Article

Distribution of Nucleosides in Populations of *Cordyceps cicadae*

Wen-Bo Zeng 1, Hong Yu 1,*, Feng Ge 2, Jun-Yuan Yang 1, Zi-Hong Chen 1, Yuan-Bing Wang 1, Yong-Dong Dai 1 and Alison Adams 3

1 Yunnan Herbal Laboratory, Institute of Herb Biotic Resources, Yunnan University, Kunming 650091, Yunnan, China; E-Mails: zengwenboherb@163.com (W.-B.Z.); icecream6973@sina.com (J.-Y.Y.); czh78@tom.com (Z.-H.C.); wangyb001@126.com (Y.-B.W.); daidiy555@gmail.com (Y.-D.D.)
2 Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, Yunnan, China; E-Mail: gefeng79@aliyun.com
3 Department of Biological Sciences, College of Engineering, Forestry and Natural Science, Northern Arizona University, Flagstaff, AZ 86011-5640, USA; E-Mail: Alison.Adams@nau.edu

* Author to whom correspondence should be addressed; E-Mail: herbfish@163.com or hongyu@ynu.edu.cn; Tel.: +86-137-006-766-33; Fax: +86-871-650-346-55.

Received: 15 January 2014; in revised form: 25 April 2014 / Accepted: 5 May 2014 / Published: 14 May 2014

**Abstract:** A rapid HPLC method had been developed and used for the simultaneous determination of 10 nucleosides (uracil, uridine, 2'-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, 2'-deoxyadenosine and cordycepin) in 10 populations of *Cordyceps cicadae*, in order to compare four populations of *Ophicordyceps sinensis* and one population of *Cordyceps militaris*. Statistical analysis system (SAS) 8.1 was used to analyze the nucleoside data. The pattern of nucleoside distribution was analyzed in the sampled populations of *C. cicadae*, *O. sinensis* and *C. militaris*, using descriptive statistical analysis, nested analysis and Q cluster analysis. The total amount of the 10 nucleosides in coremium was 1,463.89–5,678.21 µg/g in 10 populations of *C. cicadae*, 1,369.80–3,941.64 µg/g in sclerotium. The average contents of the 10 analytes were 4,392.37 µg/g and 3,016.06 µg/g in coremium and sclerotium, respectively. The coefficient of variation (CV) of nucleosides ranged from 8.36% to 112.36% in coremium of *C. cicadae*, and from 10.77% to 155.87% in sclerotium of *C. cicadae*. The CV of the nucleosides was wide within *C. cicadae* populations. The nested variation analysis by the nine nucleosides’ distribution indicated that about 42.29% of the nucleoside variability in coremium was attributable to the differentiation among populations, and the remaining 57.71% resided in the populations. It
was also shown that about 28.94% of the variation in sclerotium was expressed between populations, while most of the variation (71.06%) corresponded to the populations.

**Keywords:** Cordyceps cicadae; nucleosides; distribution

## 1. Introduction

*Cordyceps cicadae* X. Q. Shing (Figure 1), named “Chan Hua”, belongs to the genus *Cordyceps* (family Clavicipitaceae, Ascomycotina), and its anamorph is *Isaria cicadae* Miq [1], which is a major parasitic fungus growing on the nymph of *Cicada flammata* Distant, *Platyleura kaempferi* Fabricius, *Crytotympana pustulata* Fabricious [2], *Platyomia pieli* Kato [3] and *Oncotympana maculatiedoillis* Motsch (Figure 2). *Cordyceps cicadae* has been used as a Traditional Chinese Medicine and food for about 1,500 years in China [2], much longer than *Ophicordyceps sinensis* (Berk.) G. H. Sung, J. M. Sung [4,5].

**Figure 1.** *Cordyceps cicadae* used for this study.

**Figure 2.** The nymph and adult of *Oncotympana maculatiedoillis*, as one host of *C. cicadae*, collected from Kunming in Yunnan (Pop CCKSG).
Furthermore, *C. cicadae* has been used as a substitute for *O. sinensis*. Its putative active functions include: (1) treatment of childhood convulsions; (2) antitumor activity [6,7]; (3) analgesic activity and sedative function [3,8]; (4) amelioration of renal function [9]; (5) anti-fatigue effects [10]; (6) immunomodulatory effects [7].

*Cordyceps cicadae* is a cosmopolitan species in many regions of the World, and its habitat demands are less strict than those of *O. sinensis*. The distribution of *C. cicadae* had been surveyed in China (Table 1). It has also been recorded in South Asia, Europe, North America [11] and Jeju Island in South Korea [12,13].

Table 1. Distribution of *C. cicadae* in China.

| Province  | Location                                                                 |
|-----------|--------------------------------------------------------------------------|
| Yunnan    | Mojiang, Fengyang [13] Lanping, WeiXi, Xianggelila, Zhaotong and Kunming |
| Sichuan   | Mount Emei, Qingcheng mountain and Qingyun mountain [13] and Xiangcheng   |
| Guizhou   | Fanjing mountain, Libo karst geopark, Guiyang forest park and Huaxi [13,14] |
| Jiangsu   | Yixing                                                                   |
| Guangxi   | Leye [12]                                                                |
| Hainan    | Wuzhi mountain [12]                                                     |
| Fujian    | Wushan in Fuzhou [13]                                                   |
| Shanghai  | Tianma mountain [15]                                                    |
| Zhejiang  | Hangzhou [16]                                                           |

Guangdong [11] — __ a

Hunan [11] — __

Hubei [11] — __

*No details.*

According to Traditional Chinese Medicine, *C. cicadae* had been considered as a drug similar to *O. sinensis*, with its effective composition of amino acids, polysaccharides, and mannitol being similar to those of *O. sinensis* [17]. Several components, such as nucleosides, polysaccharides, ergosterol and mannitol, had been used as markers for quality control of *Cordyceps* and its products [4]. The following chemical constituents have been isolated from *C. cicadae*: polysaccharides [18,19], galactomannan [20], adenosine, uridine, inosine, guanosine [21], ISP-1(myriocin) [22,23] and ergosterol peroxide [24].

Previous studies showed that the most important bioactive constituents in *O. sinensis* and its analogs were soluble nucleosides. Since cordycepin (3’-deoxyadenosine) with antitumor activity was isolated from cultured *C. militaris* in 1950 [25], nucleosides in *Cordyceps* have become a focus of research. To date, more than ten nucleosides were detected or isolated from this group, such as adenine, adenosine, 2’-deoxyadenosine, 3’-deoxyadenosine, uracil, uridine, 2’-deoxyuridine, guanine, cytosine, guanosine, hypoxanthine, inosine, thymine and thymidine [4,26,27]. Adenosine plays a key role in the pharmacological effects, as it depressed the excitability of CNS neurons and inhibited the release of various presynaptic neurotransmitters [28,29], and adenosine has been used as a marker for quality control of *O. sinensis* in the Chinese Pharmacopoeia [30]. Inosine, the major biochemical metabolite resulting from oxidative deamination of adenosine, stimulated axon growth *in vitro* and the adult central nervous system [31]. Cordycepin, one of the main compounds found in *C. militaris*, had also
shown multiple pharmacological activities [32–34]. However, whether or not natural and cultured *O. sinensis* contain cordycepin is still controversial [27,35–37]. In addition, nucleosides were reported to play a role in growth and differentiation of the gastrointestinal tract, as well as to play a role in the maintenance of the immune response [38,39]. So far, several methods, including HPLC [27,35–37,40,41], LC–MS [26,36,42,43], CE–MS [44], CE [45], CEC [46], ultra-performance liquid chromatography (UPLC) [47], and CZE [48], had been established to determine nucleosides in *O. sinensis* and related species.

*Cordyceps cicadae* is recorded as one of the most valued Traditional Chinese Medicines [17]. It consists of the dried fungus *Isaria cicadae* growing on the nymphs of cicadas. The fruiting body (coremium) and the nymph (sclerotium) of *C. cicadae* have been applied together in Traditional Chinese Medicine and food. Up to now, the nucleosides of coremium and sclerotium have not been determined in populations of *C. cicadae*. In this study, a simple and convenient HPLC method was used to analyze the nucleosides in coremium and sclerotium of *C. cicadae* populations, comparing with those of *O. sinensis* and *C. militaris*. This method included a system of 10 nucleosides, *i.e.*, uracil, uridine, 2'-deoxyuridine, inosine, guanosine, adenine, thymidine, adenosine, 2'-deoxyadenosine and cordycepin (3'-deoxyadenosine). The nucleoside distribution patterns in populations of *C. cicadae* were revealed, and these compounds could become as useful markers for the authentication and quality control of *C. cicadae*.

2. Results and Discussions

Statistical analysis system (SAS) 8.1 was used to analyze the contents of nucleosides in *C. cicadae*, *O. sinensis* and *C. militaris*. Descriptive statistical analysis, nested analysis and Q cluster analysis (average cluster) of the data are presented in this paper.

2.1. Descriptive Statistical Analysis

The mean content and coefficient of variation (*CV*) of 10 nucleosides in coremium and sclerotium of *C. cicadae*, *O. sinensis* and *C. militaris* are shown in Tables 2 and 3. In coremium, the content of uracil was 279.84–444.47 µg/g, the *CV* was 22.23%–40.87%, and the average content was 344.60 µg/g. The content of uridine was 363.30–1,928.73 µg/g, the *CV* was 12.93%–81.78%, the average content of uridine was 1,468.78 µg/g. The content of 2'-deoxyuridine was 49.89–350.41 µg/g, the *CV* was 33.31%–95.22%, the average content was 171.24 µg/g. The content of inosine was 79.65–1,166.62 µg/g, the *CV* was 10.33%–82.36%, the average content was 456.03 µg/g. The content of guanosine was 351.44–1,483.06 µg/g, the *CV* was 8.36%–58.24%, the average content was 1,016.53 µg/g. The content of adenine was 33.63–166.17 µg/g, the *CV* was 32.36%–66.30%, the average content was 70.17 µg/g. The content of thymidine was 17.73–83.81 µg/g, the *CV* was 29.20%–91.67%, the average content was 38.96 µg/g. The content of adenosine was 201.54–1,153.78 µg/g, the *CV* was 10.33%–56.48%, the average content was 797.92 µg/g. The content of 2'-deoxyadenosine was 13.01–54.62 µg/g, the *CV* was 25.13%–112.36%, the average content was 28.13 µg/g.
Table 2. The content of 10 nucleosides in 10 populations of *C. cicadae*, four populations of *O. sinensis* and one population of *C. militaris*.

| Pop | Position | Uracil | Uridine | 2'-Deoxyuridine | Inosine | Guanosine | Adenine | Thymidine | Adenosine | 2'-deoxyadenosine | Cordycepin | Total amount (µg/g) |
|-----|----------|--------|---------|-----------------|---------|-----------|---------|-----------|-----------|------------------|------------|------------------|
| CCKSG | coremium | 444.47/33.73 | 1577.49/19.00 | 350.41/95.22 | 1166.62/10.33 | 733.03/42.09 | 166.17/22.36 | 83.81/29.20 | 837.00/39.93 | 54.62/25.13 | — | 5413.62 |
| | sclerotium | 774.72/41.32 | 57.24/49.62 | 198.44/77.06 | 314.61/48.99 | 99.15/34.95 | 75.02/39.66 | 510.99/74.40 | 52.94/24.67 | — | 2398.75 |
| CCLHH | coremium | 334.54/27.49 | 1587.46/12.93 | 119.21/40.45 | 330.38/12.14 | 72.39/34.95 | 857.36/21.13 | 32.81/28.25 | — | — | 4668.47 |
| | sclerotium | 149.32/26.57 | 62.34/81.92 | 98.06/44.89 | 138.32/126.94 | 742.77/36.52 | 120.84/155.87 | — | — | — | 3463.90 |
| CCLHQ | coremium | 371.92/38.41 | 1560.08/29.22 | 299.71/70.19 | 198.44/77.06 | 490.96/65.31 | 33.06/53.97 | 857.36/21.13 | 15.61/26.47 | — | 4565.36 |
| | sclerotium | 266.09/29.50 | 83.81/29.20 | 62.09/43.60 | 575.99/16.42 | 43.49/84.05 | 22.74/47.99 | — | — | — | 3078.23 |
| CCLHX | coremium | 334.54/27.49 | 1587.46/12.93 | 119.21/40.45 | 330.38/12.14 | 72.39/34.95 | 857.36/21.13 | 32.81/28.25 | — | — | 4668.47 |
| | sclerotium | 149.32/26.57 | 62.34/81.92 | 98.06/44.89 | 138.32/126.94 | 742.77/36.52 | 120.84/155.87 | — | — | — | 3463.90 |
| CCLHQ | coremium | 371.92/38.41 | 1560.08/29.22 | 299.71/70.19 | 198.44/77.06 | 490.96/65.31 | 33.06/53.97 | 857.36/21.13 | 15.61/26.47 | — | 4565.36 |
| | sclerotium | 266.09/29.50 | 83.81/29.20 | 62.09/43.60 | 575.99/16.42 | 43.49/84.05 | 22.74/47.99 | — | — | — | 3078.23 |
### Table 2. Cont.

| Pop   | Position | Contents (mean (µg/g)/CV (%)) | Total amount (µg/g) |
|-------|----------|-------------------------------|---------------------|
|       |          | Uracil | Uridine | 2'-Deoxyuridine | Inosine | Guanosine | Adenine | Thymidine | Adenosine | 2'-deoxyadenosine | Cordycepin |                  |
| OSNBE | stroma   | 149.76/11.15 | 2765.61/2.92 | 7.70/19.07 | 580.85/17.06 | 2130.34/3.59 | 113.59/21.67 | 58.34/26.80 | 2544.76/4.02 | 46.77/24.73 | —        | 8397.72          |
|       | sclerotium | 127.95/15.59 | 1599.98/6.53 | 9.92/27.35 | 2073.63/18.43 | 1131.88/4.76 | 154.86/11.56 | 177.09/9.04 | 1081.60/8.63 | 106.28/17.61 | —        | 6463.19          |
| CMSMB | stroma   | 319.18/19.51 | 1900.92/11.02 | 5.01/24.88 | 85.08/20.14 | 1215.38/16.31 | 313.75/20.12 | 69.41/14.69 | 1613.28/13.51 | 58.18/19.15 | 659.29/19.11 | 6239.49 |
|       | sclerotium | 332.93/20.04 | 1743.60/13.87 | 12.77/18.48 | 189.93/13.14 | 1075.99/16.58 | 264.18/20.17 | 68.87/16.74 | 1655.93/12.37 | 76.98/15.84 | 4173.57/13.81 | 9594.75 |

### Table 3. The average content (µg/g) and CV (%) of 10 nucleosides in *C. cicadae*, *O. sinensis* and *C. militaris*.

| Species | Position | Mean content (µg/g)/CV (%) | Total amount (µg/g) |
|---------|----------|----------------------------|---------------------|
| *C. cicadae* | coremium | 344.60/34.66 | 1468.78/38.58 | 171.24/104.35 | 456.03/79.35 | 1016.53/46.92 | 70.17/65.99 | 38.96/66.95 | 797.92/42.21 | 28.13/64.84 | — b | 4392.37 |
|         | sclerotium | 226.34/40.18 | 1186.46/37.05 | 134.28/60.32 | 611.69/36.79 | 62.07/59.85 | 70.54/106.70 | 619.45/40.64 | 49.56/142.54 | — | 3016.06 |
| *O. sinensis* | stroma | 182.23/75.88 | 2000.60/31.48 | 3.59/157.80 | 243.48/86.32 | 1596.76/23.20 | 120.00/56.37 | 49.57/40.12 | 1809.55/26.14 | 46.27/23.07 | — | 6052.06 |
|         | sclerotium | 126.61/68.28 | 1439.14/16.90 | 6.67/192.88 | 879.97/83.68 | 1011.20/20.51 | 101.84/38.79 | 129.71/34.04 | 740.26/45.62 | 66.56/41.41 | — | 4501.96 |
| *C. militaris* | stroma | 319.18/19.51 | 1900.92/11.02 | 5.01/24.88 | 85.08/20.14 | 1215.38/16.31 | 313.75/20.12 | 69.41/14.69 | 1613.28/13.51 | 58.18/19.15 | 659.29/19.11 | 6239.49 |
|         | sclerotium | 332.93/20.04 | 1743.60/13.87 | 12.77/18.48 | 189.93/13.14 | 1075.99/16.58 | 264.18/20.17 | 68.87/16.74 | 1655.93/12.37 | 76.98/15.84 | 4173.57/13.81 | 9594.75 |

b Not detected; * Number of population.
In sclerotium, the content of uracil was 149.32–315.63 µg/g, the CV was 20.18%–62.24%, the average content was 226.34 µg/g. The content of uridine was 520.61–1587.68 µg/g, the CV was 17.04%–41.32%, the average content was 1,186.46 µg/g. The content of inosine was 74.61–198.44 µg/g, the CV was 16.39%–77.06%, the average content was 134.28 µg/g. The content of guanosine was 260.00–859.40 µg/g, the CV was 10.77%–48.99%, the average content was 611.69 µg/g. The content of adenine was 33.93–99.15 µg/g, the CV was 29.13%–100.84%, the average content was 62.07 µg/g. The content of thymidine was 29.55–138.32 µg/g, the CV was 39.66%–126.94%, the average content was 70.54 µg/g. The content of adenosine was 230.20–961.26 µg/g, the CV was 11.29%–74.40%, the average content was 619.45 µg/g. The content of 2'-deoxyadenosine was 9.68–120.84 µg/g, the CV was 26.47%–155.87%, the average content was 49.56 µg/g.

Analysis of the nucleosides revealed obvious differences between coremium and sclerotium in populations of *C. cicadae*. The contents of uracil, uridine, 2'-deoxyuridine, inosine, guanosine, adenine and adenosine in coremium were higher than those in sclerotium. The coefficient of variation in coremium was 34.66%–104.35%, and the CV in sclerotium was 36.79%–142.54%, with a great variation of nucleosides content in populations of *C. cicadae*. The wide variation of nucleosides in *C. cicadae* populations may mainly be derived from the genetic differences of the *C. cicadae* population, being affected by different location, geography, climate, maturation of the *C. cicadae*. Furthermore, Li et al. reported that after storage of *O. sinensis* at 75% relative humidity and 40 °C for 10 days, the contents of uridine, guanosine and adenosine in natural *O. sinensis* were markedly increased about one to four fold [49], implying that the storage conditions might be another factor affecting the variation of nucleosides in *C. cicadae*.

Nucleosides were believed to be the active components in *Cordyceps*-like fungi [50], indeed, *Cordyceps*-like fungi contained a higher concentration of nucleosides [51], and some unique nucleosides, such as cordycepin, 2'-deoxyuridine and 2'-deoxyadenosine were detected in *Cordyceps*-like fungus [26,27,45,47,51–53], which could be used as markers for distinguishing *Cordyceps*-like fungi from their counterfeits.

Cordycepin in natural *O. sinensis* was found in very low amounts [36,46,54], about several tens of micrograms per gram [55]. However, in this study, cordycepin was not detected in *C. cicadae* and *O. sinensis*, and cordycepin in *C. militaris* was high, up to 659.29 µg/g in stroma and 4173.57 µg/g in sclerotium, in accordance with the reports of Guo et al., and Yang and Li [36,37]. 2'-Deoxyadenosine was detected in *C. cicadae*, i.e., 28.13 µg/g in coremium and 49.56 µg/g in sclerotium. Cordycepin and 2'-deoxyadenosine are isomers of each other, and there are a lot of reports about the pharmacological activities of cordycepin [32–34], while the pharmacological activities of 2'-deoxyadenosine in *Cordyceps*-like fungi are worth studying further.

Li et al. reported that the levels of adenosine, guanosine and uridine were very similar in stroma and sclerotium of *O. sinensis* [54]. The average content of nucleosides of 10 populations of *C. cicadae*, four populations of *O. sinensis* and one population of *C. militaris* are shown in Table 3. Several nucleosides such as uracil, uridine, guanosine, adenine and adenosine in coremium were higher than those in sclerotium of *C. cicadae*. On the contrary, the content of thymidine and 2'-deoxyadenosine in coremium were lower than those in sclerotium of *C. cicadae*. The average...
content of inosine in coremium (456.03 µg/g) was 3-fold higher than that in sclerotium (134.28 µg/g) of *C. cicadae*.

Hsu et al. reported that the content of adenosine in stroma was approximately 6-fold higher than that in sclerotium of *O. sinensis* [56]. However, the content of adenosine in coremium was approximately 1.5 times that in sclerotium of *C. cicadae*. The total content of the 10 nucleosides in coremium was approximately 1.5 times that in sclerotium in *C. cicadae*. On the contrary, the content of the 10 analytes in stroma was approximately 1.5 times that in sclerotium of *C. militaris*. The distribution of the 10 nucleosides in *C. cicadae* was similar to that in *O. sinensis*, being different from the distribution pattern of the 10 analytes in *C. militaris*.

### 2.2. Nested Analysis

Nested analysis was used to analyze the uracil, uridine, 2'-deoxyuridine, inosine, guanosine, adenine, thymidine, adenosine and 2'-deoxyadenosine in *C. cicadae*, investigating the percent of total variance of the nine analytes between populations and individuals.

The result of nested variation analysis by the nine nucleosides’ distributions is shown in Table 4. It was indicated that about 42.29% of the variation in coremium was attributed to the differentiation among populations, and the remaining 57.71% was resided among individuals within populations. It was also showed that about 28.94% of the variation in sclerotium was expressed between populations, while most of the variation 71.06% was resided among individuals within populations.

**Table 4. Nested analysis of nine nucleosides in coremium and sclerotium of *C. cicadae*.**

| Position | Analyte             | Percent of total variance (%) | Percent of population variance (%) | Percent of individual variance (%) | F Value | Pr > F |
|----------|---------------------|-----------------------------|-----------------------------------|-----------------------------------|----------|--------|
| coremium | uracil              | 100.00                      | 9.13                              | 90.87                             | 2.00     | 0.0477 |
|          | uridine             | 100.00                      | 52.45                             | 47.55                             | 12.03    | <0.0001|
|          | 2'-deoxyuridine     | 100.00                      | 23.83                             | 76.17                             | 4.13     | 0.0002 |
|          | inosine             | 100.00                      | 54.48                             | 45.52                             | 12.97    | <0.0001|
|          | guanosine           | 100.00                      | 45.25                             | 54.75                             | 9.27     | <0.0001|
|          | adenine             | 100.00                      | 53.57                             | 46.43                             | 12.54    | <0.0001|
|          | thymidine           | 100.00                      | 44.82                             | 55.18                             | 9.12     | <0.0001|
|          | adenosine           | 100.00                      | 46.69                             | 53.31                             | 9.76     | <0.0001|
|          | 2'-deoxyadenosine   | 100.00                      | 50.39                             | 49.61                             | 11.16    | <0.0001|
|          | Mean                | 100.00                      | 42.29                             | 57.71                             | ——       | ——     |
| sclerotium| uracil              | 100.00                      | 19.25                             | 80.75                             | 3.38     | 0.0013 |
|          | uridine             | 100.00                      | 47.52                             | 52.48                             | 10.06    | <0.0001|
|          | 2'-deoxyuridine     | 100.00                      | 19.19                             | 80.81                             | 3.37     | 0.0013 |
|          | inosine             | 100.00                      | 22.04                             | 77.96                             | 3.83     | 0.0004 |
|          | guanosine           | 100.00                      | 63.70                             | 36.30                             | 18.55    | <0.0001|
|          | adenine             | 100.00                      | 16.48                             | 83.52                             | 2.97     | 0.0038 |
|          | thymidine           | 100.00                      | 9.45                              | 90.55                             | 2.04     | 0.0432 |
|          | adenosine           | 100.00                      | 47.44                             | 52.56                             | 10.03    | <0.0001|
|          | 2'-deoxyadenosine   | 100.00                      | 15.38                             | 84.62                             | 2.82     | 0.0058 |
|          | Mean                | 100.00                      | 28.94                             | 71.06                             | ——       | ——     |
2.3. Q Cluster Analysis

*O. sinensis*, one of the most precious Traditional Chinese Medicines grows in a very restricted habitat, and is usually found in the soil of prairies or fir forests at an altitude from 3,500 to 5,000 m, mainly in provinces like Sichuan, Qinghai, Yunnan, Tibet and Gansu in China. In Nepal, Bhutan and India, *O. sinensis* is collected as well. In China, this fungus is usually called “Dong Chong Xia Cao”. *O. sinensis* has been used for the treatment of hyperglycemia, respiratory and liver diseases, renal dysfunction, renal failure and has antioxidant properties [57,58]. It was initially recorded in Ben-Cao-Bei-Yao by Wang Ang in 1694. Because of its scarcity in nature and high price, some studies have been carried out in order to find substitutes for *O. sinensis* [49,59,60]. *C. militaris* have been used as the main substitute for *O. sinensis* [50,61], and Traditional Chinese Medicine considers *C. cicadae* to be a drug similar to *O. sinensis*, as these two species have similar active components and medicinal value [17], however, little scientific information about the proximate composition and bioactive ingredients of *C. cicadae* and *O. sinensis* is available. Q cluster analysis (average linkage) was used to analyze the 10 nucleosides in *C. cicadae*, *O. sinensis* and *C. militaris* (Figures 3 and 4).

*Figure 3*. Q-Cluster of 10 nucleosides assayed in coremium (stroma) of 10 populations of *C. cicadae*, four populations of *O. sinensis*, and one population of *C. militaris*, using the average linkage method.

Figure 3 shows the 15 populations of *C. cicadae*, *O. sinensis* and *C. militaris* separated into three branches; clade A includes population CCKSG, clade C includes population CMSMB, and clade B includes all other populations. The populations of *C. cicadae* collected at Lanping and Weixi county clustered as one subclade, which showed the geological differences. In Figure 4, 15 populations of *C. cicadae*, *O. sinensis* and *C. militaris* also separate into three branches; clade D includes 10 populations of *C. cicadae* and three populations of *O. sinensis*, clade E includes population OSNBE, and clade F includes population CMSMB.
**Figure 4.** Q-Cluster of 10 nucleosides assayed in sclerotium of 10 populations of *C. cicadae*, four populations of *O. sinensis*, and one population of *C. militaris* by the average linkage method.

The four populations of *O. sinensis* could not be separated into a single clade, indicating that the nucleosides in *O. sinensis* had no obvious differences from those of *C. cicadae*. The average clusters based on the average content of uracil, uridine, 2'-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, 2'-deoxyadenosine and cordycepin had been constructed, showing that *C. cicadae* should be a better substitute for *O. sinensis* than *C. militaris*.

### 3. Experimental

#### 3.1. Sample Preparation

The details of the sources of *C. cicadae*, *O. sinensis* and *C. militaris*, are shown in Table 5. The samples, divided into the fruiting body (coremium or stroma) and the nymph or caterpillar (sclerotium), were dried at 50 C and ground into powder. These were separately weighed into a 5 mL volumetric flask, 20% methanol was added to the flask to about 90% of its volume, and after sonication for 90 min, the mixture was diluted to the mark with 20% methanol. After centrifugation at 25 C for 10 min at 4,000 rpm/min, sample solutions were passed through a 0.45 μm membrane filter. Duplicate analytical samples were prepared for each sample. The HPLC chromatograms of *C. cicadae* and mixed standards are shown in Figure 5.
Table 5. Localities of the 10 populations of *C. cicadae*, four populations of *O. sinensis* and one population of *C. militaris*.

| Species | NO. of populations | Sample size | Locus of extraction | Locality |
|---------|---------------------|-------------|---------------------|----------|
| *C. cicadae* | | | | |
| CCKSG 10 | Coremium | Gelezicun, Shuanglong township, Kunming City, Yunnan |
| CCKSG 10 | Coremium | Hedongqingcun, Hexi township, Lanping county, Yunan |
| CCLHQ 10 | Coremium | Qidenglongcun, Hexi township, Lanping county, Yunan |
| CCLSH 10 | Coremium | Huilongcun, Shideng township, Lanping county, Yunan |
| CCLTD 10 | Coremium | Deqingcun, Tongdian township, Lanping county, Yunan |
| CCLLL 10 | Coremium | Lianqiaoshacun, Tongdian township, Lanping county, Yunan |
| CCLZD 10 | Coremium | Datujicun, Zhongpai township, Langping county, Yunnan |
| CCWYJ 10 | Coremium | Juxiangcun, Yongchun township, Weixi county, Yunnan |
| CCYTS 10 | Coremium | Sanzhou Mountain, Taihua town, Yixing city, Jiangsu |
| *O. sinensis* | | | | |
| OSDQI 5 | Stroma | Deqin, county, Yunnan |
| OSMNI 5 | Stroma | Manicun, Lengda township, Jiacha county, Tibet |
| OSLTA 5 | Stroma | Litang county, Sichuan |
| OSNBE 5 | Stroma | Nepal |
| *C. militaris* | | | | |
| CMSMB 5 | Stroma | Baiyi township, Songming county, Yunnan |
3.2. Chemicals and Reagents

HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany). Water was purified using a Millipore Simplicity system (Billerica, MA, USA). Uracil, uridine, 2'-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, 2'-deoxyadenosine and cordycepin (purity ≥ 98.0%) were purchased from Sigma (St. Louis, MO, USA).

3.3. Liquid Chromatography Conditions

HPLC was conducted on a Dionex liquid chromatograph system (DIONEX, Sunnyvale, CA, USA) equipped with a LPG-3400A quaternary pump and a PDA-3000 photodiode array detector. The sample extracts were separated and analyzed using a Waters Symmetry® C18 column (250 mm, 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of 10% solvent A (methanol) and 90% solvent B (water). The flow rate was 1.0 mL·min⁻¹. The detecting wavelength was set between 190 and 380 nm, and the chromatographic peaks were measured at a wavelength of 260 nm for the detection of nucleosides.

3.4. Method Validation

3.4.1. Calibration Curves

Stock solutions were prepared by dissolving the standards in 20% methanol to give 1–2 mg/mL for uracil, uridine, 2'-deoxyuridine, inosine, guanosine, adenine, thymidine, adenosine, 2'-deoxyadenosine and cordycepin respectively. Further dilution with 20% methanol was performed to prepare the standard solutions for calibration curves. At least six concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The results were shown in Table 6.
Table 6. Linear regression data, LOD, and LOQ of 10 nucleosides at 260 nm.

| Analyte           | $\lambda_{\text{max}}$ (nm) | Linear Regression Equation | $r^2$ | Test Range (µg/mL) | LOD (µg/mL) | LOQ (µg/mL) |
|-------------------|-------------------------------|-----------------------------|-------|--------------------|-------------|-------------|
| uracil            | 260.1                         | $y = 0.0145x - 0.0076$      | 0.9999| 8.00–240.00        | 0.006       | 0.018       |
| uridine           | 263.1                         | $y = 0.0233x + 0.008$       | 0.9999| 8.00–240.00        | 0.008       | 0.024       |
| 2’-deoxyuridine   | 263.2                         | $y = 0.0234x - 0.0016$      | 0.9999| 7.00–140.00        | 0.008       | 0.024       |
| inosine           | 249.8                         | $y = 0.0368x - 0.0479$      | 0.9995| 7.60–380.00        | 0.015       | 0.045       |
| guanosine         | 254.2                         | $y = 0.023x + 0.029$        | 0.9997| 8.00–400.00        | 0.008       | 0.024       |
| adenine           | 261.5                         | $y = 0.0108x$               | 0.9999| 8.00–400.00        | 0.004       | 0.012       |
| thymidine         | 268.2                         | $y = 0.0315x + 0.0009$      | 0.9999| 8.60–172.00        | 0.012       | 0.036       |
| adenosine         | 261.0                         | $y = 0.0178x - 0.0009$      | 0.9999| 8.40–420.00        | 0.006       | 0.018       |
| 2’-deoxyadenosine | 261.1                         | $y = 0.0171x + 0.0025$      | 0.9999| 8.40–420.00        | 0.006       | 0.018       |
| cordycepin        | 261.2                         | $y = 0.0189x - 0.0005$      | 0.9999| 8.40–420.00        | 0.007       | 0.021       |

3.4.2. Limits of Detection and Quantification

The stock solution containing ten reference compounds was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The LOD and LOQ data for each compound investigated were shown in Table 6. The identification of investigated compounds was carried out by comparison of their retention times and UV spectra with those obtained injecting standards in the same conditions or by spiking *Cordyceps* samples with stock standard solutions.

3.4.3. Reproducibility and Accuracy

Reproducibility and accuracy were determined for 10 standard samples at a certain concentration, which was described in Table 7. The intra-day coefficients of variation for the 10 analytes were 0.65%–2.49%. The inter-day coefficients of variation for the 10 analytes were 1.08%–2.12%. The accuracy (%) of the method was expressed as the mean deviation of all repetitions from the nominal value. The intra-day accuracy for the 10 analytes was 98.98%–101.38%. The inter-day accuracy for the 10 analytes was 98.88%–101.53%.

Table 7. Reproducibility and accuracy analysis of 10 nucleosides (n = 5).

| Analyte           | Nominal Concentration (µg/mL) | Assay Value (mean ± SD) (µg/mL) | Coefficient of Variation (%) | Accuracy (%) |
|-------------------|-------------------------------|---------------------------------|-----------------------------|--------------|
| intra-day         |                               |                                 |                             |              |
| uracil            | 40.00                         | 39.84 ± 0.50                    | 1.26                        | 99.60        |
| uridine           | 40.00                         | 39.65 ± 0.88                    | 2.22                        | 99.13        |
| 2’-deoxyuridine   | 70.00                         | 70.71 ± 1.37                    | 1.94                        | 101.01       |
| inosine           | 38.00                         | 37.65 ± 0.80                    | 2.12                        | 99.08        |
| guanosine         | 40.00                         | 40.55 ± 1.01                    | 2.49                        | 101.38       |
| adenine           | 40.00                         | 39.59 ± 0.97                    | 2.45                        | 98.98        |
| thymidine         | 86.00                         | 85.32 ± 1.15                    | 1.35                        | 99.21        |
Table 7. Cont.

| Analyte        | Nominal Concentration (µg/mL) | Assay Value (mean ± SD) (µg/mL) | Coefficient of Variation (%) | Accuracy (%) |
|----------------|-----------------------------|---------------------------------|-----------------------------|--------------|
| adenosine      | 42.00                       | 41.65 ± 0.27                    | 0.65                        | 99.17        |
| 2'-deoxyadenosine | 40.00                   | 39.77 ± 0.62                    | 1.56                        | 99.43        |
| cordycepin     | 42.00                       | 42.39 ± 0.54                    | 1.27                        | 100.93       |
| Inter-day d    | uracil                     | 40.00                           | 39.71 ± 0.48                | 1.21         | 99.28        |
|               | uridine                    | 40.00                           | 39.55 ± 0.73                | 1.85         | 98.88        |
| 2'-deoxyuridine| inosine                    | 38.00                           | 37.62 ± 0.74                | 1.97         | 99.00        |
|               | guanosine                  | 40.00                           | 40.61 ± 0.93                | 2.29         | 101.53       |
|               | adenine                    | 40.00                           | 39.63 ± 0.68                | 1.72         | 99.08        |
|               | thymidine                  | 86.00                           | 85.17 ± 1.27                | 1.49         | 99.03        |
|               | adenosine                  | 42.00                           | 41.57 ± 0.45                | 1.08         | 98.98        |
| 2'-deoxyadenosine| 40.00                    | 39.62 ± 0.84                    | 2.12                        | 99.05        |
| cordycepin     | 42.00                       | 42.49 ± 0.69                    | 1.62                        | 101.17       |

d The sample was analyzed five times within one day (intra-day) and over two consecutive days (inter-day).

3.4.4. Extraction Recoveries

Recoveries and reproducibility of the proposed methods for target compounds were calculated using the C. cicadae (population CCLTL) mixture sample as a representative. The extraction recovery was performed by adding a known amount of individual standards into a 0.50 g of C. cicadae sample. Three replicates were performed for the test. The mixture was extracted and analyzed using the method mentioned above. Table 8 shows the recoveries of 10 nucleosides.

Table 8. Recoveries for the assay of 10 nucleosides in C. cicadae (n = 3).

| Analyte        | Original Amount (µg) | Spiked Amount (µg) | Found (µg ± SD) | Recovery (%) | Coefficient of Variation (%) |
|----------------|---------------------|--------------------|-----------------|-------------|-----------------------------|
| uracil         | 145.35              | 140.00             | 282.08 ± 4.89   | 98.85       | 1.73                        |
| uridine        | 714.12              | 700.00             | 1398.28 ± 11.60 | 98.88       | 0.83                        |
| 2'-deoxyuridine| 63.85               | 60.00              | 126.87 ± 2.36   | 102.44      | 1.86                        |
| inosine        | 140.62              | 140.00             | 276.66 ± 2.19   | 98.59       | 0.79                        |
| guanosine      | 415.96              | 400.00             | 806.39 ± 3.99   | 98.83       | 0.50                        |
| thymidine      | 39.61               | 40.00              | 80.55 ± 1.28    | 101.18      | 1.59                        |
| adenine        | 31.96               | 30.00              | 61.05 ± 0.70    | 98.53       | 1.14                        |
| adenosine      | 375.96              | 370.00             | 736.62 ± 4.81   | 98.75       | 0.65                        |
| 2'-deoxyadenosine| 25.31              | 30.00              | 54.20 ± 0.76    | 97.99       | 1.40                        |
| cordycepin     | — g                 | 150.00             | 152.12 ± 0.78   | 101.41      | 0.51                        |

e The data were present as an average of three determinations; f Recovery (%) = 100 × ((amount found − original amount)/amount spiked); g Not detected.

3.5. Statistical Analysis

The data were statistically analyzed using the Statistical Analysis System (SAS) 8.1 software.
4. Conclusions

Simple and convenient HPLC methods for the determination of the content of nucleosides in C. cicadae populations were described. The method might be used for fast determination of the nucleosides in Cordyceps materials.

Chemical constituents of natural crude drugs, including C. cicadae occurring in Nature, are affected by location, geography, climate and microenvironment. The variance of nucleosides was large in natural C. cicadae, and might be derived from genetic differences. The genetic differentiation of C. cicadae populations by DALP and EST-SSR will be discussed in future papers.

The use of C. cicadae as a Traditional Chinese Medicine and tonic food has been appreciated for more than 1,500 years, and it has been used as a substitute for O. sinensis. The content and distribution of nucleosides in C. cicadae were similar to those in O. sinensis, and the medicinal effectiveness of C. cicadae was also similar to that of O. sinensis. Furthermore, the habitat demands of C. cicadae are less strict than those of O. sinensis, and its resource distribution and reserves were much larger than those of O. sinensis. The price of C. cicadae was about 2,000 yuan per kilogram in 2013, which was 1/100 of that of O. sinensis. It was suggested that C. cicadae should be used as substitute for O. sinensis.

Acknowledgments

This work was supported by the Specialized Research Fund for the Doctoral Program of Higher Education (20125301110001) and Yunnan Natural Science Foundation of China (2008CC019).

Author Contributions

Wen-Bo Zeng, Hong Yu and Feng Ge designed research; Wen-Bo Zeng, Jun-Yuan Yang, Zi-Hong Chen, Yuan-Bing Wang and Yong-Dong Dai performed experiments and analyzed the data; Wen-Bo Zeng, Hong Yu, Yuan-Bing Wang and Yong-Dong Dai collected the Cordyceps materials, Wen-Bo Zeng, Hong Yu and Alison Adams wrote the paper. All authors read and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Luangs-Ard, J.J.; Hywel-Jones, N.L.; Manoch, L.; Samson, R.A. On the relationships of Paecilomyces sect. Isarioidea species. Mycol. Res. 2005, 109, 581–589.
2. Li, B.L. Herbal textuals research on “Chan Hua”. Chin. J. Med. Appl. Pharm. 1993, 10, 21–22.
3. Chen, Z.A.; Liu, G.Y.; Hu, S.Y. Study on cultivation of Paecilomyces cicadae and its pharmacological function. Acta Mycol. Sin. 1993, 12, 138–144.
4. Li, S.P.; Yang, F.Q.; Tsim, K.W.K. Quality control of Cordyceps sinensis, a valued traditional Chinese medicine. J. Pharm. Biomed. 2006, 41, 1571–1584.
5. Sung, G.H.; Hywel-Jones, N.L.; Sung, J.M.; Luangs-Ard, J.J.; Shrestha, B.; Spatafora, J.W. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. Stud. Mycol. 2007, 57, 5–59.
6. Ukai, S.; Kiho, T.; Hara, C.; Morita, M.; Goto, A.; Imaizumi, N.; Hasegawa, Y. Polysaccharides in Fungi. XIII. Antitumor activity of various polysaccharides isolated from Dictyophora indusiata, Ganoderma japonicum, Cordyceps cicadae, Auricularia auricula-judae and Auricularia species. Chem. Pharm. Bull. 1983, 31, 741–744.

7. Weng, S.C.; Chou, C.J.; Lin, L.C.; Tsai, W.J.; Kuo, Y.C. Immunomodulatory functions of extracts from the Chinese medicinal fungus Cordyceps cicadae. J. Ethnopharmacol. 2002, 83, 79–85.

8. Liu, G.Y.; Hu, S.Y. Comparison of sedative and analgesic effects between Cordyceps cicadae and its cultured product. Chin. J. Med. Appl. Pharm. 1991, 8, 4–8.

9. Zhu, R.; Chen, Y.P.; Deng, Y.Y.; Zheng, R.; Zhong, Y.F.; Wang, L.; Du, L.P. Cordyceps cicadae extracts ameliorate renal malfunction in a remnant kidney model. J. Zhejiang Univ. Sci. B 2011, 12, 1024–1033.

10. Wang, Y.; Zhao, Z.J.; Tang, F.D. Primary exploring on pharomic effect of Cordyceps cicadae. Zhejiang J. Chin. Tradiit. Med. 2001, 36, 219–220.

11. Bi, S.Z.; Liu, B.; Ying, J.Z.; Shao, L.P.; Huang, N.L.; Zhang, D.Z.; Xie, Z.X.; Zang, M.; Wei, R.Q. Edible Fungal Flora of China; China Forestry Publishing House: Shanghai, China, 1991; p. 13.

12. Liu, A.Y.; Li, Z.; Zhou, X.; Zhao, H.J.; Hu, H.Y.; Tan, A.J. Research and Application of Cordyceps cicadae Resources in China; Guizhou science and Technology Press: Guiyang, China, 2012; pp. 34–52.

13. Liu, A.Y.; Zhou, X.; Zhao, H.J.; Liang, Z.Q.; Tan, A.J.; Zheng, Q.Y. Biological diversity of Paecilomyces cicadae I. Morphological diversity of cicadae flower and Paecilomyces cicadae. Guizhou Agric. Sci. 2007, 35, 9–11.

14. Zhang, Y.J.; Liu, A.Y.; Liang, Z.Q. Formation and regeneration of protoplasts from Paecilomyces cicadae. Guizhou Agric. Sci. 1998, 26, 1–4.

15. Feng, L.C. Study on TianMa Hill Cordyceps cicadae. J. Shanghai Inst. Technol. 2002, 2, 125–127.

16. Chen, D.Q.; Ding, Z.S.; Lin, A.M.; Pan, P.L.; Chen, Y.T. Isolation and fermentation culture of fungi from Cordyceps cicadae. J. Chin. Med. Mater. 2006, 29, 99–101.

17. Wang, Q.; Liu, Z.Y. Advances in studies on medicinal fungi Cordyceps cicadae. Chin. Tradit. Herb. Drugs 2004, 34, 469–471.

18. Kiho, T.; Ito, M.; Nagai, K.; Hara, C.; Ukai, S. Polysaccharides in fungi. X XII. a water soluble polysaccharide from the alkaline extract of the insect-body portion of Chan hua (fungus: Cordyceps cicadae). Chem. Pharm. Bull. 1988, 36, 3032–3037.

19. Kim, H.S.; Kim, J.Y.; Ryu, H.S.; Shin, B.R.; Kang, J.S.; Kim, H.M.; Kim, Y.O.; Hong, J.T.; Kim, Y.; Han, S.B. Phenotypic and functional maturation of dendritic cells induced by polysaccharide isolated from Paecilomyces cicadae. J. Med. Food. 2011, 14, 847–856.

20. Kiho, T.; Nagai, K.; Miyamoto, I.; Watanabe, T.; Ukai, S. Polysaccharides in fungi. XXV. Biological activities of two galactomannans from the insect-body portion of Chan hua (fungus: Cordyceps cicadae). Yakugaku Zasshi 1990, 110, 286–288.

21. Ge, F.; Xia, C.R.; Li, C.R.; Ding, T.; Shao, Y.; Fan, M.Z. Analysis of the chemical compositions of Paecilomyces cicadae fermented mycelia and Cordyceps cicadae fruit body. Mycosystema 2007, 26, 68–75.
22. Osuchowski, M.F.; Johnson, V.J.; He, Q.R.; Sharma, R.P. Myriocin, a serine palmitoyltransferase inhibitor, alters regional brain neurotransmitter levels without concurrent inhibition of the brain sphingolipid biosynthesis in mice. *Toxicol. Lett.* **2004**, *147*, 87–94.

23. Yu, J.W.; Xu, H.J.; Mo, Z.H.; Zhu, H.L.; Mao, X.B. Determination of myriocin in natural and cultured *Cordyceps cicadae* using 9-fluorenylmethyl chloroformate derivatization and high-performance liquid chromatography with UV-detection. *Anal. Sci.* **2009**, *25*, 855–859.

24. Kuo, Y.C.; Weng, S.C.; Chou, C.J.; Chang, T.T.; Tsai, W.J. Activation and proliferation signals in primary human T lymphocytes inhibited by ergosterol peroxide isolated from *Cordyceps cicadae*. *Br. J. Pharmacol.* **2003**, *140*, 895–906.

25. Cunningham, K.G.; Manson, W.; Spring, F.S.; Hutchinson, S.A. Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. *Nature* **1950**, *166*, 949.

26. Fan, H.; Li, S.P.; Xiang, J.J.; Lai, C.M.; Yang, F.Q.; Gao, J.L.; Wang, Y.T. Qualitative and quantitative determination of nucleosides, bases and their analogues in natural and cultured *Cordyceps* by pressurized liquid extraction and high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS). *Anal. Chim. Acta* **2006**, *567*, 218–228.

27. Ikeda, R.; Nishimura, M.; Sun, Y.; Wada, M.; Nakashima, K. Simple HPLC-UV determination of nucleosides and its application to the authentication of *Cordyceps* and its allies. *Biomed. Chromatogr.* **2008**, *22*, 630–636.

28. Coradetti, R.; Conte, G.L.; Moroni, F.; Passani, M.B.; Pepeu, G. Adenosine decreases aspartate and glutamate release from rat hippocampal slices. *Eur. J. Pharmacol.* **1984**, *104*, 19–26.

29. Schmidt, C.; Bellingham, M.C.; Richter, D.W. Adenosinergic modulation of respiratory neurones and hypoxic responses in the anaesthetized cat. *J. Physiol.* **1995**, *483*, 769–781.

30. The Pharmacopoeia Commission of PRC. *Pharmacopoeia of the People’s Republic of China*, 9th ed.; Chemical Industry Publishing House: Beijing, China, 2010; pp. 106.

31. Benowitz, L.I.; Goldberg, D.E.; Irwin, N. Inosine stimulates axon growth in vitro and in the adult CNS. *Prog. Brain Res.* **2002**, *137*, 389–399.

32. Zhou, X.X.; Meyer, C.U.; Schmidtke, P.; Zepp, F. Effect of cordycepin on interleukin-10 production of human peripheral blood mononuclear cells. *Eur. J. Pharmacol.* **2002**, *453*, 309–317.

33. Ahn, Y.J.; Park, S.J.; Lee, S.G.; Shin, S.C.; Choi, D.H. Cordycepin: Selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against *Clostridium* spp. *J. Agric. Food Chem.* **2000**, *48*, 2744–2748.

34. Kodama, E.N.; McCaffrey, R.P.; Yusa, K.; Mitsuya, H. Antileukemic activity and mechanism of action of cordycepin against terminal deoxynucleotidal transferase-positive (TdT+) leukemic cells. *Biochem. Pharmacol.* **2000**, *59*, 273–281.

35. Li, S.P.; Li, P.; Lai, C.M.; Gong, Y.X.; Kan, K.K.W.; Dong, T.T.X.; Tsim, K.W.K.; Wang, Y.T. Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurised liquid extraction and high-performance liquid chromatography. *J. Chromatogr. A* **2004**, *1036*, 239–243.

36. Guo, F.Q.; Li, A.; Huang, L.F.; Liang, Y.Z.; Chen, B.M. Identification and determination of nucleosides in *Cordyceps sinensis* and its substitutes by high performance liquid chromatography with mass spectrometric detection. *J. Pharm. Biomed.* **2006**, *40*, 623–630.
37. Yang, F.Q.; Li, S.P. Effects of sample preparation methods on the quantification of nucleosides in natural and cultured Cordyceps. J. Pharm. Biomed. 2008, 48, 231–235.
38. Uauy, R.; Stringel, G.; Thomas, R.; Quan, R. Effect of dietary nucleosides on growth and maturation of the developing Gut in the Rat. J. Pediatr. Gastroenterol. Nutr. 1994, 10, 497–503.
39. Carver, J.D.; Walker, W.A. The role of nucleotides in human nutrition. J. Nutr. Biochem. 1995, 6, 58–72.
40. Wang, S.; Yang, F.Q.; Feng, K.; Li, D.Q.; Zhao, J.; Li, S.P. Simultaneous determination of nucleosides, myriocin, and carbohydrates in Cordyceps by HPLC coupled with diode array detection and evaporative light scattering detection. J. Sep. Sci. 2009, 32, 4069–4076.
41. Wang, Z.B.; Li, N.; Wang, M.; Wang, Y.; Du, L.; Ji, X.F.; Yu, A.M.; Zhang, H.Q.; Qiu, F.P. Simultaneous determination of nucleosides and their bases in Cordyceps sinensis and its substitutes by matrix solid-phase dispersion extraction and HPLC. J. Sep. Sci. 2013, 36, 2348–2357.
42. Yang, F.Q.; Li, D.Q.; Feng, K.; Hu, D.J.; Li, S.P. Determination of nucleotides, nucleosides and their transformation products in Cordyceps by ion-pairing reversed-phase liquid chromatography-mass spectrometry. J. Chromatogr. A 2010, 1217, 5501–5510.
43. Huang, L.F.; Liang, Y.Z.; Guo, F.Q.; Zhou, Z.F.; Cheng, B.M. Simultaneous separation and determination of active components in Cordyceps sinensis and Cordyceps militaris by LC/ESI-MS. J. Pharm. Biomed. 2003, 33, 1155–1162.
44. Yang, F.Q.; Ge, L.Y.; Yong, J.W.H.; Tan, S.N.; Li, S.P. Determination of nucleosides and nucleobases in different species of Cordyceps by capillary electrophoresis-mass spectrometry. J. Pharm. Biomed. 2009, 50, 307–314.
45. Gong, Y.X.; Li, S.P.; Li, P.; Liu, J.J.; Wang, Y.T. Simultaneous determination of six main nucleosides and bases in natural and cultured Cordyceps by capillary electrophoresis. J. Chromatogr. A 2004, 1055, 215–221.
46. Yang, F.Q.; Li, S.P.; Li, P.; Wang, Y.T. Optimization of CEC for simultaneous determination of eleven nucleosides and nucleobases in Cordyceps using central composite design. Electrophoresis 2007, 28, 1681–1688.
47. Yang, F.Q.; Guan, J.; Li, S.P. Fast simultaneous determination of 14 nucleosides and nucleobases in cultured Cordyceps using ultra-performance liquid chromatography. Talanta 2007, 73, 269–273.
48. Ling, J.Y.; Sun, Y.J.; Zhang, H.; Lv, P.; Zhang, C.K. Measurement of cordycepin and adenosine in stroma of Cordyceps sp. by capillary zone electrophoresis (CZE). J. Biosci. Bioeng. 2002, 94, 371–374.
49. Li, S.P.; Li, P.; Ji, H.; Zhang, P.; Dong, T.T.X.; Tsim, K.W.K. The contents and their change of nucleosides from natural Cordyceps sinensis and cultured Cordyceps mycelia. Acta Pharm. Sin. 2001, 36, 436–439.
50. Rao, Y.K.; Chou, C.H.; Tzeng, Y.M. A simple and rapid method for identification and determination of cordycepin in Cordyceps militaris by capillary electrophoresis. Anal. Chim. Acta 2006, 566, 253–258.
51. Yuan, J.P.; Zhao, S.Y.; Wang, J.H.; Kuang, H.C.; Liu, X. Distribution of nucleosides and nucleobases in Edible Fungi. J. Agric. Food Chem. 2008, 56, 809–815.
52. Gu, Y.X.; Wang, Z.S.; Li, S.X.; Yuan, Q.S. Effect of multiple factors on accumulation of nucleosides and bases in Cordyceps militaris. Food Chem. 2007, 102, 1304–1309.
53. Yu, L.; Zhao, J.; Li, S.P.; Fan, H.; Hong, M.; Wang, Y.T.; Zhu, Q. Quality evaluation of *Cordyceps* through simultaneous determination of eleven nucleosides and bases by RP-HPLC. *J. Sep. Sci.* **2006**, *29*, 953–958.

54. Li, S.P.; Su, Z.R.; Dong, T.T.X.; Tsim, K.W.K. The fruiting body and its caterpillar host of *Cordyceps sinensis* show close resemblance in main constituents and anti-oxidation activity. *Phytomedicine* **2002**, *9*, 319–324.

55. Xie, J.W.; Huang, L.F.; Hu, W.; He, Y.B.; Wong, K.P. Analysis of the main nucleosides in *Cordyceps sinensis* by LC/ESI-MS. *Molecules* **2010**, *15*, 305–314.

56. Hsu, T.H.; Shiao, L.H.; Hsieh, C.; Chang, D.M. A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom DongChongXiaCao, its counterfeit and mimic, and fermented mycelium of *Cordyceps sinensis*. *Food Chem.* **2002**, *78*, 463–469.

57. Zhu, J.S.; Halpern, G.M.; Jones, K. The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: Part I. *J. Altern. Complement. Med.* **1998**, *4*, 289–303.

58. Zhu, J.S.; Halpern, G.M.; Jones, K. The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*: Part II. *J. Altern. Complement. Med.* **1998**, *4*, 429–457.

59. Li, S.P.; Li, P.; Dong, T.T.X.; Tsim, K.W.K. Determination of nucleosides in natural *Cordyceps sinensis* and cultured *Cordyceps* mycelia by capillary electrophoresis. *Electrophoresis* **2001**, *22*, 144–150.

60. Jiang, H.; Liu, K.; Meng, S.; Chu, Z.Y. Chemical constituents of the dry sorophore of *Cordyceps militaris*. *Acta Pharm. Sin.* **2000**, *35*, 663–668.

61. Yu, H.M.; Wang, B.S.; Huang, S.C.; Duh, P.D. Comparison of protective effects between cultured *Cordyceps militaris* and natural *Cordyceps sinensis* on oxidative damage. *J. Agric. Food Chem.* **2006**, *54*, 3132–3138.

*Sample Availability*: Not available.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).