the included outgroups, but the results agree with previous studies (Han and Ro, 2009) supporting a clade with Tephritinae (only members of Tephritini were included) as sister group of Anastrepha + Toxotrypana (other taxa in the clade in Han and Ro, 2009, fig. 3) not included here), although with low support (BS = 37; PP = 0.83). Members of the Dacinae (Ceratitis and Bactrocera) form a clade (BS = 73; PP = 0.90), as do members of the tribes Campomyni (Zonosomata and Rhagoletis; BS = 90; PP = 0.83) and Trypetinae (Strauzia and Parastenopa; BS = 47; PP = 0.98) of the Trypetinae, although some with low bootstrap support. The Trypetinae is resolved as paraphyletic in this analysis, with the Dacinae and Tephritinae arising among taxa currently included in this subfamily.

Phylogenetic analyses

We used two different methods of phylogenetic analysis, Maximum Likelihood (ML) and Bayesian inference (BI). For both analyses, the molecular data set was divided into 16 partitions: first, second and third codon positions of CAD, COI, EF1a, PER and PGD, and 16S gene. We determined the best choice of model for each partition using jModelTest 2.1.5 (Darriba et al., 2012) under the Akaike Information Criterion (AIC), as recommended by Posada and Buckley (2004), and analyzed the data under the recommended models (see Table 3). ML analyses were performed using the Genetic Algorithm for Rapid Likelihood Inference, Garli v2.1 MPI (Zwickl, 2006, 2013), on the Smithsonian Institution High Performance Cluster (SI/HPC), known as Hydra-3. Twenty-four independent runs (24 different runs with the command searchres = 1) were conducted using the score/thresh/for/term = 0.05 and significanttopochange = 0.001 settings and using the automated stopping criterion, terminating the search when the ln score remained constant for 50,000 consecutive generations. Bootstrap support values (BS) were estimated from 1000 replicates using the same independent models in Garli.

Phylogenetic estimation using the Markov Chain Monte Carlo algorithm as implemented in MrBayes 3.2.5 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was performed using a parallelized version of the software. Data were divided into the above 16 partitions and each partition has its own set of parameters. Priors were applied with default values. Six runs, with four chains each (one “cold” chain and three heated chains; temp = 0.5), were performed simultaneously for 30,000,000 generations which were sufficient to bring the convergence (average standard deviation) to a value <0.1 (Ronquist et al., 2005), sampling trees every 5000 generations. The program Tracer 1.5 (Drummond and Rambaut, 2007; Rambaut and Drummond, 2007) was used to check convergence and acceptable mixing. The initial 1500 trees (25%) were discarded as burn-in and Bayesian posterior probabilities (PP) were calculated using a 50% majority-rule consensus tree inferred from the data.

All trees were drawn with the aid of FigTree v.1.3.1 (Rambaut, 2009), Adobe Illustrator® and Adobe Photoshop® CS5.

3. Results

A total of 160 Anastrepha and six Toxotrypana specimens were successfully sequenced, representing 146 Anastrepha and four Toxotrypana species (13 Anastrepha species with two terminals of the same species, and A. obliqua and T. liitoralis with three terminals each). The aligned data matrix had a total 4712 characters with 561 bp for the aligned 16S, 793 bp for CAD, 1430 bp for COI, 977 bp for EF1a, 451 bp for PER, and 500 bp for PGD. Sequences of all molecular markers were not possible to amplify for all studied taxa. Levels of missing data were greater in nuclear than mitochondrial regions (nuclear markers: PGD: 66%; EF1a: 50.8%; PER: 43.5%; CAD: 37.1%; mtDNA: 16S: 6.8%; COI-3: 6.3%; COI-5: 0.5%), but there was no apparent taxonomic bias in the distribution of missing data. We included every species with at least three gene regions in our analysis, as this approach has been shown to improve phylogenetic accuracy if the missing data are distributed evenly and are not concentrated in a few taxa (Wiens, 1998, 2006; Prevosti and Chemisquy, 2009; Jiang et al., 2014). The only exceptions made for this dataset were Anastrepha fuscata Norbom & Korytkowski, A. grandicula Norbom, A. hypercantha Norbom & Korytkowski, and Hexachaeta amabilis (Loew) to two gene regions each.

Table 3

<table>
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<th>Genetic marker</th>
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<td>16S</td>
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<td>1st codon position</td>
<td>2nd codon position</td>
</tr>
<tr>
<td>CAD</td>
<td>TVM + I + G</td>
</tr>
<tr>
<td>COI</td>
<td>GTR + I + G</td>
</tr>
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<td>EF1a</td>
<td>TMM2 + I + G</td>
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<tr>
<td>PER</td>
<td>TriNef + I + G</td>
</tr>
<tr>
<td>PGD</td>
<td>GTR + I + G</td>
</tr>
</tbody>
</table>

Toxotrypana species (13 Anastrepha species with two terminals of the same species, and A. obliqua and T. liitoralis with three terminals each). The aligned data matrix had a total 4712 characters with 561 bp for the aligned 16S, 793 bp for CAD, 1430 bp for COI, 977 bp for EF1a, 451 bp for PER, and 500 bp for PGD. Sequences of all molecular markers were not possible to amplify for all studied taxa. Levels of missing data were greater in nuclear than mitochondrial regions (nuclear markers: PGD: 66%; EF1a: 50.8%; PER: 43.5%; CAD: 37.1%; mtDNA: 16S: 6.8%; COI-3: 6.3%; COI-5: 0.5%), but there was no apparent taxonomic bias in the distribution of missing data. We included every species with at least three gene regions in our analysis, as this approach has been shown to improve phylogenetic accuracy if the missing data are distributed evenly and are not concentrated in a few taxa (Wiens, 1998, 2006; Prevosti and Chemisquy, 2009; Jiang et al., 2014). The only exceptions made for this dataset were Anastrepha fuscata Norbom & Korytkowski, A. grandicula Norbom, A. hypercantha Norbom & Korytkowski, and Hexachaeta amabilis (Loew) to two gene regions each.

The likelihood score for the best maximum likelihood tree was −87576.370208 (Figs. 1–3). The 50% majority-rule consensus tree resulted from MrBayes analysis was consistent and concordant with the ML tree and only the Bayesian posterior probabilities are shown from the Bayesian inference together with the bootstrap support values in Figs. 2 and 3. The present analysis was not designed to study the included outgroups, but the results agree with previous studies (Han and Ro, 2009) supporting a clade with Tephritinae (only members of Tephritini were included) as sister group of Anastrepha + Toxotrypana (other taxa in the clade in Han and Ro, 2009, fig. 3) not included here), although with low support (BS = 37; PP = 0.83). Members of the Dacinae (Ceratitis and Bactrocera) form a clade (BS = 73; PP = 0.90), as do members of the tribes Campomyni (Zonosomata and Rhagoletis; BS = 90; PP = 0.83) and Trypetinae (Strauzia and Parastenopa; BS = 47; PP = 0.98) of the Trypetinae, although some with low bootstrap support. The Trypetinae is resolved as paraphyletic in this analysis, with the Dacinae and Tephritinae arising among taxa currently included in this subfamily.

Surprisingly the genus Hexachaeta is not supported as monophyletic, although bootstrap values are low for the clades in which its species groups are included (Fig. 2). The represented species of Hexachaeta are included with those of Pseudophorellia and Alujamya in a clade with moderate support (BS = 63; PP = 0.98), and not as the sister group of the other Toxotrypanini (Anastrepha + Toxotrypana). The species of the socialis group of the subgenus Costamyia are grouped with the species of the eximia group of Hexachaeta s. str., and these taxa are grouped with the species of Pseudophorellia, whereas the clade forming the sister group of these taxa is comprised of the species of the amabilis group of the subgenus Costamyia grouped with the species of Alujamya. Support values are high for Alujamya and Pseudophorellia (each with BS = 100; PP = 1) as well as for the socialis species group (BS = 97; PP = 1) and amabilis species group (BS = 100; PP = 1), but not for the eximia species group (BS = 45; PP = 0.57).

Regarding the ingroup, Anastrepha + Toxotrypana, the overall evolutionary scenario depicted in our results is a well-supported group with many internal nodes with low or very low support, or with very short branches, although some groups have high support values (Fig. 1). The Anastrepha + Toxotrypana clade is well supported (BS = 100; PP = 0.83), as is Toxotrypana (BS = 100; PP = 1), but the latter is placed within Anastrepha as sister group of the three studied members of the cryptostrepha clade of the robusta species group (BS = 100; PP = 1) (Fig. 2). The cryptostrepha clade + Toxotrypana forms the sister group of the single studied species of the tripunctata species group (BS = 84; PP = 0.67). The sister
Fig. 1. Maximum Likelihood tree (ln L = –87,576.370208) based on the combined dataset using Garli 2.1. Bootstrap support values (left) and Bayesian posterior probabilities (right) are depicted at the nodes (≥50% or 0.5, respectively). Asterisk (*) denotes bootstrap support values of 100% or Bayesian posterior probabilities of 1.0; a dash (−) indicates support values below 50% for bootstrap values or below 0.5 for Bayesian posterior probabilities. *Anastrepha* and *Hexachaeta* species groups are indicated using colors, even for undescribed species. Images [mostly from Norrbom et al., 2012] (right to left, top to bottom): *Hexachaeta amabilis* [credit D. Guzman], *Anastrepha robynae*, *Toxotyrpana curvicauda*, *A. paradentata*, *A. daciformis*, *A. robusta*, *A. magna*, *A. superflua*, *A. cocorae*, *A. lanceola*, *A. zeteki*, *A. ramosa*, *A. grandis*, *A. serpentina*, *A. manihoti*, *A. pseudoparallela*, *A. grandicula*, *A. fraterculus*, and *A. obliqua*. 
Fig. 2. Upper half (arbitrary) of the Maximum Likelihood tree (Fig. 1) based on the combined dataset using Garli 2.1. Bootstrap support values (left) and Bayesian posterior probabilities (right) are depicted at the nodes (>50%). *Anastrepha* and *Hexachaeta* species groups are indicated using colors.
Fig. 3. Lower half (arbitrary) of the Maximum Likelihood tree (Fig. 1) based on the combined dataset using Garli 2.1. Bootstrap support values (left) and Bayesian posterior probabilities (right) are depicted at the nodes (>50%). *Anastrepha* species groups are indicated using colors. The box is an enlargement of the *fraterculus* group so that the relationships and support values are legible.
group relationship between the daciformis and dentata species groups is strongly supported (BS = 97; PP = 1.0). The large clade comprising the pseudoparallelia + spatulata + ramosa + doryphoros + grandis + serpentina + striata + fraterculus groups recognized by Norrbom et al. (1999b) is recovered but without strong statistical support (BS = 73; PP = 0.72) (Fig. 3), although the inclusion of the doryphoros group, which is not represented in this study, could not be tested, and the inclusion of A. bella Norrbom & Korytkowski (previously in robusta group, but here considered unplaced) is unexpected. Relationships among the other species groups are not clearly resolved.

Within Anastrepha, of the 21 species groups recognized by Norrbom et al. (2012), two groups are not represented in this study (caudata and doryphoros groups) and four groups are represented by single species. Of the remaining 15 groups, seven are supported as monophyletic, whereas eight are recovered as non-monophyletic, although several of the latter are nearly monophyletic (except for the placement of single or a few species) (Fig. 1).

The hastata, punctata, schausi and tripunctata species groups were represented by single species in this study, thus their monophyly could not be tested. The two specimens of A. cocorea Norrbom & Korytkowski (hastata group) included in the analysis do cluster together and are placed as the sister group of Anastrepha hamadryas Stone, a species previously of unknown affinity (Fig. 3). The single species of the tripunctata group, A. tehuacana Norrbom, is placed as sister group of the cryptostrepha clade + Toxotrypana, as noted above (Fig. 2). The single included species of the schausi group, A. fuscicauda Norrbom & Korytkowski, is placed in a strongly-supported clade (BS = 99; PP = 1) with A. dryas Stone (pseudoparallelia group) and A. breviapex Norrbom, a species tentatively placed in the mucronata group (Norrbom et al., 2015) (Fig. 2). Another species of the schausi group, A. iutea Stone (= A. bellicauda Norrbom), which was not included in this study, was grouped with A. dryas in the 16S study by McPheron et al. (1999).

The single included species of the punctata group, A. punctata Hendel, is placed within a clade with high support values (BS = 98; PP = 1) otherwise comprised by the species of the panaensis group (Fig. 3).

The daciformis and dentata species groups are each strongly supported as monophyletic (both with BS = 100; PP = 1) (Fig. 2). Regarding the relationships within the daciformis group, the results of this study agree in some aspects with those of the morphological analysis by Norrbom (1998), e.g., the monophyly of the macrocaria complex represented by A. avispa Norrbom, A. bicolor (Stone), and A. macarra Hendel. They do not support the monophyly of the daci- formis complex, however, as A. katiyari Norrbom is grouped with A. pallens Coquillet rather than A. daciformis Hendel. The placement of A. antillensis Norrbom as sister group of the other species group agrees with one of the hypotheses of Norrbom (1998) regarding its relationship (see his Fig. 3).

Other species groups inferred as monophyletic with high support include: the leptozona group (BS = 90; PP = 0.83); the raveni group (BS = 100; PP = 1); and the striata group (BS = 99; PP = 1) (Figs. 2 and 3). The species of the raveni group are grouped with four species of the mucronata group, including the three species of the megacantha clade. They may be derived members of that group. The species of the serpentina group included in this study also form a monophyletic clade, although with relatively low support (BS = 30; PP = 0.95) (Fig. 3). A new species from Bolivia, Anastrepha sp. [BOL-02], is inferred as its sister group with weak support (BS = 70; PP < 0.5). The serpentina and striata groups are not recovered together, thus their combination into one group by Norrbom (2002) is not supported. The striata group is placed as sister group of the fraterculus group, although with low support (BS = 38; PP = 0.84).

One of the largest and most economically important species groups, the fraterculus group, is well represented in this study by 20 species (Fig. 3) and is supported as monophyletic with the exception of A. annonae Norrbom, which was tentatively placed in this group (Norrbom et al., 2015). This species is placed with a group of species of unknown affinity. The other 19 species form a relatively well supported cluster (BS = 75; PP = 0.90).

The mucronata group, the largest species group within Anastrepha, is well represented in this study by 24 species. The monophyly of the mucronata group was not supported and its members were placed in seven lineages, although the higher clades containing those lineages have low support. The majority of the species of the group (15 species) are included in a well-supported clade (BS = 85; PP = 0.97) placed as the sister group of A. flavipennis Greene (Fig. 3). Four species, including the three species representing the megacantha clade, are placed with the raveni group in a clade with low support. Two species (A. galbina Stone + A. aphelocentema Stone) form a well-supported clade (BS = 100; PP = 1) as the sister group of A. intermedia Norrbom & Korytkowski (benjamini group) (Fig. 2), whereas two others (A. simillis Greene + A. atrox (Aldrich)) form a weakly supported clade (BS = 14; PP = 0.84). The final two species, A. robynae Norrbom and A. breviapex Norrbom, which were tentatively included in the mucronata group, but apparently do not belong, are placed with other species. Within the mucronata group, the representatives of the lanceola clade (Norrbom et al., 2015), A. ericki Norrbom, A. lanceola Stone, A. latilanceola Norrbom, A. nr. tumbalai, and A. sp. [Peru 45], form a highly supported cluster (BS = 100; PP = 1) along with A. minuta Stone, a somewhat similar species that previously had not been suggested to belong to the clade (Fig. 3).

The pseudoparallelia group was well represented in this study, with ten previously included species and several more added on the basis of this study. Except for A. dryas Stone, these species are placed in the same clade, although it has low support (BS = 7; PP = 0.67) (Fig. 3). The clade also includes three undescribed species that belong to the group and three species, A. buscki Stone, A. nigripalpis Hendel and A. rosilioid Blanchard, that were not included in the group by Norrbom et al. (2012). Examination of fresh specimens of A. rosilioid and A. nigripalpis, versus the brief original descriptions used as the basis for the Norrbom et al. (2012) classification, indicates that these species also fit within the morphological definition of the group (Norrbom, personal observation), but A. buscki differs considerably from the other species in the form of the aculeus tip, which is nonserrate, strongly tapered, and dorsally angled. Its placement is surprising. The species of the pallidipennis complex (Norrbom, 1997), namely A. curitis Stone, A. pallida Norrbom and A. pallidipennis Greene, form a clade with good support (BS = 59; PP = 0.96).

The robusta species group is not supported as monophyletic in this study, but the subclasses of the group recognized by Norrbom and Korytkowski (2009) that are represented are supported. The cryptostrepha clade, represented by A. cryptostrepha Hendel, A. cryptostrephoides Norrbom & Korytkowski and A. cordata Aldrich, the nigra clade, represented by A. nigra Norrbom & Korytkowski and A. partita Norrbom & Korytkowski, the speciosa clade, represented by A. rafaeli Norrbom & Korytkowski and A. speciosa Stone, the robusta clade, represented by A. fenesetrae Norrbom & Korytkowski, A. sp. nr. pittieri, A. furcata Lima, A. fuscata Norrbom & Korytkowski, A. nigricascia Stone, and A. robusta Greene, are each strongly supported (BS = 100; PP = 1). The cryptostrepha clade forms the sister group of Toxotrypana (Fig. 2). The nigra clade is the sister group of A. robynae, and that clade and the speciosa clade are the first branches within Anastrepha, although with low support (Fig. 2). The robusta clade forms the sister group of the lone representative of the lambda clade, A. nigriivittata Norrbom & Korytkowski (BS = 94; PP = 1). The two species that Norrbom and
Korytkowski (2009) left unplaced, A. bella Norrbom & Korytkowski and A. concava Greene, are placed in separate positions on the tree and do not appear to be closely related to any of the other species (Fig. 3).

Very interesting is the placement of the two studied members of the ramosa group, A. ramosa Stone and A. subramosa Stone, which are placed in a highly supported clade (BS = 99; PP = 1) with two species of the spatulata group, however, each species of the ramosa group is closer to one of the species of the spatulata group (Fig. 3).

The monophyly of the spatulata group is not supported by this analysis, and the members of this species group are placed in four different clades. The species feeding on the plant genus Manihot Mill. (Euphorbiaceae), i.e., A. manihoti Lima, A. montei Lima, and A. pickeli Lima, are clustered together (BS = 70; PP = 0.99). Two of the Olacaceae-feeding species (A. alveata Stone and A. spatulata Stone) are grouped with the species of the ramosa group (Fig. 3). Surprisingly, A. interrupta Stone and A. spatulata, which are difficult to distinguish morphologically and have the same host plant, are not inferred to be sister species.

The benjami and grandis species groups appear to be polyphyletic. The three species of the benjami group and the four species of the grandis group included in this study each arise in separate lineages on the tree (Figs. 2 and 3).

Some Anastrepha species included in this analysis were not classified in a species group by Norrbom et al. (2012, 2015). Of these 15 species, three are placed within the pseudoparallela group on the tree (see discussion of that group), but the rest are dispersed and none is resolved within any other recognized species groups. Most of the groupings in which these species are placed have low support values, thus their inferred relationships should only be considered as hypotheses to be further tested. One exception is the grouping of A. camba Norrbom and A. sylvicola Stone (BS = 90; PP = 1) (Fig. 3).

4. Discussion

4.1. The tribe Toxotrypanini

Han and McPheron (1997) inferred Hexachaeta as the possible sister group of Anastrepha + Toxotrypana, and Norrbom et al. (1999a) subsequently included it in the tribe Toxotrypanini. Conversely, Korneyev (1994) placed Hexachaeta in the tribe Hexachaetini, to which Korneyev (1999) added Callistomyia Bezzi and Alinoccallistomyia Hardy. Korneyev (1999) also considered that the Hexachaetini were possibly related to the Xarnutini (Xarnuta Walker and Platystomopsis Hering) based mainly on nine morphological characters. All of these morphological characters are also present in some species of Alujamyia Norrbom, Mlynocoeoila Giglio-Tos, or Pseudophorella Lima (the three genera of the Mlynocoeila group). But none of these characters seems to be a unique synapomorphy of these three genera, Callistomyia, Alinoccallistomyia, the Aedramini and/or the Toxotrypanini, nor of those taxa plus the Xarnutini, as none of these characters are present in all of the species (Norrbom, 2006).

Norrbom (2006) suggested three hypotheses regarding the relationships of the Mlynocoeila group. The first is that the group is most closely related to the Paleotropical genera Callistomyia and Alinoccallistomyia, the two genera that Korneyev (1999) grouped with Hexachaeta in Hexachaetini. The second hypothesis is that the Mlynocoeila group is more closely related to the Aedramini, based on the usual presence of setulae on the katepimeron, a presumably apomorphic state within the Trypetinae. The third hypothesis is a close relationship between the Mlynocoeila group and the Toxotrypanini.

The results of this study suggest that Hexachaeta is more closely related to at least two genera of the Mlynocoeila group, Pseudophorella and Alujamyia, than to Anastrepha and Toxotrypana (Figs. 1 and 2). On the other hand, the tribe Toxotrypanini sensu Hancock (1986; as the subfamily Toxotrypaninae) is strongly supported as monophyletic in agreement with previous morphological (Norrbom et al., 1999b) and molecular studies (Han and McPheron, 1997; McPheron et al., 1999; Han and Ro, 2009). For this reason we recognize the tribe Toxotrypanini comprising only Toxotrypana and Anastrepha. Further studies and a more complete sampling of molecular markers are needed to infer the placement of Hexachaeta, Alujamyia, Pseudophorella and the Mlynocoeila group. Our results are not conclusive as several genera (Mlynocoeila, Callistomyia and Alinoccallistomyia) were not available for this study and we were unable to sequence some nuclear markers for some of the included species.

4.2. The monophyly of Anastrepha

The results of this study strongly support the monophyly of Anastrepha + Toxotrypana and of the genus Toxotrypana. However, Toxotrypana is resolved within the large radiation of Anastrepha species. Thus, our results agree with previous studies (Norrbom et al. 1999b; McPheron et al. 1999; Barr et al. 2005) that do not support the monophyly of Anastrepha as currently recognized. The results indicate that Toxotrypana and Anastrepha should be considered synonyms. Anastrepha includes more species of economic importance and should be the valid name so as not to destabilize nomenclature for the broad community. However since Toxotrypana is the older name, this nomenclatural action would need to be validated by the ICZN. For this reason, we refrain from making a change here and intend to formalize the synonymy in a separate paper, once our application (in preparation) is approved by the ICZN.

4.3. Relationships among Anastrepha species groups

The results of this analysis agree to a large extent with previous hypotheses and the species group classification based on morphological characters (Norrbom et al., 1999b, 2012, 2015; Norrbom and Korytkowski, 2009). In general, clades that have strong morphological support are also supported here, and those with weak support are not supported by the molecular data. Most of the early branches of the cladogram are weakly supported, thus the relationships among the species groups remain largely uncertain. Exceptions include: the placement of Toxotrypana as sister group of the cryptostrepha clade (BS = 100; PP = 1), with this taxon in turn the sister group of the tripunctata group (or at least A. tehuacana, the single representative of that group included in this study (BS = 84; PP = 0.67)); the sister group relationship between the daciformis and dentata groups (BS = 97; PP = 1); and the large clade comprising the pseudoparallela + spatulata + ramosa + grandis + serpentina + striata + fraterculus groups (BS = 80; PP = 0.69). The relationships of the daciformis and dentata species groups and the monophyly of each of those groups are strongly supported by morphological characters (Norrbom et al. 1999b) and are very highly supported in this study. Study of the missing sequences of the current data set, additional DNA regions and addition of more species are needed to further resolve the relationships among the species groups.

Of the 15 species groups whose monophyly could be tested in this study, seven are resolved as monophyletic, whereas eight are not, although several of the latter would be monophyletic with slight adjustments (e.g., removal of a single species).

The results strongly support the monophyly of the daciformis, dentata, leptozona, raveni, and striata species groups, and with
lower support, monophyly of the serpentina group. The raveni group may comprise derived members of the mucronota group. The hypothesis of Norrbom (2002) that the serpentina and striata groups may be closely related is not supported.

The inferred close relationship of the species of the punctata and panamensis groups is a novel result, although the single represented species of these groups in the 16S study of McPherson et al. (1999) are sister taxa in their cladogram. These groups are morphologically similar, differing by the presence/absence of paired brown markings near the posterior margin of the scutum, thus it is not surprising that molecular data support their close relationship. The position of A. punctata among species of the panamensis groups suggests that these groups might be combined. More species of the punctata group and further studies are needed to resolve this relationship.

The results of this analysis also support the monophyly of each of two large species groups, the fraterculus and pseudoparallela groups, with minor modification. The present study supports the notion that Anastrepha fraterculus (Wiedemann) as currently conceptualized is polyphyletic within the fraterculus species group, consistent with previous studies (Smith-Caldas et al., 2001; Hernández-Ortiz et al., 2012 and references therein; Scally et al., 2016). Our results also confirm that A. barbiellini Lima should not be included in the fraterculus group, in agreement with Smith-Caldas et al. (2001). It was tentatively included in the group by Norrbom et al. (1999b), but not by Norrbom et al. (2012). Also, A. amonae Norrbom probably should be removed from the fraterculus group.

For the pseudoparallela group, additional modifications are recommended. Five species (A. nigripalpis and A. rosiloi, and three undescribed species) which fit the morphological diagnosis of the pseudoparallela group, should be added. Our results also suggest that A. buscki, which differs morphologically, also should be included in that group, but A. dryas should be removed. Species of the pseudoparallela group breed almost exclusively in species of Passiflora L. Discovery of the hosts of A. buscki would be useful to test if it truly belongs in this group. Within the group, our results support the monophyly of the pallidipennis complex.

The mucronota group, as currently conceived, is not supported as monophyletic, although 15 of the 24 species included in this study do form a well-supported clade, and four other species are placed in a weakly supported clade along with species of the raveni group, which suggests that the latter may be derived members of the mucronota group. Five other species are placed in three places on the tree relatively close to the previous two clades in a section of the tree where the branches generally are weakly supported, thus the relationships of the mucronota group remain poorly resolved. Further study is needed to test if it is monophyletic, or perhaps paraphyletic (e.g., the raveni group and a few currently unplaced species, such as A. flavipennis Greene, should be added).

One species, A. robusta, is more distantly placed and appears not to belong to the mucronota group. Within the mucronota group, our results support the monophyly of the lanceola clade with the addition of A. minuta, but the status of the megacantha clade is uncertain.

The robusta species group is not recovered as monophyletic in this study, but the subclades of the group are highly supported. Our results indicate that the cryptostrepha clade, the nigra clade, the speciosa clade, and presumably the binodosa clade (the latter not represented here) should be treated as separate species groups. The robusta group should be restricted to the species of the lambda and robusta clades, which are sister groups. The two unplaced species of the robusta group, A. bella and A. concava, should be treated as unplaced to species group.

The ramosa and spatulata groups were not supported by this analysis. The three Manihot-feeding species of the spatulata group that were included formed a clade. The species of the ramosa group were placed in a well-supported clade with two species of the spatulata group. Further study is needed to clarify the composition and relationships of these two groups, which are based mainly on the shape of the aculeus. The relationships of a number of unplaced species with somewhat similar aculeus tips (e.g., A. rhediae Stone, A. nascimentoi Zucchi) also need to be evaluated.

The benjamini species group appears to be polyphyletic as the three species of the group included in this study arise in three separate positions on the tree. Norrbom (1997), Norrbom et al. (1999b), and Norrbom and Korytkowski (2012) previously indicated that morphological support for the group is weak. It is based mainly on a character state (produced facial carina) that may not be homologous in all of the species (the part of the carina that is produced varies) and also occurs in several species in other species groups. Nevertheless, some species within the group may be closely related, such as the species with dense microtrichia surrounding the lobe of cell cu (Norrbom et al., 2015), but none of these species were available for this study. Norrbom and Korytkowski (2012) also hypothesized that A. gigantea Stone, A. intermedia Norrbom & Korytkowski, and A. neogigantea Norrbom & Korytkowski are closely related, but only A. intermedia was available for this study, so the monophyly of this clade still needs to be tested with molecular data.

The grandis species group also appears to be polyphyletic. The four species of the group included in this study arise in four separate positions on the tree. Norrbom et al. (1999b) indicated that morphological support for the group is weak.

The relationships of most of the 15 Anastrepha species included in this analysis that were not classified in a species group by Norrbom et al. (2012, 2015) remain uncertain as they were placed in groupings with low support values. Exceptions include: three species that belong in the pseudoparallela group, and A. camba and A. sylvicola, which form a well-supported clade.

This study provides new data, analysis, and context for a wide sampling of diversity within Anastrepha (including Toxotrypana), the most economically important fruit fly genus in the Western Hemisphere. The challenge of continuing this work, to place all of the more than 300 species of Anastrepha and Toxotrypana into a predictive phylogenetic framework is a formidable task, however, and may perhaps never be satisfied completely. Yet our understanding of species group boundaries and their relationships becomes more important as new species of these tephritids continue to be discovered and described. Analyses of additional taxa and data are expected to improve our understanding of the relationships within this group and provide improved assessments of the potential commercial impacts of these pests.

Acknowledgements

The research by the senior author was conducted primarily during a fellowship funded by the USDA-ARS Postdoctoral Research Associate Program, project “Molecular characterization and analysis of pest fruit flies (Diptera: Tephritidae).” That of the second author was conducted starting with a fellowship funded by USDA Specialty Crops program project (CDFA#806052) to the last author, “A multi-faceted study of fruit flies (Diptera: Tephritidae) of economic importance to California”.

This study would not have been possible without the extensive samples provided by a myriad of sources. Some samples were collected via the USDA Farm Bill project “Enhancement of fruit fly immature stage identification and taxonomy” to FDACS-DPI; project numbers 3.0342 (2012), 13-8131-0291-CA (2013), and 3.0295.01 (2014). We also are indebted to all of the individuals and institutions who provided samples, particularly the following: Jorge López (Programa MoscaMed, Guatemala), Don Thomas


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