Israeli acute paralysis virus (IAPV) of honeybees is a Dicistrovirus that was first described in 2004 in Israeli colonies that had suffered from heavy losses (Maori et al., 2007). This virus gained prominence after a report in 2007 associated it with the colony collapse disorder (CCD) in the USA, where IAPV presence was considered a statistically significant marker of CCD (Cox-Foster et al., 2007). IAPV has since been described in numerous countries such as China (Xun et al., 2009), Canada and Australia (Palacios et al., 2008), or Argentina (Reynaldi et al., 2011). In Europe, IAPV has been found in France (Blanchard et al., 2008) and more recently in Spain (Kukielka & Sánchez-Vizcaíno, 2010) and Poland (Pohorecka et al., 2011). However, the high frequencies found in some countries (up to 41% in Argentina) and lack of obvious disease symptoms in most sampled colonies suggest that IAPV is a widespread virus that usually appears in covert infections, like most Dicistroviruses (De Miranda et al., 2010). Criteria identification to differentiate covert from overt infections in honeybee colonies is essential to identify risk factors of honeybee diseases, including CCD, where viruses may play an important role. Viral load may influence the development of disease symptoms, thus studies that differentiate the virus load causing covert and overt infections at the colony level are required. Some studies have quantified IAPV loads in experimental infections (Maori et al., 2007, 2009) and have observed high mortality upon inoculation and feeding with purified viral particles. However, none of these studies has assessed IAPV loads in field colonies (commercial hives) until recently (Martin et al., 2012). Another explanation for the covert infection of IAPV is the virulence of the isolate. Most published isolates are low-virulent, as IAPV normally persists in the colony in covert infections, showing no obvious symptoms at the individual or colony level (De Miranda et al., 2010). In Spain, only one sequence has been phylogenetically analysed from one healthy colony in Valencia (Kukielka & Sánchez-Vizcaíno, 2010) despite...
a report of 18% IAPV frequency in 2006 (Garrido-
Bailón et al., 2010) and 13% and 25.7% in 2006 and
2007, respectively (Antúnez et al., 2012). This Valen-
cian IAPV isolate was surprisingly clustered with
isolates from collapsed colonies from geographically
far countries. Thus, new phylogenetic analyses of IAPV
are required in Spain to confirm its phylogeny in rela-
tion to virulence as compared with other isolates from
different countries.

This study aimed to: assess the possible relationship
between IAPV presence of honeybees and disease symp-
toms at the colony level; describe IAPV load in commer-
cial hives; and illustrate the phylogenetic relationships
between IAPV isolates by focusing on Andalusia, an
important Spanish beekeeping region.

This work studied Andalusia (south-east Spain),
which ranks first in hive censuses and is the second
most important Spanish honey-producing region. Tech-
nicians from the Coordination of Agricultural and
Livestock Organisations (COAG) and the Beekeeping
Reference Centre of Andalusia (CERA, University of
Córdoba) sampled 96 honeybee colonies from all pro-
vinces of Andalusia in winter 2010/2011. The study
was designed to describe frequencies of honey bee vi-
ruses in this autonomous community. Assuming the
worse prevalence scenario (50%), sample size was cal-
culated by considering hive censuses with an expected
error of 10% and a 95% of confidence interval with
WinEpiscope v2.0 (De Blas et al., 2000). One hundred
different aged adult bees (nurse bees, guard bees,
foragers) were collected per colony and frozen imme-
diately afterwards. Epidemiological data were also
collected during sampling by surveys to assess sanitary
conditions. No clear CCD symptoms, collapse nor se-
vere winter losses were found in the sampled colonies.
Thus the present study focused on the potential asso-
ciation of IAPV with colony weakening represented
by colonies with worse health status. Worse health sta-
tus indicators included presence of clear IAPV disease
symptoms, such as paralysis, loss of hair or inability
to fly, depopulation, kleptoparasitism of stronger
neighbouring colonies, diagnosed disease potentially
associated with immune depletion such as chalkbrood
(Glinski & Buczek, 2003; Aronstein & Murray, 2010); and
problems to control Varroa destructor – mite infes-
tation might lead to immunosuppression (Yang & Cox-
Foster, 2005), which may facilitate IAPV replication,
as Varroa destructor has been described as an IAPV
vector (Di Prisco et al., 2011). Sanitary status data
were collected from the epidemiological surveys con-
ducted by technicians upon sampling. Colonies were
classified as “healthy” or “weak” depending on the
above-described symptoms appearing or not. Each
analytical sample consisted of 50 bees, which were
homogenized with 6 mL of sterile phosphate-buffered
saline (PBS) using a mortar and pestle. RNA extraction
was carried out using the column-based Nucleospin II
Virus® kit (Macherey Nagel) following the manufactu-
rer’s instructions.

Samples were analysed for IAPV presence by ampli-
fying virus-specific nucleic acid. One-step real-time
reverse transcription polymerase chain reaction (RT-
qPCR) was carried out using SYBR-Green dye. Pri-
mers targeting a 220-bp region of the gene ORF-2 were
used (Palacios et al., 2008), and amplification was
done following the thermocycler protocol described
by Palacios et al. (2008).

Absolute quantification of IAPV-positive samples
was performed to determine their viral load. The ORF-
2 fragment of IAPV was cloned into a PGemT® TA
cloning vector (Promega) following the manufacturer’s
instructions. The standard curve was constructed with
triplicates of serial dilutions of known amounts of plas-
mid DNA. The viral load results were expressed in
genome equivalent copies (GEC) per bee (Gauthier et al.,
2007). One sample from Valencia, previously
analysed for IAPV presence in 2010 (Kukiela & Sánchez-Vizcaíno, 2010), was included in the samples
quantification with identical procedures because its
viral load was not quantified in the previous study. This
sample was also included in the phylogenetic analysis
as it was the first IAPV isolate found in Spain.

After confirming IAPV presence, RNA samples
were first re-amplified by conventional RT-PCR target-
ing of a 705-bp sequence, which contains the 3’ end
of ORF1 (including part of the RdRp polymerase), the
intergenic region (IGR) (183-bp) and the 5’ end of the
ORF2 region -including part of viral protein 2 (VP2)-
of the IAPV genome (Palacios et al., 2008), and were
then sequenced. This fragment contains a variable re-
gion considered adequate to perform the IAPV phylo-
genetic analyses, as the highly conserved nature of the
RdRp region used in other bee virus phylogenetic ana-
lyses can produce cross-reactivity between close KBV
and IAPV viruses (Palacios et al., 2008). The result-
ing 705-bp sequences were utilised to construct a phy-
logeny of the identified isolates. Complete sequen-
ces of the amplified region were aligned with other
IAPV sequences from different countries using MEGA
4 (Tamura et al., 2007) (Table 1). A phylogenetic
The viral diagnosis results showed that IAPV was present in 13 of the 96 samples (13.5%) with \(4.9 \times 10^5\) GEC/bee on average (minimum-maximum GEC/bee: \(8.6 \times 10^3 – 1.2 \times 10^7\)). The IAPV load of the Valencian sample was \(1.43 \times 10^5\) GEC/bee. The Andalusian IAPV frequency results were similar to those previously obtained (Garrido-Bailón et al., 2010) with IAPV present in 18 out of 100 samples from 33 Spanish provinces; and more recently, to those of a retrospective study (Antúnez et al., 2012) describing IAPV frequencies of 13% and 25.7% in 2006 and 2007, respectively. Other than IAPV frequency in Andalusia, viral quantification showed that IAPV was present in substantial loads (up to \(10^7\) GEC/bee). Another study on adult honeybees at the colony level found viral loads of six different honeybee viruses in the order of \(10^8\) and \(10^9\) GEC/bee (Gauthier et al., 2007). More recently, Martin et al. (2012) reported similar results on the Hawaiian islands with IAPV loads in the order of \(10^7\) GEC/bee in covert infections not associated with colony collapse, but with lower prevalences (only 3 colonies of 293 were infected with IAPV). Based on the recent description of the *V. destructor* mite as a transmitter of IAPV (Di Prisco et al., 2011), future studies should also determine IAPV loads in pupae and *V. destructor* to better understand the biology of the virus. In this study, only the effect of IAPV presence and load was considered on weakening colonies in Andalusia. Future weakening colonies research should also contemplate other pathogens, the effect of co-infections and the host-pathogen-environment interaction. Given the IAPV frequency and load in Andalusia, these results suggest that IAPV presence is not an anecdotic finding in Andalusia.

According to the epidemiological surveys, 32 colonies were classified as “weak” and showed one or more above-described disease symptoms. No colony reported CCD or collapse. Eight of these weak colonies were positive to IAPV (25%), but IAPV presence was not

| GenBank accession No. | Country | Virus | Reference |
|-----------------------|---------|-------|-----------|
| AF150629              | UK      | ABPV  | Govan et al., 2000 |
| AY275710              | USA     | KBV   | de Miranda et al., 2004 |
| EU122347              | Australia | IAPV | Cox-Foster et al., 2007 |
| EU122348              | Australia | IAPV | Cox-Foster et al., 2007 |
| EU122349              | Australia | IAPV | Cox-Foster et al., 2007 |
| EU122350              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU122356              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU122357              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU122358              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU122361              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU122362              | USA     | IAPV  | Cox-Foster et al., 2007 |
| EU436427              | Canada  | IAPV  | Palacios et al., 2008 |
| EU436432              | USA     | IAPV  | Palacios et al., 2008 |
| EU436443              | USA     | IAPV  | Palacios et al., 2008 |
| EU436446              | Australia | IAPV | Palacios et al., 2008 |
| EU436447              | Australia | IAPV | Palacios et al., 2008 |
| EU436448              | Australia | IAPV | Palacios et al., 2008 |
| EU436449              | Israel  | IAPV  | Palacios et al., 2008 |
| EU436455              | Australia | IAPV | Palacios et al., 2008 |
| EU436456              | Australia | IAPV | Palacios et al., 2008 |
| EU604006              | France  | IAPV  | Blanchard et al., 2008 |
| EU604007              | France  | IAPV  | Blanchard et al., 2008 |
| EU604008              | France  | IAPV  | Blanchard et al., 2008 |
| EU604009              | France  | IAPV  | Blanchard et al., 2008 |
| EU604010              | France  | IAPV  | Blanchard et al., 2008 |
| FJ754324              | China   | IAPV  | Xun et al., 2009 |
| EU218534              | USA     | IAPV  | Chen Y., published in Genbank, 2009 |
| FJ821506              | Spain   | IAPV  | Kukielka & Sánchez-Vizcaíno, 2010 |
| JQ435732-JQ435744     | Spain   | IAPV  | Present study |
Figure 1. “Neighbour-joining” phylogenetic tree of IAPV sequences. Triangles indicate sequences from Andalusia and the diamond indicates the sequence from Valencia. Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) sequences were included as the out-group. Genbank accession numbers, sampling location and year are shown per sequence. The number of each node represents the bootstrap values as the result of 1,000 replicates. Bootstrap values of < 50% were omitted.
associated with weakening (χ² test, p > 0.05). No specific symptom contemplated in the epidemiological surveys was statistically associated with IAPV presence. Besides, the highest viral loads were present in both healthy and weak colonies, indicating that IAPV loads in the order of 10⁷ GEC/bee do not produce obvious disease symptoms at the colony level themselves. However, it would be interesting to perform molecular and histopathological analyses of individual bees to describe the proportion of infected bees per colony as pools of bees were used, thus we cannot ensure that these viral loads do not produce clinical symptoms in individual bees. Future studies should consider this issue to establish the IAPV load baseline in healthy commercial hives.

The phylogenetic analysis revealed two main lineages of IAPV (Fig. 1). The first one grouped all the IAPV sequences from Andalusia in the present study with IAPV isolates mostly from colonies suffering severe winter losses in France. The Valencian isolate (Kukielka & Sánchez-Vizcaíno, 2010) was, however, grouped with the second lineage, which included isolates from collapsed colonies in USA, China, Australia, Israel and Canada. These results confirm the great genetic diversity of IAPV sequences identified in Spain and suggest at least two differentiated evolutionary IAPV lineages in Spain. Andalusian samples may relate more closely to French samples given geographical proximity since natural bee migration between Spain and France can occur in lower-altitude areas of the Pyrenees, near the Atlantic and Mediterranean coasts. Hence, colonies separated by approximately 1,000 kilometres, like those in Andalusia and the northern Spain, can come into contact when bee hives are moved to pollinate crops during transhumance, a common practice in Spain where 30.2% (7,323 of 24,251) of colonies are transhumant (MAGRAMA, 2012). The Valencian isolate is phylogenetically divergent from the IAPV isolates described in Andalusia and relates instead to isolates from geographically remote regions. Trade is the most likely explanation for the spread of IAPV between countries; imports of IAPV-positive honeybees from Australia to the USA have been reported (Palacios et al., 2008). The fact that IAPV isolates from healthy and symptomatic hives cluster together suggests that the symptoms development may be linked with viral replication activation and presence of high viral loads. This fact has been suggested (Ribière et al., 2008), but is still to be verified in IAPV. Since IAPV has been found in many Spanish regions, further phylogenetic analyses are recommended in future studies on IAPV in honeybees. To confirm the results described herein, these future analyses should include the whole genome of the studied IAPV isolates.

Here the results suggest that IAPV is no sporadic finding in Andalusia because its frequency is 13.5% and IAPV presence does not significantly associate with disease symptoms presence at the colony level in this region. Therefore, we report IAPV loads in the order of up to 10⁷ GEC/bee in healthy commercial hives, which indicates that presence of these viral loads is not indicative of overt infection at the colony level. Therefore, the development of obvious disease symptoms at this level must be produced by higher viral loads, probably in conjunction with other factors and especially in immunosuppressed colonies. Moreover, Andalusian IAPV isolates are phylogenetically distant from the Valencian isolate, suggesting that Andalusian IAPV has at least two different evolutionary lineages in Spain. Spanish isolates from healthy colonies relate to isolates from colonies suffering depopulation or collapse in other countries, thus further investigation into isolates virulence in field samples is required. Indeed, phylogenetic analysis may help investigate relevant honeybee virus epidemiology aspects, such as their spread via trade routes, which must be considered when implementing control measures.

In conclusion, this study reports the IAPV frequency in an important Spanish beekeeping region, Andalusia, and lack of association between IAPV presence and disease symptoms at the colony level. It is also the first quantification of IAPV load in Spain and it has established phylogenetic similarities between Andalusian IAPV isolates and those mainly from France by identifying at least two different evolutionary IAPV lineages in Spain.

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